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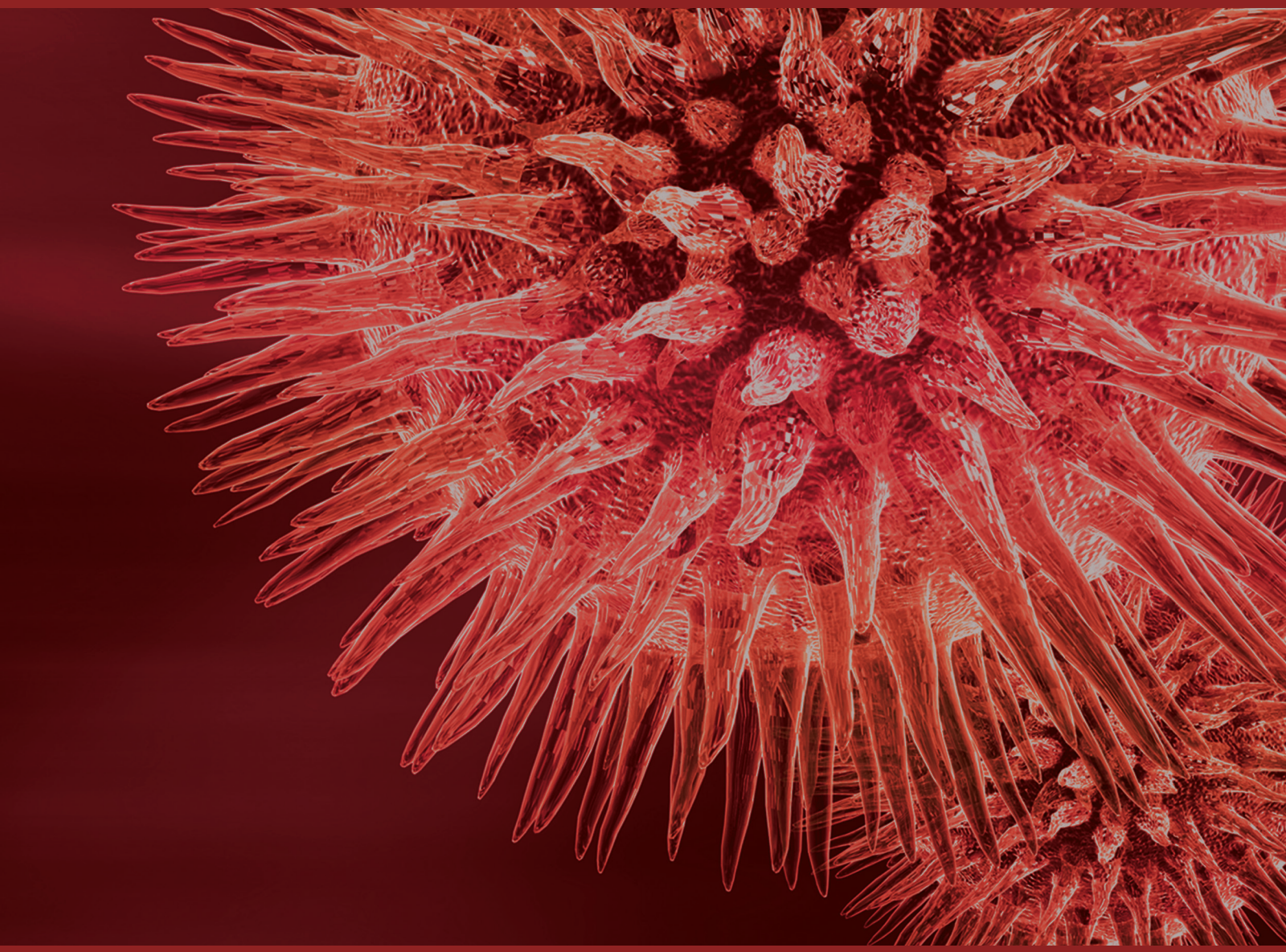
Guest Editors: Akio Tomoda, Yoshinori Marunaka, Douglas C. Eaton, and Anuwat Dinudom



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Editorial

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Membrane transport plays a critical role in producing ionic environments in both intracellular and extracellular spaces that are necessary for cellular signal transduction. The importance of membrane transport implies that any abnormality may lead to pathophysiological conditions and often to specific diseases. This also means that membrane transporters, including ion channels and transporters, are very important therapeutic targets in several diseases such as cardiovascular disorders, hypertension, dementia, metabolic syndrome associated with diabetes mellitus, and cancer. Thus, many researchers have been investigating regulatory mechanisms of membrane transport with an aim to develop new therapies for treating transport disorders found in various diseases.

A specific example of a transport defect associated with serious pathology involves abnormalities in total body sodium balance. Na^+ that is transported via the epithelial Na^+ channel (ENaC) [1–3] determines the body fluid volume, blood pressure, and the amount of fluid in the alveolar space. The positive potential gradient generated by transepithelial Na^+ movement (reabsorption) drives movements of anions. The osmotic gradient produced by movement of salt promotes water reabsorption [4]. Patients with Liddle's syndrome have a gain of function mutation in ENaC leading to hypertension due to excess body fluid volume, caused by abnormally high ENaC-mediated Na^+ reabsorption [5], showing that the activity of ENaC is involved in the regulation of blood pressure. ENaC-mediated Na^+ reabsorption is mainly determined by the number of ENaC expressed at the apical cell membrane, which is regulated by intracellular trafficking of ENaC proteins. In addition, the activity of ENaC is also regulated by protease-mediated cleavage of ENaC subunits [4]. Of course, biosynthesis of ENaC proteins is one of the most important regulatory factors that determine total activity. Several humoral agents including vasopressin, catecholamine, osmolarity, and aldosterone are known to regulate expression of ENaC at the plasma (apical) membrane [4, 6, 7].

Epithelial Cl^- transport (secretion) has been known to play an important role in the regulation of fluid volume in the lung [8–10]. Epithelial Cl^- transport is a two-step process: (1) the Cl^--uptake from the interstitium into the cytosolic space across the basolateral membrane, which is mediated by the Na^+-K^+-$2Cl^-$ cotransporter (NKCC), and (2) the Cl^--releasing step across the apical membrane via apical Cl^- channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and the Ca^{2+}-activated Cl^- channels. Activities of NKCC and Cl^- channels are known to be regulated by various factors, including catecholamines and insulin [10].

Cytosolic Cl^- is involved in various cellular functions such as gene expression, neuron elongation, and cancer cell

growth [11, 12]. In addition, mRNA expression of ENaC is regulated by cytosolic Cl^-. Quercetin (a flavonoid) that elevates cytosolic Cl^- concentration by activating NKCC has been known to downregulate mRNA expression of ENaC. Furthermore, elongation of neurites depends on the concentration of cytosolic Cl^-. It has been reported that Cl^- enhances tubulin polymerization by inhibiting activity of GTPase contained in tubulin molecule. Moreover, changes in the activity of ion transporters and channels regulate cancer cell growth by modulating the cytosolic Cl^- concentration via control of MAPK-mediated signaling pathways.

pH is one of the most important factors that regulate cell function and enzyme activity [13–15]. It is well established that the intracellular pH is finely regulated by various types of H^+ transporters, such as the Na^+/H^+ exchanger. Although the physiological role of extracellular (interstitial) pH has not been studied extensively, extracellular (interstitial) pH has recently been recognized as an essential factor in several pathophysiological conditions and insulin resistance in diabetes mellitus.

Many investigators have studied cell volume regulatory mechanisms. The physiological and pathophysiological significance of cell volume regulation including regulatory cell volume decrease (RVD) have recently been described [16]. Chemosensing process and signal transduction of immune cells are mediated via membrane transport. More detail is available in this issue.

The roles of membrane transporters in the regulation of cellular functions described above has addressed some, but not by any means all, of the significance of the transporters as key factors in maintaining the homeostasis of body compartments and functions. Membrane transporters, including ion channels and transporters, are known targets for treatment of various diseases. We hope that readers of this special issue will find not only new data on membrane transport and its roles and updated reviews on membrane transport mechanisms, but also concepts and new ideas for developing new therapies for various types of diseases.

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Research Article

Inactivation of Src-to-Ezrin Pathway: A Possible Mechanism in the Ouabain-Mediated Inhibition of A549 Cell Migration

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Ouabain, a cardiac glycoside found in plants, is primarily used in the treatment of congestive heart failure and arrhythmia because of its ability to inhibit Na^+/K^+-ATPase pump. Recently ouabain has been shown to exert anticancer effects but the underlying mechanism is not clear. Here, we explored the molecular mechanism by which ouabain exerts anticancer effects in human lung adenocarcinoma. Employing proteomic techniques, we found 7 proteins downregulated by ouabain in A549 including p-ezrin, a protein associated with pulmonary cancer metastasis in a dose-dependent manner. In addition, when the relative phosphorylation levels of 39 intracellular proteins were compared between control and ouabain-treated A549 cells, p-Src (Y416) was also found to be downregulated by ouabain. Furthermore, western blot revealed the ouabain-mediated downregulation of p-FAK (Y925), p-paxillin (Y118), p130CAS, and Na^+/K^+-ATPase subunits that have been shown to be involved in the migration of cancer cells. The inhibitory effect of ouabain and Src inhibitor PP2 on the migration of A549 cells was confirmed by Boyden chamber assay. Anticancer effects of ouabain in A549 cells appear to be related to its ability to regulate and inactivate Src-to-ezrin signaling, and proteins involved in focal adhesion such as Src, FAK, and p130CAS axis are proposed here.

1. Introduction

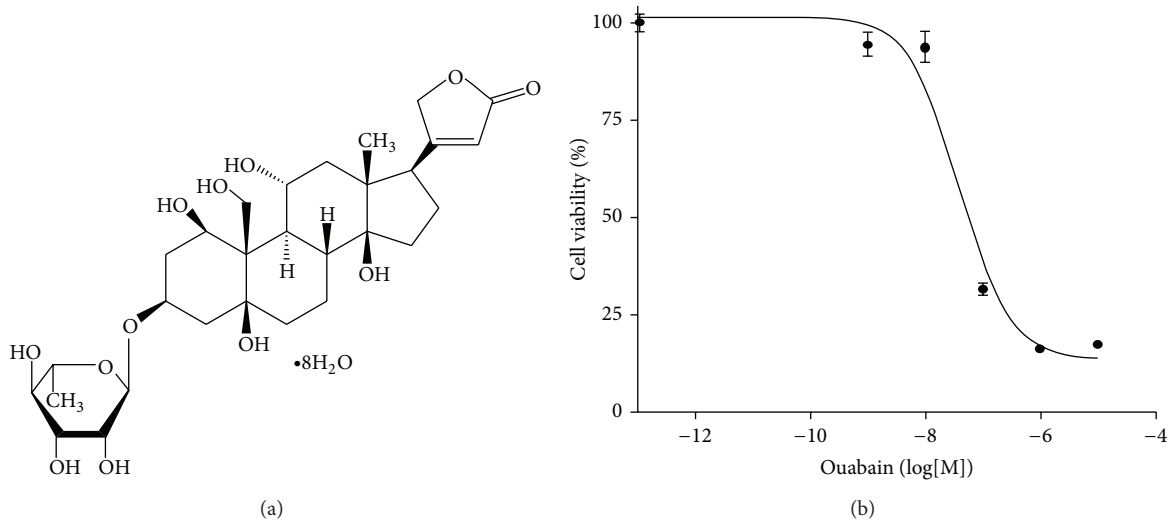
Ouabain (Figure 1(a)) is a cardiac glycoside found in plants and is primarily used in the treatment of congestive heart failure and cardiac arrhythmia because it inhibits the Na^+/K^+-ATPase pump leading to a sequence of events including increase in the level of calcium ions and cardiac contractile force. A recent unexpected epidemiological finding that cancer patients receive cardiac glycosides showed significantly lower mortality rates sparked new interest in possible anticancer properties of cardiac glycosides [1–4]. Prassas and Diamandis [3] confirmed that cardiac glycosides exert antiproliferative and/or apoptotic effects on breast, prostate, lung, renal, pancreatic, melanoma, leukemia, neuroblastoma, and myeloma cancer cells in vitro. But the underlying molecular pathways have not been clarified.

Most of the previous studies of proteomic profile changes resulting from ouabain treatment focused on Na^+/K^+-ATPase suppression and were conducted in vascular smooth muscle cells (VSMCs) or in the endothelial cells (ECs) in order to identify the proteins involved in ouabain-induced regulation of cell proliferation and apoptosis and vascular remodeling [5–8] but not the proteins involved in ouabain's anticancer effects.

In this context we conducted a proteomic analysis of human lung adenocarcinoma A549 cells, treated with ouabain to identify the proteins altered when ouabain exhibits its anticancer effects, and thus it is possibly responsible for its anticancer effects.

2. Methods

2.1. Materials. Ouabain octahydrate and PP2 (Src inhibitor) were purchased from Sigma (MO) and Calbiochem EMD Millipore (Darmstadt, Germany), respectively. Sources of other chemicals and reagents are indicated as they appear in the text.



(a) (b)

Figure 1: Structure of ouabain and its effect on viability of A549 cells. (a) Structure of ouabain. (b) The effect of ouabain on the viability of A549 cells. Cells (1×10^4 cells/well) in a 96-well plate were incubated with ouabain for 24 h and cell viability was measured by counting.

2.2. Cell Culture. Human lung adenocarcinoma A549 cells were cultured in Dulbecco's modified Eagles's medium (DMEM, HyClone, UT) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/mL of penicillin, and 100 μg/mL Streptomycin (HyClone) in humidified atmosphere of 5% CO_2 at 37°C. The culture medium was changed every 3 days.

2.3. Cell Viability Assay. Cells in a 96-well plate (1×10^4 cells/well) were treated with ouabain octahydrate for 24 h and cell viability was determined in triplicate by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's protocol.

2.4. Sample Preparation and 2-Dimensional Electrophoresis (2-DE). These were performed as essentially as described by Park et al. [9]. In brief, cells were treated with ouabain for 24 h in DMEM with 10% FBS. The cells were harvested and samples were suspended in 0.5 mL of 50 mM Tris buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 16 μL protease inhibitor cocktail (Roche Molecular Biochemicals, IN) and sonicated on ice. The sonicates were homogenized and centrifuged at 12,000 ×g for 15 min. Its protein content was quantitated by the Bradford method (Bio-Rad, CA). Fifty units of benzonase (250 units/μL; Sigma, MO) was added to the stock and stored at −80°C until use. For 2-DE analysis, pH 3–10 nonlinear IPG strips (Habersham Biosciences) were rehydrated in a swelling buffer containing 7 M urea, 2 M thiourea, 0.4% (w/v) DTT, and 4% (w/v) CHAPS. The protein lysates (300 μg) were cup-loaded into the rehydrated IPG strips using a Multiphor II apparatus (Amersham Biosciences) set for a total of 57 kVh. The 2D separation was performed on 12% SDS-polyacrylamide gels. Following fixation of the gels for 1 h in 40% (v/v) methanol containing 5% (v/v) phosphoric acid, the gels were stained with colloidal Coomassie Blue G-250 solution (Proteome-Tech, South Korea) for 5 h. They were then destained in 1% (v/v) acetic acid for 4 h and imaged using a GS-710 imaging calibrated densitometer (Bio-Rad). Protein spot detection and 2D pattern matchings were carried out using ImageMaster 2D Platinum software (Amersham Biosciences).

2.5. In-Gel Digestion of Protein Spots with Trypsin and Extraction of Peptides. The procedure for in-gel digestion of protein spots from Coomassie Blue stained gels was carried out as described in [9]. In brief, protein spots were excised from stained gels and cut into pieces. The gel pieces were washed for 1 h at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile (ACN) and dehydrated in a SpeedVac for 10 min and rehydrated in 10 μL (20 ng/μL) of sequencing grade trypsin solution (Promega, WI). After incubation in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight, the tryptic peptides were extracted with 5 μL of 0.5% TFA containing 50% (v/v) ACN for 40 min with mild sonication. The extracted solution was reduced to 1 μL in a vacuum centrifuge. The resulting peptide solution was desalted using a reversed-phase column [10] and subjected to mass spectrometric analysis. A GEloader tip (Eppendorf, Hamburg, Germany) constricted was packed with Poros 20 R2 resin (PerSpective Biosystems, MA). After an equilibration with 10 μL of 5% (v/v) formic acid, the peptide solution was loaded on the column and washed with 10 μL of 5% (v/v) formic acid. The bound peptides were eluted with 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) (5 mg/mL in 50% (v/v) ACN/5% (v/v) formic acid) and dropped onto a MALDI plate (96 × 2; Applied Biosystems, CA).

2.6. Analysis of Peptides Using MALDI-TOF MS and Identification of Proteins. The masses of the tryptic peptides were determined with a Voyager-DE STR mass spectrometer (PerSpective Biosystems) in reflectron positive ion mode as

described in [11]. External calibration was performed for every four samples with mixtures of adrenocorticotropic fragment 18–39 (monoisotopic mass, 2465.1989), neurotensin (monoisotopic mass, 1672.9175), and angiotensin I (monoisotopic mass, 1296.6853) as standard calibrants, and mass spectra were acquired for the mass range of 900–3500 Da. The proteins were identified through matches in Swiss-Prot and NCBI databases, using the search program ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe), MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF), or MS-Fit (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard, University of California San Francisco, Version 4.0.5). The following mass search parameters were set: peptide mass tolerance, 50 ppm; a mass window between 0 and 100 kDa, allowance of missed cleavage, 2; consideration for variable modifications such as oxidation of methionine and propionamides of cysteines. Only significant hits as defined by each program were considered initially with at least 4 matching peptide masses.

2.7. Western Blotting. Cells (2×10^5 cells/well) placed in a 6-well plate were treated with ouabain octahydrate for the indicated time, washed twice with cold PBS, and scraped into a lysis buffer containing 50 mM Tris-HCl (pH 7.4) 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF, 0.25% deoxycholate, 1% Triton X-100, and a protease inhibitor cocktail tablet (Roche, Diagnostics, Mannheim, Germany). The cell lysate was centrifuged at 15,000 ×g for 10 min at 4°C. The protein level of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples (20 μg) were mixed with sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, and 0.01% bromophenol blue) and incubated at 95°C for 10 min. To detect the Na^+/K^+-ATPase α1 and β1, samples were incubated at 75°C for 15 min. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Equivalent amounts of the proteins separated on gels were transferred onto nitrocellulose membranes (Whattman, Germany) and stained with Ponceau S to confirm efficiency of transfer. Membranes were washed, blocked with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 3% BSA, for 1 h at room temperature, and probed with primary antibody overnight at 4°C. They were washed three times with TBST for 30 min, incubated with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., TX) for 2 h, and washed three times with TBST for 30 min. Antibodies against p-ezrin (Y353), ezrin, p-Src (Y416), Src, p-paxillin (Y118), paxillin, p-FAK (Y925), and FAK, respectively, were purchased from Cell Signal Technology (MA). Antibodies against Na^+/K^+-ATPase α1 and β1 were purchased from Upstate, Merck Millipore (MA), and antibodies against Na^+/K^+-ATPase α2 and α3 were purchased from Santa Cruz Biotechnology and Thermo Fisher Scientific (MA), respectively. Antibodies against p130CAS and actin were purchased from R&D System and Santa Cruz Biotechnology, Inc., respectively. Membranes were developed with Amersham ECL Plus (GE Healthcare Bio-sciences, Sweden) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan).

2.8. Phosphokinase Antibody Array Analysis. Phosphokinase array analysis was performed using Proteome Profiler Human Phosphokinase Array Kit (R&D systems) according to the manufacturer's procedure. Briefly, cells (5×10^5 cells) in a 60 mm^2 dish were treated with ouabain octahydrate for 24 h, washed twice with cold PBS, lysed with the lysis buffer 6 in the kit, and centrifuged at 14,000 ×g for 5 min. The protein levels of the supernatants were assayed using BCA kit (Pierce, Rockford, IL, USA). For Human Phosphokinase Array assay, preblocked nitrocellulose membranes were incubated with 300 μg of cellular extracts overnight at 4°C on a rocking platform. The membranes were washed three times with 1x wash buffer in the kit to remove unbound proteins and then incubated with a mixture of biotinylated antibodies and streptavidin-HRP antibodies. Amersham ECL Plus was applied to determine spot densities. Array images were analyzed using Multi Gauge 3.0 (Fuji Photo Film Co., Ltd., Japan).

2.9. Cell Migration Assay. Cell migration assay was performed as described previously with some modification [12]. A549 cells (5×10^5 cells) were treated with various concentrations of ouabain (1, 10, and 100 nM) for 4 h in a 60 mm culture dish and harvested with trypsin EDTA. After recounting the harvested cells, their immigrations were assayed in a Boyden chamber (Neuro Probe, MD, USA) as follows. The lower compartment of a 48-well Boyden chamber was filled with 30 μL of DMEM containing 0.1 and 10% FBS. An 8.0 μm pore polycarbonate membrane (Neuro Probe) was coated with gelatin solution (0.01% gelatin and 0.1% acetic acid in distilled water) for 24 h and a 50 μL volume of a cell (2×10^4 cells) suspension was introduced into the upper compartment of the chamber, and the chamber was incubated at 37°C for 6 h. The membrane was fixed and stained with Diff-Quik solution (Dade Behring, DE, USA) and placed on a microscope slide. The invading cells were counted using a light microscope at 100x magnification. The data are presented as means ± standard deviation of 4 fields from each well of triplicate samples. Statistical significance was determined using Student's t-test and differences of $P < 0.05$ were considered significant.

3. Results

3.1. Ouabain Decreased the Viability of A549 Cells in a Dose Dependent Manner and Changes the Expression of some Cellular Proteins. The effect of ouabain on the viability of A549 cells was assessed by counting viable cells (Figure 1(b)) and changes in protein expression in the cells were assessed using two-dimensional (2D) gel electrophoresis. IC_{50} of ouabain on the viability of A549 cells was about 40 nM (Figure 1(b)). In order to identify the proteins that might be involved in the anticancer activity of ouabain, we performed a comparative proteomic analysis of lysates of control A549 cells and cells treated with 100 nM ouabain. Of over 500 protein spots that

Table 1: MALDI-TOF-based identification of ezrin.

Protein name	NCBI BLAST	Number of matched peptides	Sequence coverage (%)	Theoretical Mr(Da)/pI	Score	Expect
Ezrin	gi|46249758	21	30	69199/5.94	116	$6.1e-07$

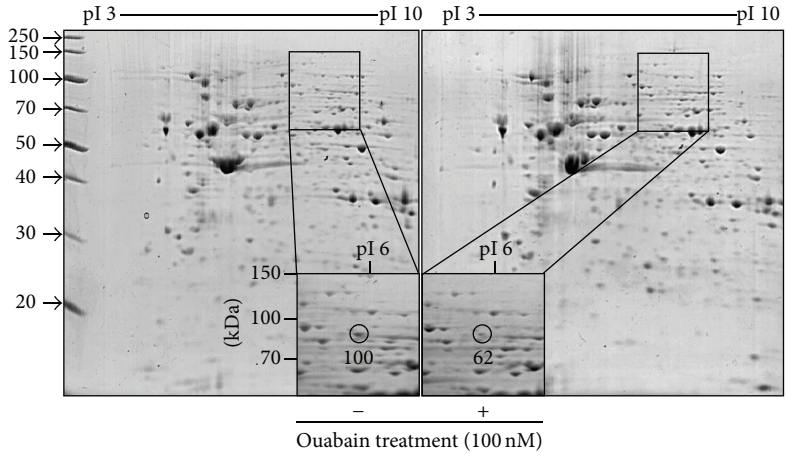


Figure 2: 2-DE analysis. The relative volume of circle-indicated spot was analyzed by ImageMaster 2D Platinum software. MALDI-TOF-MS spectrum of the circled peptide spot after in-gel digestion.

appeared in the 2-DE gel, two spots showed increases in proteins and 7 spots showed decreases in proteins (Figure S1 and Table S1 in the Supporting Information available online at http://dx.doi.org/10.1155/2014/537136). The circled spot in Figure 2 shows a 62% decrease in ouabain-treated A549 cells compared to control. This circled spot was digested in gel with trypsin and subjected to peptide mass fingerprinting (PMF).

3.2. Ouabain Decreased the Expression of Ezrin. Based on the PMF, the estimated pI and molecular weight by 2-DE map, the circle-indicated protein in the 2-DE gel was identified as ezrin. These characteristics are listed in Table 1. Ouabain-induced decrease in the ezrin signal in 2-DE gel was further differentiated by Western blot analysis. As shown in Figure 3(a), ouabain dose-dependently decreased the level of phosphoezrin (Y353), but not that of total ezrin (Figure 3(a)). Further, we carried out phosphokinase array analysis to investigate molecular pathways that potentially contribute to ouabain-mediated cell death. We found that p-Src (Y416) was downregulated by ouabain in 39 intracellular proteins in the control and ouabain-treated A549 cells (Figure 3(b)). Ouabain-mediated decrease of p-Src (Y416) was also confirmed by Western blot analysis (Figure 3(c)).

Since ezrin protein family and Src are known to play roles in membrane-cytoskeleton interactions and focal adhesion, respectively, we further investigated the effect of ouabain on the expression and activation levels of other molecules related to focal adhesion in A549 cells by Western blot analysis. These included focal adhesion kinase (FAK) and cytoskeletal proteins such as p130CAS and paxillin. Figure 3(c) shows that ouabain decreased the phosphorylation of FAK (Y925) and paxillin (Y118) and the expression of p130CAS.

3.3. Ouabain Inhibited Src-Mediated Cell Migration. Human A549 cells have been reported to be highly metastatic, and cardiac glycosides have been reported to inhibit the migration of cancer cells by the specific inhibition of the Na^+/K^+-ATPase $\alpha 1$ subunit [13, 14]. Therefore, we examined the effect of ouabain on the expression of Na^+/K^+-ATPase subunits and the migration of A549 cells using the Boyden chamber analysis. As shown in Figure 3(c), ouabain treatment for 1 day of A549 cells strongly inhibited the expression levels of Na^+/K^+-ATPase $\alpha 1$ and $\beta 1$. Furthermore, it decreased the migration of A549 cells in a dose-dependent manner even when it was exposed to A549 cells for 4 h before migration (Figures 4(a) and 4(b)).

Binding of cardiac glycosides to Na^+/K^+-ATPase is known to activate several downstream signaling pathways, including phospholipase C (PLC), mitogen-activated protein kinase (MAPK), phosphatidyl-inositol-3-kinase (PI3 K), and Src kinase [15–17]. To determine whether ouabain exerts its antimigration effect by inactivating ezrin and paxillin via Src inhibition, the phosphorylation levels of signaling molecules including Src, FAK, paxillin, and ezrin were assessed by Western blotting. As shown in Figure 4(c), ouabain decreased the phosphorylated level of FAK in 30 min and its decrease was maintained up to 6 hr. The phosphorylated levels of Src and ezrin, but not of paxillin, were shown to be decreased by ouabain in 6 hr.

The involvement of Src in the antimigration activity of ouabain was further confirmed by the pharmacologic inhibition study. Src inhibitor, PP2, also exhibited significant

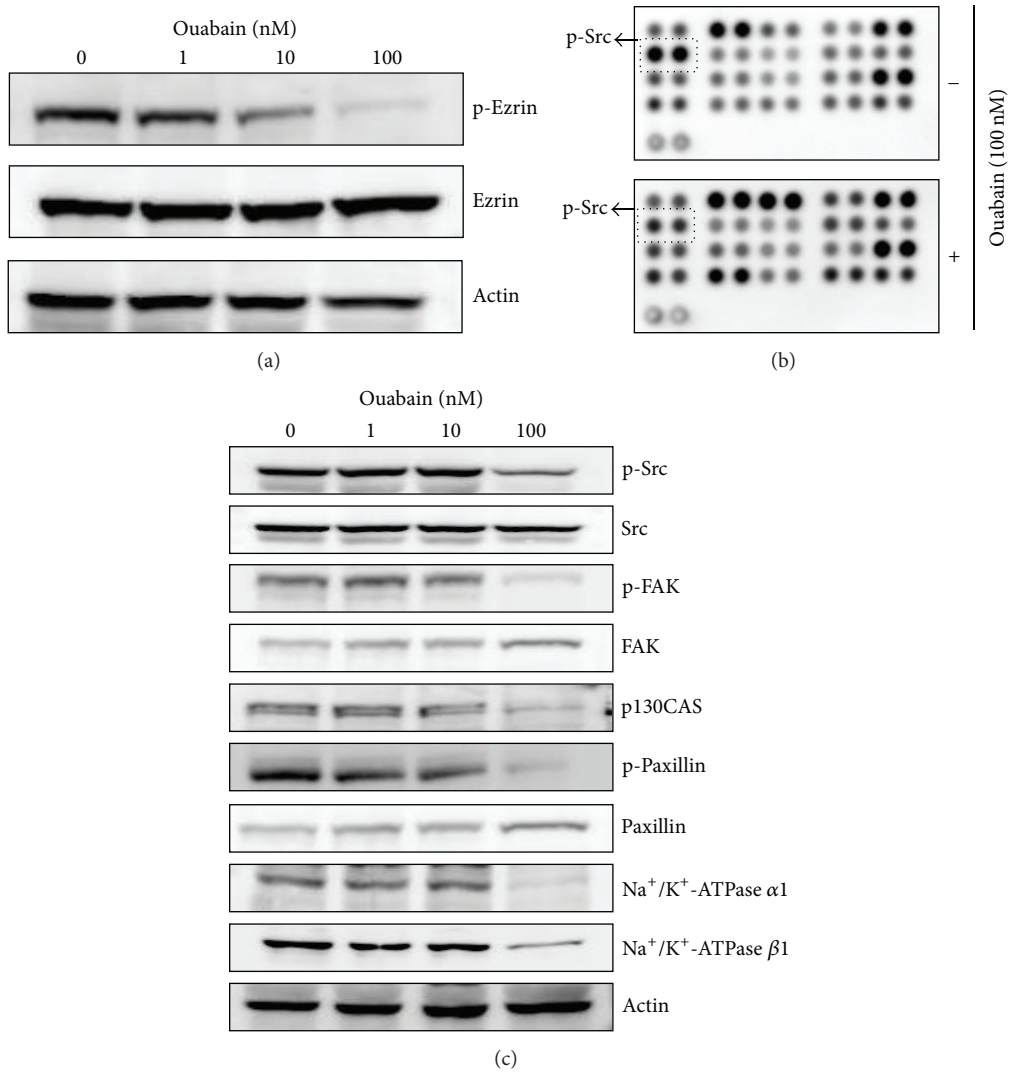


Figure 3: Proteome profiler array analysis of phosphokinase and validation. (a) Effects of ouabain on the expression and phosphorylation of ezrin were evaluated by Western blot analysis. Actin was used as an internal control. (b) For phosphokinase array study, 300 μg of proteins obtained from A549 cells (5×10^5 cells in a 60 mm^2 dish) treated with vehicle (DMSO) or ouabain octahydrate for 24 h in the membranes was probed. (c) Effects of ouabain on the expression and/or phosphorylation of Src, FAK, p130CAS, paxillin, and Na^+/K^+-ATPase subunits were evaluated by Western blot analysis.

inhibition effect in the migration of A549 cells from the top to the bottom chamber at 6 h, in a dose-dependent fashion when several doses of PP2 (3, 10, and 30 μM) were administered in the bottom chamber (Figures 5(a) and 5(b)). Additionally, A549 cells were treated with PP2 for 30 min and after 24 h the phosphorylation of signaling molecules including Src, ezrin, and paxillin was assessed by Western blotting. As shown in Figure 5(c), PP2-induced inhibition of Src resulted in reduced phosphorylation of ezrin and paxillin. These results suggest that ouabain exerts its antimigration effect by inactivating ezrin and paxillin via Src inhibition.

4. Discussion

Anticancer effect of ouabain has been reported in several cancer cells including A549 cells [18]. This study confirmed that ouabain exerts strong antiproliferative activity on A549 cells at nanomolar concentrations (IC_{50}, 40 nM). Also our results are in agreement with previous reports that ouabain *per se* significantly inhibits the growth of A549 cells by inducing cell arrest, but not by apoptosis at nanomolar concentrations which correlate with the inhibition of Na^+/K^+-ATPase [18, 19]. Furthermore, the finding that ouabain-induced inhibition of tumor growth accentuates irradiation damage led to the suggestion that ouabain may have clinical application in radiotherapy [20].

In this study we employed proteomics technology to identify proteins that change in A549 cells in response to ouabain and possibly inhibit the cancer cells. Recently, starvation-induced autophagy has been suggested to account for the growth inhibitory effect of ouabain in A549 [18]. In our study, A549 cells were treated with ouabain in the

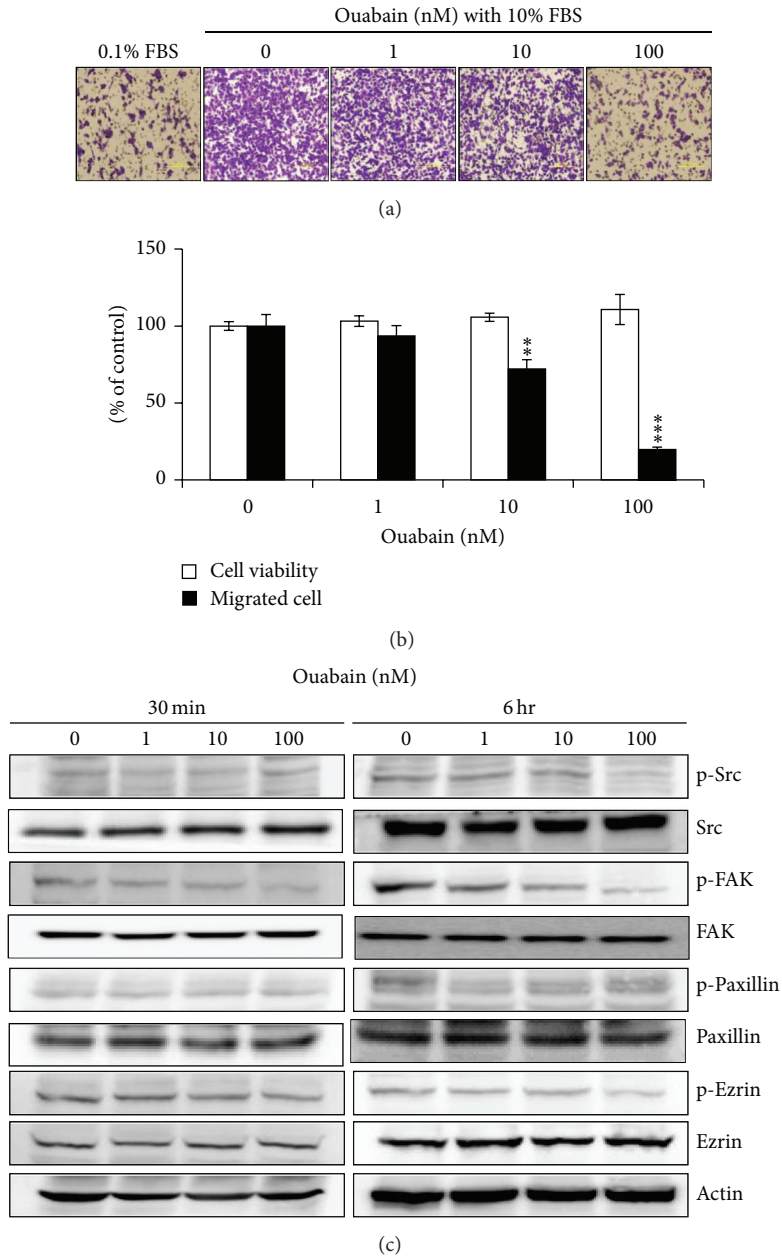


Figure 4: Effect of ouabain on migration of A549 cells. (a) In vitro migration assay was performed twice in triplicate using a 48-well Boyden chamber with a gelatin-coated polycarbonate membrane. DMEM containing either 0.1% FBS or 10% FBS was added into the bottom chamber and cells were loaded into the upper chamber and incubated at 37°C for 6 h. The cells on the upper side of the membrane were removed, and the cells on the bottom of the filter membrane were stained with Diff-Quick solution. (b) The numbers of migrated cells were counted under a light microscope. The data are presented as mean ± standard deviation ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

presence of FBS and eliminated the possibility that we may be identifying proteins regulated by cell culture conditions including starvation-induced autophagy. Among 9 proteins regulated by ouabain in A549, p-ezrin was confirmed to be regulated by ouabain in a dose-dependent manner.

Ezrin is a member of the cytoskeleton-associated protein family and is involved in a wide variety of cellular processes [21]. Importantly, ezrin has been reported to play an important role in the metastasis of lung cancers [22, 23]. The expression and clinical significance of ezrin in lung cancers have been related to phosphoezrin protein expression in tumor tissues found to be higher in precancerous tissues and in benign pneumonic tissues [24]. Levels of phosphoezrin were found to correlate with the invasiveness of tumors in several types of cancers [25]. Phosphoezrin (Y353) required for transmitting a survival signal during epithelial differentiation has been suggested to be a potent prognosis predictor for pancreatic cancer [26, 27]. In this study, we found that

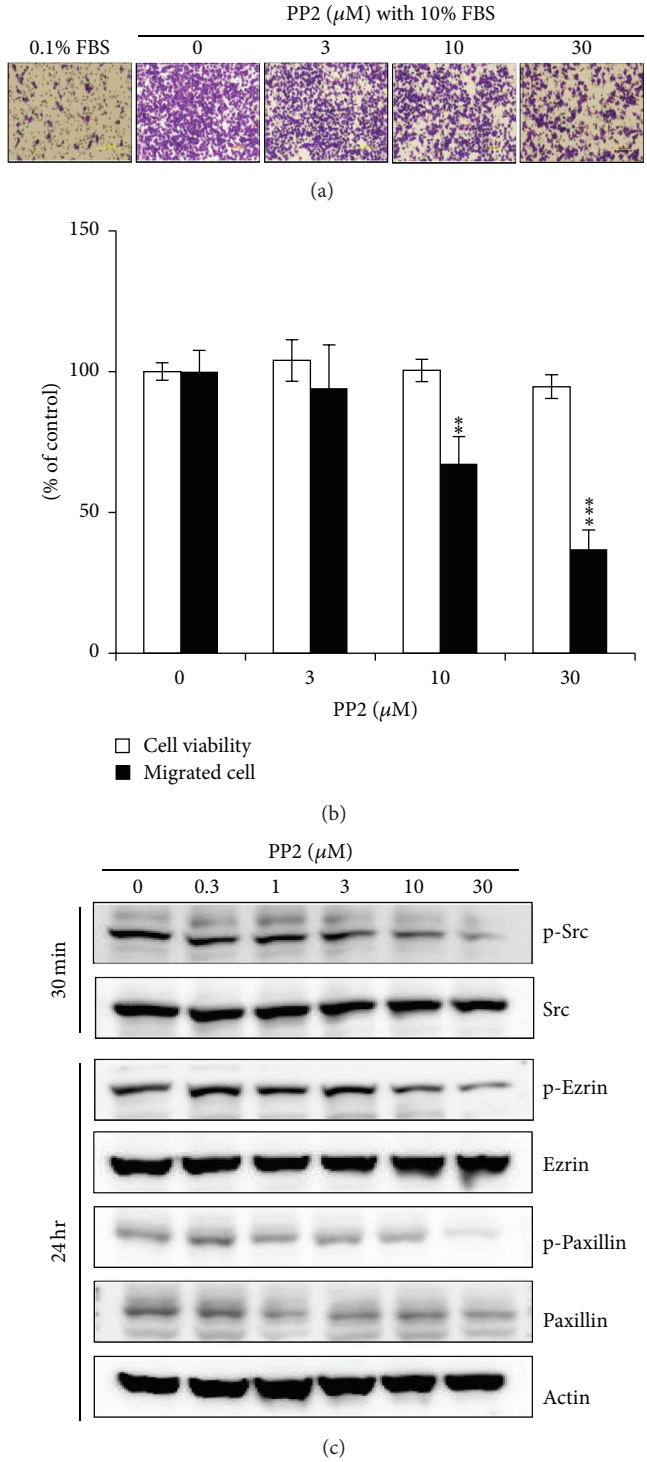


FIGURE 5: Effects of Src inhibitor, PP2, on cell migration and on the phosphorylation of ezrin and paxillin. (a) In vitro migration assay was performed twice in triplicate using a 48-well Boyden chamber with a gelatin-coated polycarbonate membrane. Serially diluted PP2 was added into the bottom chamber and cells were loaded into the upper chamber. Following incubation at 37°C for 6 h, the cells on the upper side of the membrane were removed, and the cells on the bottom of the filter membrane were stained with Diff-Quick solution. (b) The numbers of migrated cells were counted under a light microscope. The data are presented as mean ± standard deviation ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (c) Src inhibitor, PP2, was treated for indicated time and then Western blot analysis was performed as described in Materials and Methods.

ouabain dose-dependently decreased the level of phospho-ezrin (Y353).

Phosphorylation of ezrin at Y353 was found to be mediated through Src tyrosine kinase in prostate cancer [28], and it is a crucial element of Src-induced features in malignant cells [29]. The phosphorylation of Src at Y416 in the activation loop of the kinase domain upregulates the enzymatic activity of Src. Interestingly, exposure of human breast MDA-MB-435s cells to 100 nM ouabain caused rapid and transient activation of Src kinase in 5 min (but not in 15 min) and increased the coimmunoprecipitations of Src and Na^+/K^+-ATPase α subunit with epidermal growth factor receptor when ouabain was incubated for 5 min [20]. However, in this study, we found ouabain-mediated decreases of p-Src (Y416) and Na^+/K^+-ATPase subunits in A549 cells after 1-day incubation. These results suggest that ouabain transiently induces the activation of Src kinase and its binding to Na^+/K^+-ATPase, but, after this transient activation, both Src and Na^+/K^+-ATPase subunits are downregulated by long-term exposure of ouabain.

Ezrin deficiency in highly metastatic human lung carcinoma 95D cells caused the reduction of the cell migration and invasion [30], and Src-FAK signaling is known to regulate the migration of cancer cells. Furthermore, ouabain has recently been shown to inhibit migration of A549 and human lung cancer H292 cells, via suppressing FAK signaling [31, 32], and the involvement of Na^+/K^+-ATPase in the migration of cancer cells has been also reported [13, 14]. These results suggest a possible mechanism that involves ouabain-mediated inhibition of A549 cell migration via inactivation of Src-to-ezrin signaling axis.

In many tumor cells, Src forms a complex with FAK to generate signals leading to tumor growth and metastasis [30, 33]. Within this complex, Src transphosphorylates FAK within C-terminal domain (Y925) and provides a binding site for the Grb2/SH2 domain and triggers a Ras-dependent activation of MAP kinase pathway [31]. In Src-transformed cells, Ras signal transduction pathway may be constitutively activated by FAK Y925 phosphorylation. FAK is overexpressed in a variety of cancers and its overexpression in lung cancer leads to the cancer migration and invasion [32]. Additionally, the overall survival was better in FAK-negative than in FAK-positive patients with lung adenocarcinoma [30]. In this study, when A549 cells were incubated with ouabain for 30 min, p-FAK (Y925), but not p-Src, was downregulated. Furthermore, the downregulation of p-Src and p-FAK observed after 6 hr was maintained up to 24 hr.

FAK-Src complex binds to and phosphorylates various adaptor proteins such as paxillin. Here, we found that ouabain decreased the phosphorylation of paxillin (Y118), which has been shown to occur at Y118 [31]. Phosphopaxillin (Y118) can provide a docking site for recruitment of other signaling molecules to focal adhesions [34] and contribute to the control of Src-induced anchorage-independent growth by FAK and adhesion [35].

We also found that another cytoskeletal protein, p130CAS, was also downregulated by ouabain in A549 cells. p130CAS is also a substrate of FAK and plays an important role in regulating focal adhesion, driving cell migration. Recently, the overexpression of p130CAS has been observed in 61.9% of lung cancers suggesting that p130CAS may impact a variety of clinicopathological features of lung cancer and may influence the prognosis of lung cancer patients [23].

Our ongoing studies include the investigation on the ouabain-induced signaling such as Src, ezrin, FAK, and p130CAS in other lung carcinoma cells including H460 cells. To dissect the mechanism of ERM proteins in the signaling described herein, effects of local phosphorylation of ERM proteins by ouabain are worthy of investigation using the immunofluorescence experiments or phosphospecific antibodies. Also, it was reported, recently, that small molecule inhibitors of ezrin inhibit lung metastasis of ezrin-sensitive cells as well as invasive phenotype of osteosarcoma cells suggesting that novel targeted therapy that directly or indirectly inhibits the function of ezrin might be a rational approach to prevent tumor metastasis [36]. In this regard, studies on the inhibitory effects of an ezrin inhibitor, NSC668394, on the ezrin-related signaling such as Src and paxillin and on the migration of cancer cells are underway in our researches.

5. Conclusions

We showed here that the anticancer effects of ouabain in A549 cells are related to its ability to regulate ezrin, Na^+/K^+-ATPase subunits, and proteins involved in the signaling of focal adhesion such as Src, FAK, and p130CAS. Furthermore, we also proposed that a possible mechanism for ouabain-mediated inhibition of A549 cell migration is inactivation of Src-to-ezrin signaling axis. The present study suggests that molecules involved in Src-to-ezrin signaling axis offer a new target for lung cancer therapy.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Hye Kyoung Shin, Seong Hwan Kim, and Kyunglim Lee designed and directed the study. Hye Kyoung Shin, Byung Jun Ryu, and Sik-Won Choi performed the experiments. Hye Kyoung Shin, Byung Jun Ryu, Seong Hwan Kim, and Kyunglim Lee analyzed and interpreted data. Hye Kyoung Shin, Seong Hwan Kim, and Kyunglim Lee wrote the paper. All authors read and approved the final paper. Hye Kyoung Shin and Byung Jun Ryu contributed equally to this work.

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Review Article

Thyroid Hormone and P-Glycoprotein in Tumor Cells

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P-glycoprotein (P-gp; multidrug resistance pump 1, MDR1; ABCB1) is a plasma membrane efflux pump that when activated in cancer cells exports chemotherapeutic agents. Transcription of the P-gp gene (*MDR1*) and activity of the P-gp protein are known to be affected by thyroid hormone. A cell surface receptor for thyroid hormone on integrin $\alpha v\beta 3$ also binds tetraiodothyroacetic acid (tetrac), a derivative of L-thyroxine (T_4) that blocks nongenomic actions of T_4 and of 3,5,3′-triiodo-L-thyronine (T_3) at $\alpha v\beta 3$. Covalently bound to a nanoparticle, tetrac as nanotetrac acts at the integrin to increase intracellular residence time of chemotherapeutic agents such as doxorubicin and etoposide that are substrates of P-gp. This action chemosensitizes cancer cells. In this review, we examine possible molecular mechanisms for the inhibitory effect of nanotetrac on P-gp activity. Mechanisms for consideration include cancer cell acidification via action of tetrac/nanotetrac on the Na^+/H^+ exchanger (NHE1) and hormone analogue effects on calmodulin-dependent processes and on interactions of P-gp with epidermal growth factor (EGF) and osteopontin (OPN), apparently via $\alpha v\beta 3$. Intracellular acidification and decreased H^+ efflux induced by tetrac/nanotetrac via NHE1 is the most attractive explanation for the actions on P-gp and consequent increase in cancer cell retention of chemotherapeutic agent-ligands of MDR1 protein.

1. Introduction

P-glycoprotein (P-gp; multidrug resistance protein 1, MDR1; ABCB1) is a plasma membrane efflux pump with broad ligand specificity in normal cells and in cancer cells [1]. A glycoprotein ATPase is responsible in cancer cells for the outward transport of a variety of chemotherapeutic agents and thus is a critical vehicle of chemoresistance. P-gp is subject to pharmacologic inhibition with a variety of agents, for example, the calcium channel blocker, verapamil [1, 2], and tyrosine kinase inhibitors [3]. The search for effective P-gp inhibitor drugs is active [4, 5]. Thyroid hormone, L-thyroxine (T_4) or 3,3′,5-triiodo-L-thyronine (T_3), is known to induce transcription of P-glycoprotein (*MDR1*) gene [6–8] and P-gp function [8]. We have shown that a thyroid hormone antagonist, tetraiodothyroacetic acid (tetrac), acting at the thyroid hormone-tetrac receptor on plasma membrane integrin $\alpha v\beta 3$, increases the intracellular residence time of doxorubicin in chemoresistant (doxorubicin-resistant) human breast cancer cells [9]. This is an index of inhibition of P-gp activity. Thus, in cancer cells, this function of the hormone supports drug resistance, whereas in nonmalignant cells, this action of the hormone may stimulate desirable efflux of toxic substances accumulated by the cells. In this review, we examine the mechanisms by which thyroid hormone, tetrac and nanoparticulate tetrac formulation (nanotetrac) that acts exclusively at integrin $\alpha v\beta 3$, may regulate P-gp function in cancer cells. The integrin is also known to regulate P-gp by other mechanisms [10].

2. Integrin $\alpha v\beta 3$ and Nongenomic Actions of Thyroid Hormone

Integrins are structural proteins of the plasma membrane that bind extracellular matrix (ECM) proteins and are integral to

cell-cell adhesion and cell-ECM protein interactions. Among ECM protein ligands of various integrins are fibronectin, vitronectin, osteopontin (OPN), and von Willebrand factor [11]. Of more than 20 integrins, only $\alpha v\beta 3$ contains a receptor site for thyroid hormone [12]. $\alpha v\beta 3$ is amply expressed by tumor cells and rapidly dividing endothelial cells usually found supporting cancers. We have described cancer cell proliferation *in vitro* in response to T_4 and T_3 in a variety of human cells [13–15] and these hormones are proangiogenic by a variety of mechanisms [16–18]. Both actions are wholly dependent upon the hormone-tetrac receptor on integrin $\alpha v\beta 3$. Such actions of T_4 and T_3 at the integrin are termed nongenomic because they do not primarily require the interaction of nuclear thyroid hormone receptors (TRs) with T_3, the definition of the genomic mechanism of hormone action [19]. T_4 is active at the integrin and the affinity of the hormone receptor on $\alpha v\beta 3$ is higher for T_4 than for T_3; in contrast, T_4 in genomic actions is a prohormonal source of T_3 via deiodination.

Tetrac and nanotetrac inhibit binding of agonist thyroid hormone to the receptor on the ectodomain of $\alpha v\beta 3$. But, in the absence of T_4 and T_3, nanotetrac and tetrac have a set of novel proapoptotic and antiangiogenic actions [17]. These involve modulation of crosstalk between the integrin and adjacent vascular growth factor receptors, the promotion of apoptosis, and the disordering of transcription of genes important to cell survival pathways [17, 30]. Specifically, there is crosstalk between $\alpha v\beta 3$ and receptors for vascular endothelial growth factor (VEGF) [17] and epidermal growth factor (EGF) [17] that may be relevant to the $\alpha v\beta 3$-mediated effects of thyroid hormone on cellular retention of chemotherapeutic agents (see next section).

From the integrin, T_4 can also alter intracellular trafficking and state of serine phosphorylation of TRs, of estrogen receptor-α (ERα), of signal transducing and activator of transcription (STAT) proteins, and of p53 [17]. These phosphorylation steps are dependent upon mitogen-activated protein kinase (MAPK; ERK1/2) and represent an interesting adjunctive interface of nongenomic actions with genomic actions of thyroid hormone. In human lung carcinoma cells that express ERα, T_4 may be estrogen-like, supporting cell proliferation that is ER-dependent [15]. Migration of endothelial cells toward a vitronectin cue is also stimulated by T_4 via $\alpha v\beta 3$ [18]. Fibroblast migration in an *in vitro* model of wound-healing is also stimulated by T_4 at the cell surface hormone receptor (SA Mousa: unpublished observations). The state of the actin cytoskeleton is nongenomically regulated by T_4 [31, 32], in part reflecting action of the hormone to increase the amount of fibrous (F) actin from the pool of available soluble actin.

Finally, thyroid hormone can nongenomically regulate the activities of several plasma membrane transport systems, including the sodium/proton (Na^+/H^+) exchanger (NHE1) or antiporter [33, 34], Na^+, K^+-ATPase [35, 36], and the glucose transport system [37]. The action on NHE1 contributes to regulation of intracellular pH (pHi). Inhibition of this integrin-mediated effect of thyroid hormone decreases cellular pHi and may permit modulation of activity of enzymes whose pH optima are physiologic or slightly alkaline. Increased activity of NHE1 will also decrease extracellular pH (pHe), an effect that may reduce cell uptake of certain chemotherapeutic agents [21]. The plasma membrane calcium pump (Ca^{2+}-ATPase) is another ATPase whose transport activity is activated nongenomically by T_4 [38–40].

3. Possible Mechanisms by Which Tetrac and Agonist Thyroid Hormone Cause Tumor Cell Retention of Chemotherapeutic Agents

When we studied doxorubicin-resistant human breast cancer (MCF-7/dox) cells *in vitro*, we confirmed shortened intracellular residence time of labeled doxorubicin in these cells [9]. Tetrac exposure significantly increased residence time of doxorubicin in MCF-7/dox cells. The residence time of etoposide and cisplatin in neuroblastoma and osteosarcoma cell lines was also increased by tetrac. Of importance here is that doxorubicin and etoposide are P-gp substrates, whereas cisplatin is not. P-gp may influence the activities of certain apoptosis-relevant proteins such as p53 and caspase-3 and thus increase cancer cell sensitivity to agents such as cisplatin that are not P-gp substrates [41]. This indicates that tetrac may inactivate mechanisms of resistance in addition to the efflux pump. In studies we have carried out [9], we found that tetrac did not alter cellular abundance of superoxide dismutase (SOD) or glutathione-S-transferase-π (GST-π) proteins that support chemoresistance in the MCF-7/dox cell line. The P-gp protein abundance was ample in resistant cells but undetectable in wild-type MCF-7 cells. We postulated that tetrac decreased the activity of the P-gp ATPase to cause increased residence time of doxorubicin and etoposide, because agonist thyroid hormones (T_4 and T_3) nongenomically increase the activity of a variety of plasma membrane pumps—including several ATPases—and tetrac blocks nongenomic actions of T_4 and T_3, which are agonists at their receptor on $\alpha v\beta 3$.

What are the molecular mechanisms that might be modulated by tetrac to result in decreased activity of P-gp and tumor cell retention of P-gp ligands such as doxorubicin and etoposide? Tetrac will block binding of thyroid hormone to integrin $\alpha v\beta 3$ and if transcription of *MDR1* is regulated from the cell surface, as is expression of a wide variety of genes [17, 30], then this action will decrease abundance of the protein in cancer cells. Thyroid hormone does increase transcription of *MDR1* [6–8, 42]. This effect of the hormone does not involve the pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR) [42] that is usually implicated in *MDR1* gene expression, thus indicating the existence of one or more alternative pathways for regulation of *MDR1* expression. Gene expression modulation from the integrin by thyroid hormone and tetrac formulations may involve alteration of the states of phosphorylation and acetylation of certain intranuclear receptors, as well as regulation of coactivator/corepressor complex formation [17]. Thus, it is not surprising that the hormone can affect *MDR1* expression independently of PXR/SXR. Integrin $\alpha v\beta 3$ has recently been shown to affect *MDR1* expression by the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway [10] that we have implicated in a variety of actions of thyroid hormone and tetrac initiated at this integrin [17].

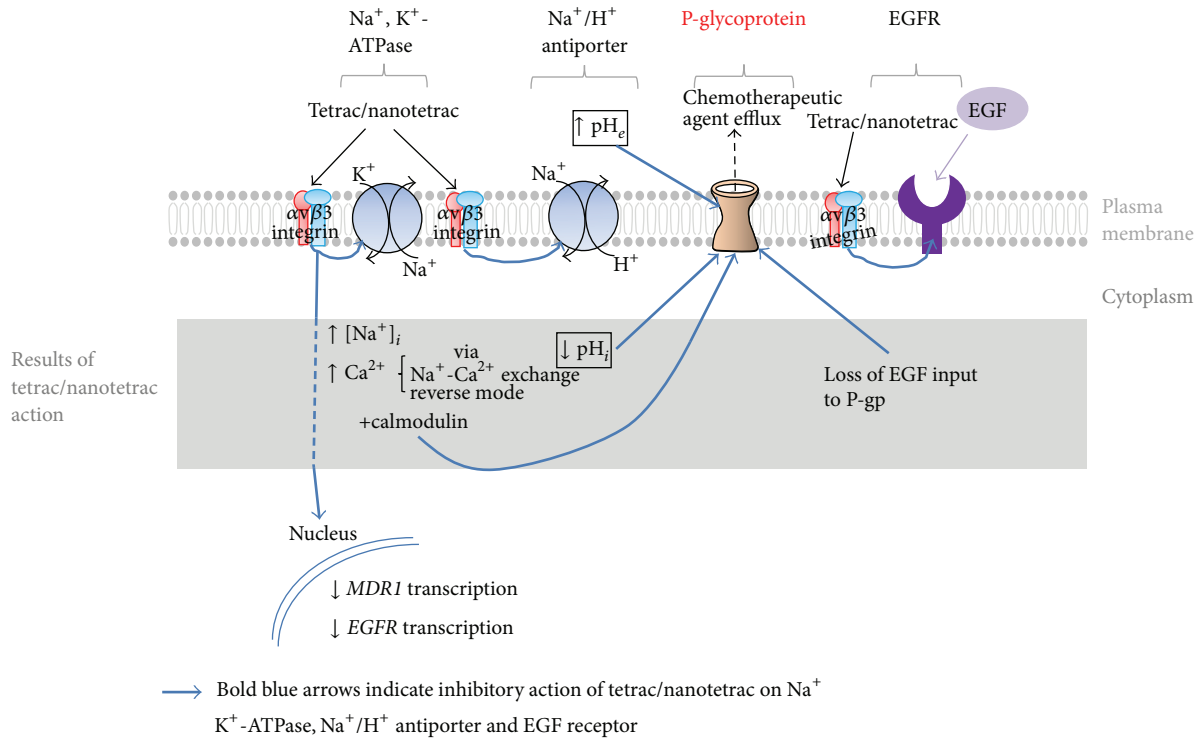

Figure 1: Schematic overview of possible mechanisms in tumor cells by which tetrac and nanotetrac may decrease function or abundance or both of P-gp. Postulated mechanisms are initiated at the thyroid hormone-tetrac receptor site on integrin $\alpha v\beta 3$ in the plasma membrane. An example shown is downregulation of the Na^+/H^+ antiporter by tetrac that results in decreased intracellular pH (pHi) and increased extracellular pH (pHe), both of which may serve to reduce P-gp function (see text). Another example is inhibition by tetrac of Na, K-ATPase, resulting in increased $[Na^+]_i$, reverse mode Na^+/Ca^{2+} exchange, and increased $[Ca^{2+}]_i$. The latter, in conjunction with calmodulin, can downregulate P-gp activity. EGF is one of several extracellular factors that supports P-gp activity. Tetrac/nanotetrac may remove any contributions of EGF to P-gp activity by disrupting function of the plasma membrane EGF receptor (EGFR) or by decreasing *EGFR* gene expression. The figure also proposes that the decreased expression of the *MDR1* gene is initiated at integrin $\alpha v\beta 3$; this possibility has not yet been explored. The figure does not include factors such as osteopontin and VEGF that are also known to regulate P-gp and whose actions might be affected by tetrac/nanotetrac. These factors are discussed in the text.

Thyroid hormone also enhances function of the P-gp protein [8], but it is not yet known whether the latter effect is nongenomic in mechanism. Another possible mechanism of tetrac action on P-gp is sustained intracellular acidification, such as that induced pharmacologically with cariporide, an NHE1 inhibitor. This results in decreased P-gp activity [20] and also causes a reduction in *MDR1* (P-gp) gene expression and *MDR1* mRNA. Thyroid hormone acutely upregulates NHE1 activity and the inhibition of this nongenomic hormonal action by tetrac may result in a significant decrease in pHi [33, 34], away from the pH optimum of the pump. In addition, a consequence of the tetrac effect on NHE1 is failure of the antiporter to support the extracellular acidosis that favors P-gp transport function [43, 44]. It is important to point out that the bovine serum-supplemented medium that cancer cells require for growth contains ample amounts of T_4 and T_3. We can conclude that one mechanism by which tetrac may downregulate activity of P-gp in tumor cells is via its $\alpha v\beta 3$-dependent action on NHE1. Recent reviews of P-gp chemistry and conceptual approaches to the inhibition of efflux pump activity have not considered acidification of P-gp-containing cells [1, 4, 5] as a strategy. This omission presumably reflects an assumption that pharmacologic acidification will affect normal cells, as well as tumor cells. This need not be the case when the pharmacologic initiation site is a protein such as integrin $\alpha v\beta 3$ whose expression/activation is primarily by tumor cells and rapidly dividing endothelial cells. A summary of molecular mechanisms by which tetrac and nanotetrac may affect P-gp function or abundance is presented in Figure 1.

As noted above, thyroid hormone action at $\alpha v\beta 3$ may also regulate activity of Na, K-ATPase. A direct influence of change in $[Na^+]_i$ or $[K^+]_i$ on P-gp activity is not proposed, but inhibition by tetrac of the sodium pump will result in increased intracellular $[Na^+]$ and decreased $[K^+]$. It is not known whether a specific change in intracellular $[K^+]$ or $[Na^+]$ affects P-gp, but inhibition by ouabain of Na, K-ATPase increases P-gp (MDR1) mRNA [45], suggesting that the monovalent cation microenvironment may directly or indirectly affect P-gp protein abundance. An indirect mechanism

would be the effect of increased $[Na^+]_i$ to increase $[Ca^{2+}]_i$ by activation of the Na^+-Ca^{2+} exchange in reverse mode [46], a factor that is relative to the discussion below of calmodulin.

Epidermal growth factor (EGF) can increase efflux activity of P-gp [25], apparently by phospholipase C-dependent phosphorylation of the pump. We have found that agonist thyroid hormone can enhance the biochemical activity of EGF [47, 48] and that tetrac blocks the capacity of thyroid hormone to potentiate EGF actions on signal transducing kinases. Thus, we expect tetrac to be capable of modifying the action of EGF on P-gp, favoring chemosensitivity. This possibility has not been experimentally tested. It is also important to note that transcription of the EGF receptor (*EGFR*) gene is inhibited by nanoparticulate tetrac [17, 30], so that the trophic effect of endogenous EGF on P-gp is unlikely to be manifested in the presence of nanotetrac.

VEGF can acutely decrease activity of P-gp, without a change in number of pumps/cell [26]. This is an interesting observation, indicating that anti-VEGF clinical strategies could increase chemoresistance of cancer cells. The action of VEGF on P-gp is decreased by nocodazole, an inhibitor of microtubule polymerization, suggesting that P-gp internalization or orientation might contribute to its efflux activity [49]. Tetrac and its nanoparticulate formulation are potent antagonists of VEGF actions by multiple pathways [16, 17]. Bevacizumab and aflibercept are VEGF-directed, clinical antiangiogenic agents that also relieve P-gp from VEGF-imposed inhibition. However, the inhibitory effect of VEGF is Src kinase-requiring [26] and tetrac is known to downregulate this kinase via the thyroid hormone/tetrac receptor on $\alpha v\beta 3$ [50]. Against this background, we may speculate that agonist thyroid hormone (T_4 or T_3) may support the action of VEGF on P-gp activity and chemoresistance, whereas tetrac and nanotetrac will oppose the effect.

OPN also increases cellular abundance of P-gp mRNA [24]. It does so via its interaction with the ectodomain of integrin $\alpha v\beta 3$. A clinical study has demonstrated that thyroid hormone increases OPN production [51]. Thus, it is possible that the thyroid hormone effect on P-gp may also have a contribution from increased availability of OPN for interaction with $\alpha v\beta 3$ that is thyroid hormone-directed. Hypoxia is another factor that serves to upregulate *OPN* gene expression [24] and thus may enhance chemoresistance.

Thyroid hormone (T_3) increases expression of the hypoxia-inducible factor 1-α (*HIF-1α*) gene via $\alpha v\beta 3$ [50]; the *HIF-1α* gene product increases transcription of the P-gp gene [23, 52, 53]. The action of T_3 on HIF-1α abundance is inhibited by tetrac [50]. Thus, thyroid hormone analogues may act on P-gp gene expression by more than one mechanism including the T_3-nuclear thyroid hormone receptor (TR) pathway [8, 54] and also through control of HIF-1α production that begins nongenomically for T_3 at $\alpha v\beta 3$.

A contribution of intracellular $[Ca^{2+}]$ to the function of P-gp is inferred by the effect of verapamil to decrease efflux pump activity. However, it is not clear that this action of verapamil relates to its prototypic calcium channel effects, since certain other channel blockers may not inhibit P-gp but can affect the multidrug resistance state of cells [55]. Thyroid hormone is a regulator of $[Ca^{2+}]_i$ via hormonal actions on plasma membrane Ca^{2+}-ATPase ("calcium pump") [39, 40]. This effect of thyroid hormone is dependent upon calmodulin. Verapamil has been shown by us to block the stimulatory effect of T_4 on the calcium pump by interfering with the interaction of calmodulin with the ATPase [39]. Calmodulin is involved in control of P-gp activity through calmodulin-dependent kinase II activity [27, 28]. Thus, the conventional experimental use of verapamil to inhibit the P-gp axis may extend to calmodulin-relevant thyroid hormone actions that are linked to the efflux pump. It is not clear whether $[Ca^{2+}]_i$ has roles in modulation of P-gp activity or the actions of tetrac/nanotetrac on the efflux pump, beyond generation of calmodulin-Ca^{2+} complexes.

A mechanism does exist by which agonist thyroid hormone (T_4 or T_3) might decrease cell P-gp activity, as tetrac appears to do via $\alpha v\beta 3$. The hormone induces cellular reactive oxygen species (ROS) generation [56, 57] and this may reduce P-gp [58, 59]. One of the coauthors of the present paper (S Incerpi) has shown that integrin $\alpha v\beta 3$ is not involved in T_3-directed generation of ROS in hepatocytes [57]. Control thyroid hormone-containing (FBS-supplemented) culture medium for tumor cells does not increase intracellular residence time of chemotherapeutic agents [9] that is clearly seen with exposure of cells to tetrac.

4. Discussion

The observation that tetrac/nanotetrac can chemosensitize tumor cells previously resistant to agents such as doxorubicin and etoposide [9] caused us to undertake the present review of molecular mechanisms that may be the basis for actions of tetrac/nanotetrac on P-gp. Tetrac/nanotetrac oppose the nongenomic actions of T_4 and T_3 at plasma membrane integrin $\alpha v\beta 3$ that regulate a variety of plasma membrane transport systems—such as the Na^+/H^+ antiporter, Na, K-ATPase, and Ca^{2+}-ATPase [60]—that may be relevant to P-gp activity or to transcription of the *MDR1* (P-gp) gene. Further, integrin $\alpha v\beta 3$ interacts with OPN and with the VEGF/VEGFR axis, offering opportunities for thyroid hormone analogues to modulate the influence of OPN and VEGF on P-gp. Table 1 summarizes a group of factors that modulate P-gp action and may be contributors to the increased intratumor intracellular residence time of chemotherapeutic agents in tetrac/nanotetrac-exposed tumor cells.

The most obvious molecular mechanism that contributes to the apparent effect(s) of tetrac/nanotetrac on P-gp is the action of these hormone analogues on intracellular pH. Tetrac acidifies cells by inhibiting the Na^+/H^+ exchanger and the P-gp efflux pump is arrested by an acid intracellular environment. Here, the importance of the generous expression of the agent's target—integrin $\alpha v\beta 3$ with the tetrac receptor—on cancer cells is critical, so that conventional and necessary activity of MDR1 in nonmalignant tissues is unimpaired in the presence of nanotetrac. Unmodified tetrac is unsatisfactory for cancer management because in the intact organism it is taken up by normal cells, as are T_4 and T_3. Within the normal cell, unmodified tetrac is a low-potency thyromimetic that can promote hypermetabolism.

Table 1: Selected intra- and extracellular factors that affect activity and/or abundance of P-glycoprotein (P-gp; MDR1).

Factor	P-gp activity	P-gp abundance	Reference
Intracellular pH (pHi)	↓	↓	[20]
Extracellular pH (pHe)	↑	NS	[21]
Hypoxia	↑	↑	[22]
Hypoxia-inducible factor 1-α (HIF-1α)	NC	↑	[23]
Thyroid hormone/analogues			
T_4, T_3	↑	↑	[6]
Tetrac/nanotetrac	↓	NC	[9]
Osteopontin (OPN)	↓	↓	[24]
Epidermal growth factor (EGF)	↑	NS	[25]
Vascular endothelial growth factor (VEGF)	↓	NC	[26]
Calcium channel blockers	↓	↓	[2]
Ouabain	NS	↑	[27]
Calmodulin antagonists E6, EBB	↓	NS	[28, 29]

T_4: L-thyroxine.
T_3: 3,5,3′-triiodo-L-thyronine.
NC: no change in parameter.
NS: parameter not investigated/recorded.
A variety of additional pharmacologic inhibitors of P-gp are reviewed in [3–5].

The plasma membrane sodium pump and calcium pump are also regulated nongenomically by thyroid hormone. Inhibition of such nongenomic actions of thyroid hormone at $\alpha v\beta 3$ by nanotetrac would serve to increase $[Na^+]_i$ and $[Ca^{2+}]_i$. Such changes are not known to directly affect P-gp, although calmodulin-Ca^{2+} complexes are involved in calmodulin kinase-mediated effects that serve to increase P-gap activity, as mentioned above.

It is also apparent that P-gp and thyroid hormone analogues share mechanistic interests in a diverse set of protein molecules. As noted above, thyroid hormone increases transcription of the *OPN* gene and the OPN protein activates P-gp. Thus, in the clinical setting, host T_3 (and T_4 as a prohormone for T_3) that acts via nuclear TR may support chemoresistance via P-gp. Nanotetrac is unlikely to affect P-gp via OPN because actions of nanotetrac are limited to $\alpha v\beta 3$ and do not directly involve TR [17].

In contrast, EGF stimulates P-gp activity [25] and we have shown that, acting at the cell surface, thyroid hormone can potentiate certain effects of EGF [47]. Acting nongenomically, tetrac can inhibit agonist thyroid hormone action on EGF. Thus, a component of the prolongation of intracellular residence time of certain chemotherapeutic agents in nanotetrac-exposed cancer cells may be due to blockade of the action of T_4 at the EGF receptor.

Recent reviews of regulation of P-gp [1, 4, 5, 61] endorse the search for new approaches to the efflux pump that are suitable for application to clinical chemoresistance. New approaches are facilitated by characterization of previously unrecognized control mechanisms for P-gp. We point out here that integrin $\alpha v\beta 3$ offers access to multiple regulatory pathways for MDR1 that may be suitable for pharmacological exploration. We have emphasized in this review the potential usefulness of the cell surface receptor on $\alpha v\beta 3$ for thyroid hormone and tetrac/nanotetrac as a regulator of P-gp. However, the specific interactions of the integrin with extracellular matrix proteins, for example, OPN or growth factors, and existence on the integrin of other small molecule receptor sites offer new opportunities to modulate efflux pump activity.

Finally, it is interesting to note two additional interactions of thyroid hormone and P-gp. First, the export of the hormone from cells is a P-gp-mediated, verapamil-inhibitable process [62, 63] and thus to the extent that thyroid hormone may increase P-gp activity—or nanotetrac may inhibit such activity—intracellular hormone levels may be affected. For purposes of efflux, the hormone is a ligand of P-gp, but regulation by the hormone of P-gp activity is likely to originate at integrin $\alpha v\beta 3$ and involve intermediary kinases implicated in transporter control [41]. Second, the extensive intracellular trafficking of P-gp among compartments is actin-dependent [64]. The integrity of the actin cytoskeleton and maintenance of F-actin is in part T_4-regulated [31, 32]. The nongenomic actions of thyroid hormone on intracellular protein trafficking are reviewed elsewhere [17, 65].

In summary, P-glycoprotein (MDR1; ABCB1) is a ubiquitous plasma membrane efflux pump capable of exporting specific pharmacologic agents. In tumor cells, P-gp substrates include chemotherapeutic agents such as doxorubicin, etoposide, and trichostatin A. Thyroid hormone is known to stimulate expression of the *MDR1* gene and activity of P-gp and thus may be seen to support chemoresistance. Tetrac is a thyroid hormone antagonist at the thyroid hormone-tetrac receptor on cell surface integrin $\alpha v\beta 3$ and exposure of tumor cells to tetrac desirably increases retention time of the cancer chemotherapeutic agents that are known substrates of P-gp. A variety of molecular mechanisms are reviewed here by which thyroid hormone/tetrac may influence P-gp

activity. Of interest is that cisplatin is not a substrate of P-gp, yet we have shown elsewhere [9] that its intracellular residence time is also increased by tetrac, raising the possibility of multiple mechanisms by which tetrac affects tumor cell handling of anticancer drugs. For example, tetrac may increase the activity of the organic cation transporter (OCT) [66] that imports (rather than exports) cisplatin, perhaps by inducing intracellular acidosis via the Na^+/H^+ antiporter, as discussed above. Thus, "intracellular residence time" of drugs in response to tetrac may reflect decreased P-gp efflux or, possibly, increased cationic transporter influx. The latter mechanism has not yet been examined.

Conflict of Interests

Coauthors Paul J. Davis and Shaker A. Mousa hold U.S. patents on nanoparticulate tetrac (nanotetrac). They receive no remuneration from ownership of these patents. The other coauthors have no conflict of interests regarding the publication of this paper.

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Review Article

New Insights into Glomerular Parietal Epithelial Cell Activation and Its Signaling Pathways in Glomerular Diseases

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The glomerular parietal epithelial cells (PECs) have aroused an increasing attention recently. The proliferation of PECs is the main feature of crescentic glomerulonephritis; besides that, in the past decade, PEC activation has been identified in several types of noninflammatory glomerulonephropathies, such as focal segmental glomerulosclerosis, diabetic glomerulopathy, and membranous nephropathy. The pathogenesis of PEC activation is poorly understood; however, a few studies delicately elucidate the potential mechanisms and signaling pathways implicated in these processes. In this review we will focus on the latest observations and concepts about PEC activation in glomerular diseases and the newest identified signaling pathways in PEC activation.

1. Introduction

The glomerulus comprises four types of intrinsic cells including endothelial cells, mesangial cells, podocytes, and parietal epithelial cells (PECs). There are numerous studies focused on the biological functions and pathogenic roles of the first three cells, whereas PEC, which lines along Bowman's capsule, until recently has aroused scientific interest leading to the exploration of its physiological and pathological effects especially in several forms of glomerular diseases, such as crescentic glomerulonephritis (CGN), focal segmental glomerulosclerosis (FSGS), and diabetic nephropathy (DN).

Quiescent PECs are very flat and inconspicuous; their cell body size ranges from 0.1 to 0.3 μm in thickness, increasing to 2.0~3.5 μm at the nucleus. Transmission electron microscopic studies indicate PECs have a "labyrinth-like" outlook with junctions present between adjacent PECs. At the glomerular urinary pole, PECs develop junctions with proximal tubular cells, while, in the vascular pole, PECs transit into podocytes [1].

Comparing to other kidney resident cells, there are a few universally accepted concepts about the physiologic functions of PECs. Several best evidence-based researches suggest they act as a selective permeability barrier to urinary filtrate [2]. Moreover, in albumin overload state, PECs can uptake albumin likely by endocytosis which eventually leads to the injury of PECs [3]. In addition, PECs may serve mechanosensing and contractile functions through their primary cilia which are constantly exposed to the urine flow from the glomerular filtrate [4].

Recently Shankland et al. [5] published an elegant review about the emerging concepts of PECs. In this review we summarize PECs activation in several kinds of glomerular diseases, particularly the signaling pathways implicated in PECs activation.

2. Parietal Epithelial Cell (PEC) Activation in Glomerular Disease

Currently, no well-defined glomerular disease is predominantly caused by abnormalities arising in PECs, unlike other glomerular diseases that occur mainly due to the injury of certain intrinsic glomerular cells, such as podocytes (minimal change disease and focal segmental glomerulosclerosis),

Table 1: Glomerular parietal epithelial cells activation in glomerular disease.

	Reference	
	Human	Animal model
CGN	[14]	[9, 14, 19]
FSGS	[14, 17, 26]	[17, 27]
DN	[29, 30]	[31, 32]

CGN: crescentic glomerulonephritis; FSGS: focal segmental glomerulosclerosis; DN: diabetic nephropathy.

mesangial cells (IgA nephropathy), and glomerular endothelial cells (thrombotic microangiopathies and pauci-immune glomerulonephritis) [6]. Nonetheless, emerging data suggest that PECs are directly involved in the pathogenesis of certain glomerular disease entities, which are featured by increased cellular activity of PECs. Activated PECs have enlarged nuclei and increased cuboidal cytoplasm, and occasionally cytoplasma vacuolation and protein resorption droplets are observed [2, 7–9]. Besides morphological alterations, CD44, phosphorylated extracellular signal regulated kinase, and several molecules are considered to be the specific markers for activated PECs [7]. After activation, the biological properties of PECs are changed, presenting as increased proliferation, migration, or extracellular matrix production [1].

The precise role of PECs in disease states remains unclear; it might be potentially detrimental or beneficial in glomerular diseases. On the one hand, the overgrown PECs could obstruct the urine flow and release chemokines and cytokines, which could impair the function of the affected glomeruli. On the other hand, several studies have suggested that PECs could perhaps migrate from Bowman's capsule to the capillary tuft and differentiate into podocytes in response to injury [10, 11]. In the latter circumstance, PECs serve a reparative and regenerative role when podocytes are lost. Several studies have shown the involvement of the activation of PECs in various glomerular diseases in humans and mouse models (Table 1).

2.1. PEC Activation in Crescentic Glomerulonephritis. CGN is the best-characterized disease in which PECs are the major culprits. Cellular crescent is the typical morphological change observed in CGN. It is defined as the multilayered accumulations of PECs and other cell types within Bowman's space. Consequently it occludes the urinary outlet and the flow of the primary urine, and later the implicated nephron is impaired.

No consensus has been reached with regard to the cell types inside the classic crescent, due to the fact that crescentic lesions often stain positive for both PEC and podocyte markers [9, 12]. However, at least three cell types contribute to the cellular components of classic crescents, either individually or in combination. Firstly, numerous studies have shown that PECs are the predominant cells in cellular crescents. Ultrastructural studies performed in the 1970s showed that crescents were largely composed of PECs and to a lesser extent podocytes [13]. In addition immunohistological staining confirmed that cellular crescents mainly expressed PECs markers, for example, CD24, glycCD133, and claudin-1 in humans and cytokeratin and UCH-L1 in rats [14]. More importantly, genetic cell-fate tracking studies elegantly demonstrated that PECs were the predominant source of cellular crescents in mice [9]. Secondly, under specific circumstances, podocytes contribute to cellular crescent formation to a much less extent in human diseases [12, 15] and animal model [16, 17]. For the latter, it is especially dependent on the experimental setting, such as the use of different anti-GBM serum to induce disease. Lastly, infiltrating macrophages have also been implicated as the potential origin of cellular crescents [18]. All in all, PECs are the predominant components of cellular crescents although they are likely to have multicellular origin.

PECs present in cellular crescents undergo epithelial-to-mesenchymal transition (EMT) potentially due to the deposition of extracellular matrix (ECM) proteins. The accumulation of ECM proteins results in the development of fibrocellular crescents. EMT in PECs is characterized by the loss of epithelial polarity and increased extracellular matrix synthesis [19], which eventually generates classical honeycomb-like lesions.

Undoubtedly, PECs are the main players in CGN, but they do not etiologically account for this pathogenic abnormality. Rather, glomerular tuft necrosis caused by vascular injury is the driving force for PECs proliferation, crescent formation, and consequent renal impairment. PECs usually settle in an environment without plasma exposure; nonetheless, when the glomerular capillary wall is ruptured the concentration of plasma within Bowman's space is increased around 20–40%. Amounting evidence proposes that plasma leakage initiates PECs activation and crescent development. The components from plasma markedly promote murine and human PECs hyperplasia in culture [20]. The plasma gradients that account for PECs activation have not been fully identified, but to date there is consistent data which shows fibrinogen, a member of the activated coagulation cascade, to be a driver for PECs activation. Accordingly, in several rodent models crescents formation was prevented in the absence of fibrinogen [20, 21].

2.2. PEC Activation in Noncrescentic Glomerulonephritis. CGN is frequently accompanied with inflammatory and necrotizing processes; thus, it is believed that inflammatory component is the main driving force for crescent formation. However, Sicking et al. [22] demonstrated that partial transgenic depletion of PECs was sufficient to trigger the activation of the remaining PECs population; eventually, these cells filled Bowman's capsule and formed cellular crescents. This suggests that cellular crescents might be developed without primary inflammatory stimulus. Consistently, crescent formation was noted in some patients with noninflammatory glomerular diseases including FSGS, DN, and membranous nephropathy (MN).

2.2.1. PEC Activation in Focal Segmental Glomerulosclerosis. Collapsing variant of focal segmental glomerulosclerosis (cFSGS) is morphologically featured by segmental to global

collapse of the capillary tuft with dramatic hyperplasia within Bowman's capsule, often termed pseudocrescents.

However, the origin of the proliferating cells in cFSGS is a debating issue. The traditional opinion believes that the overgrown cells stem from podocytes without podocyte markers due to the fact that they are dedifferentiated or dysregulated podocytes and they reentered the cell cycle to mitosis [23–25]. However in human HIV and pamidronate-associated cFSGS, the PECs markers were obviously expressed in proliferating epithelial cells within Bowman's capsule [26]. In addition, several nicely designed experiments support that hyperplastic epithelial cells are originated from PECs. The cFSGS mice model, established by cell cycle inhibitor p21 knockout, manifested progressive loss of podocytes with collapsed capillary loop and hyperplastic epithelial cells which were negative for WT-1 and nestin (both podocyte specific markers) but positive for PECs markers [27]. Podocyte lineage tracing by genetic tagging in p21 knockout mice proved that proliferating cells within Bowman's space were not podocyte-derived whereas they basically expressed PECs markers [27]. This study strongly suggests that,in cFSGS, proliferating epithelial cells originate from PECs rather than from hyperplastic podocytes *de novo* expressing the PECs phenotype. Dysregulated mitosis and cell cycle may account for the pathogenesis of cFSGS.

In other variants of FSGS the initial pathologic step is regularly the adhesion formation between the glomerular capillary tuft and Bowman's capsule caused by the synechia of the denuded glomerular basement membrane (GBM) to the PECs [28]. The naked GBM is the consequence of the impairment or loss of podocytes. Nonetheless, the role of PECs in the above process is not completely understood. It is not clear if the migration of the activated PECs along the glomerular tuft is limited to the region where podocytes have already been lost or if the invasion of PECs is essentially involved in the injury and loss of podocytes or both. In FSGS patients and three different animal models (5/6-nephrectomy plus DOCA-salt, the transgenic chronic Thy1.1 mice, and the MWF rat), Moeller et al. [17] found that the primary insult which triggered FSGS was associated with PEC activation and cellular adhesions to the capillary tuft. In more detail, activated PECs invaded the engaged capillary tuft and deposited extracellular matrix, and then podocytes were lost and mesangial sclerosis developed. Activated PECs were observed on the tuft where the podocyte marker remained. Therefore, it proposed that activated PECs may impair podocytes and thereby contribute to the initiation and progression of the disease, not just an innocent victim.

2.2.2. PEC Activation in Diabetic Nephropathy (DN). Activated PECs can sometimes be observed in patients with DN, especially in advanced stage, in our own opinion and also as reported by several other investigators [29, 30]. Occasionally, the proliferation of PECs is prominent with pseudocrescent formation.

In $BTBR^{ob/ob}$ diabetic mice, replacement of leptin restored the number and density of podocytes, accompanied with evident proliferation of PECs. Concomitantly, proteinuria and other morphologic abnormalities were significantly reversed. The authors also proposed that glomerular PECs could function as a progenitor cell niche for podocytes and under proper settings could proliferate and transdifferentiate into podocytes, which may be a pivotal factor for the regression of DN [31]. In another DN animal model, activated and proliferating PECs were observed and associated with overexpressed kidney injury molecule 1 which positively correlated with the extent of proteinuria and podocytopenia in diabetes [32]. Therefore, the pathophysiologic effect of activated PECs on DN remains controversial, probably dependent on the setting of the experiments.

The mechanism of PEC activation in DN is unclear. To our understanding, the endothelium is severely injured in the late phase of diabetic status, which leads to leakage of plasma and consequently induces PEC activation and pseudocrescent formation, partially resembling the mechanisms which account for cellular crescents development in inflammatory CGN.

2.2.3. PEC Activation in Other Glomerular Diseases. Occasionally PEC activation and pseudocrescents formation are presented in membranous nephropathy, progressive glomerulosclerosis, and thrombotic microangiopathy, among others [33, 34]. The mechanisms are not known currently; the activation of PEC may similarly be triggered by the injury of podocyte or endothelium and the seepage of plasma.

3. Signaling Pathways Involved in PEC Activation

The signaling pathways that mediate PEC activation are only partially comprehended; however, more and more related observations are emerging. Getting a closer look at them will offer us practical clues for clinical treatment. We summarize the major signaling pathways related to PECs activation in Table 2.

3.1. Notch Signaling Pathway in PEC Activation. Notch is a single-transmembrane protein. The family of Notch receptors is evolutionarily conserved through worms to humans [35]. Mammals have four types of Notch receptors (Notch1-4) and five identified Notch ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2). Each of them shows a cell and tissue-specific expression. Upon ligand binding a cascade of proteolytic cleavage events of the Notch receptor is initiated. Firstly it is lysed by disintegrin and metalloproteinase and then cleaved by the γ-secretase complex. The final cleavage product, the Notch intracellular domain, a transcription factor, binds to other transcriptional factors and induces the synthesis of Notch target genes such as *Hes* and *Hey* [36–38].

Notch signaling controls cell differentiation in diverse organ systems and also during kidney development. It is detected transiently in prospected podocytes and PECs and is essential for glomerulogenesis [39, 40]. Once glomerular development is completed, Notch activity is significantly decreased; upregulated glomerular Notch activity has been

Table 2: Signaling pathways involved in glomerular parietal epithelial cells activation.

	Human	Animal model
Notch	FSGS, DN [46]	Collapsing FSGS transgenic mice model [44], FSGS SCID mice model [46]
Wnt/β-catenin	N/A	Conditional β-catenin −/− mice [57]
HB-EGF/EGFR	CGN, FSGS [60, 61]	Anti-GBM induced CGN mice model [62]
CXCR4/SDF-1	CGN [63]	MWF rat with CGN [64], VHL −/− mice [65]
Ang II//AT1 receptor	Proliferative CGN [64]	MWF rat with CGN [75]
LAT2	N/A	Anti-GBM induced CGN rat model [78]
SSeCKS/cyclin D	N/A	SSeCKS −/− mice [82]

identified in patients with HIV nephropathy, FSGS, systemic lupus erythematous, and diabetes. These findings were also identified in animal models with different kinds of glomerular diseases [41, 42].

In rat PAN nephropathy increased Notch1 expression in podocytes was associated with apoptosis and proteinuria [43]. Nonetheless, in cFSGS transgenic mouse model, upregulated Notch1 was predominant in PECs accompanied by its apoptosis. On the other hand, this finding was not observed in podocytes [44]. Inhibition of Notch signaling markedly reduced PEC hyperplasia in cFSGS mice; conversely, proteinuria and renal morphologic alteration obviously deteriorated which underlines the potential beneficial effects of PEC activation in the setting of advanced podocyte loss. It is well known that the Notch pathway plays pivotal roles in cell migration and phenotypic transformation. For example, inhibition Notch pathway delayed wound healing by preventing cell migration in a skin scratch-scarring model [45]. Similarly, in cultured PECs, Notch inhibition suppressed its migration and mesenchymal phenotypic transition which suggests that Notch-mediated mesenchymal phenotypic alteration and cell migration may compensate for the loss of podocytes.

In addition, a strong upregulation of Notch3 was observed in $CD24^+CD133^+$ PECs in patients with lupus nephritis and FSGS by Lasagni et al. [46]. Blocking the Notch signaling in FSGS model established in SCID mice injected with adriamycin ameliorated proteinuria and prevented podocyte loss in the early stage (7 days) of glomerular insult; however, it hampered $CD24^+CD133^+$ PECs proliferation during the later reparative stage with exacerbating proteinuria and glomerulosclerosis [46]. These observations raise the possibility that the degree of glomerular injury depends on the Notch-mediated balance between podocyte death and renewal offered by PECs.

The trigger of the Notch signaling pathway in PECs in response to podocyte loss requires further investigation although it is known that TGF-β is a candidate [44]. Properly temporal and spatial modulation of Notch expression may provide an ideal strategy for glomerular diseases.

3.2. Wnt/β-Catenin Signaling Pathway in PEC Activation. Wnt/β-catenin signaling is an evolutionarily conserved and a multifunctional pathway that regulates cell proliferation and differentiation, angiogenesis, inflammation, and fibrosis. The canonical Wnt pathway through a complicated cascade of reactions prevents transcription factor β-catenin degradation and promotes its translocation and accumulation in the nucleus. In the nucleus, β-catenin modulates the transcription of Wnt target genes, including genes encoding cyclin D1, VEGF, c-Myc, and CTGF [47, 48].

In kidney organogenesis, the Wnt/β-catenin signaling controls both nephrogenesis and ureteric bud development [49, 50]. The Wnt pathway is also involved in the pathogenesis of renal cell carcinoma and Wilms' tumor [51, 52]. The connection of the Wnt signaling pathway with glomerular diseases, cystic kidney diseases, acute renal failure, renal fibrosis, and kidney cancers has been identified and highly concerned [53, 54].

The canonical Wnt/β-catenin signaling pathway is specifically involved in podocyte abnormality and proteinuria. Conditional depletion of the β-catenin gene in podocytes or the pharmacological inhibition of β-catenin by paricalcitol protected mice against proteinuria after adriamycin injection [55, 56]. On the other hand, activation of β-catenin by lithium chloride induced proteinuria in mice [55].

As to PECs, Wnt/β-catenin activity is indispensable for its lineage specification during the late stages of nephrogenesis which was demonstrated in conditional β-catenin knockout mice [57]. Recently it was recognized that developmental pathways are reactivated in injured glomeruli, including the Wnt pathway which plays pivotal roles in the regeneration and repair process. Susztak group found that the Wnt/β-catenin pathway modulated podocyte versus PEC marker expression. Increased Wnt/β-catenin signaling resulted in the loss of podocyte differentiation markers and the upregulation of PECs specific markers, whereas deletion of β-catenin promoted the expression of podocyte markers podocin and WT1 [58, 59]. Therefore Wnt/β-catenin signaling likely implicates the transition from PECs to podocytes; however, further convincing evidences are required.

3.3. HB-EGF/EGFR Signaling Pathway in PEC Activation. PECs and podocytes *de novo* express heparin-binding epidermal growth factor-like growth factor (HB-EGF) exclusively in human CGN and to a less extent in cFSGS or within the synechia lesion of FSGS [60, 61]. In contrast normal glomerular PECs do not express HB-EGF and this abnormality is not identified in other types of glomerulopathies. One of the receptors for HB-EGF, the EGF receptor (EGFR), is also expressed by PECs and podocytes. In a mouse model of

CGN, HB-EGF deficiency and genetic deletion of the HB-EGF alleles or tetracycline-inducible conditional depletion of the EGFR gene in podocytes significantly attenuated CGN development and improved survival rate [62]. This finding indicates that the HB-EGF/EGFR pathway plays an indispensable role in the induction and progression of PEC activation and crescents formation. Targeting HB-EGF/EGFR signaling pathway is a very promising therapeutic approach for CGN.

3.4. CXCR4/SDF-1 Signaling Pathway in PEC Activation. $CD133^{+}CD24^{+}$ PECs in normal human kidney have been proved to express CXCR4 although it is scarce. However, in patients with CGN the expression of CXCR4 was dramatically enhanced, particularly in the hyperplastic lesions comprised predominantly of PECs. CXCR4 overexpression in PECs was accompanied by upregulation of its ligand, stromal cell-derived factor 1 (SDF-1), in podocytes. In contrast, membranous nephropathy and DN patients with PECs proliferation show only very weak CXCR4 expression [63, 64]. It seems increased CXCR4 expression in PECs is exclusive in proliferative and inflammatory glomerular diseases.

Consistently, Ding et al. [65] reported that the CXCR4/SDF-1 axis was implicated in the interaction and activation of PECs and podocytes in the CGN mice model which is established by von Hippel-Lindaugene deletion in podocytes.

In CGN, the immune complex activates the humoral and cellular immune systems and recruits phlogogenic neutrophils and monocytes/macrophages to the glomerular tuft [66, 67]. The phlogogenic neutrophils can also be primed by anti-neutrophil cytoplasmic antibodies. The inflammatory cells infiltrating the capillary tuft release soluble cytokines and chemokines that penetrate into Bowman's space, ultimately triggering the expression of adhesion molecules and chemokine receptors such as CXCR4 on PECs [68, 69].

3.5. Ang II/AT1 Receptor Pathway in PEC Activation. Local production of angiotensin II (Ang II), the main component of the renin-angiotensin system, is upregulated in glomerular disease with proteinuria [70]. Inflammatory cells can release enzymes that produce Ang II, including angiotensin-converting enzyme (ACE) from monocytes/macrophages [71, 72] and cathepsin G in neutrophils [73]. Activation of angiotensin II type 1 (AT1) receptor by Ang II generates cytokines, chemokines, reactive oxygen species, and adhesion molecules which further maintain the inflammatory state.

Activation of the Ang II/AT1 receptor pathway plays a role in cell proliferation and migration [74]. Several studies indicated that inhibition of the ACE could reduce glomerular lesions by limiting PEC migration. Benigni et al. [75] reported that ACE inhibitor reduced the extent of crescents and glomerulosclerosis in MWF rats. The underlying mechanism was that use of the ACE inhibitor resulted in an upregulation of the activity of the cell cycle inhibitor, C/EBPδ, thereby preventing PEC proliferation. Accordingly, mitotic activity of cultured PECs was triggered by angiotensin II through blocking of C/EBPδ.

In addition, AT1 receptor blocker (ARB) significantly attenuated Ang II initiated PECs proliferation and collagen secretion in vitro. Furthermore combination treatment with ARB and CCR2 antagonist improved renal function in an anti-GBM nephritis model [76].

In human proliferative CGN, Rizzo and coworkers showed that abundant PECs expressed AT1 receptors in patients with proliferative disorders. By contrast, rare PECs detected AT1 receptor immunoreactivity in patients with membranous and diabetic glomerulopathy. Similar to CXCR4/SDF-1 axis, Ang II/AT1 receptor pathway seems only involved in inflammatory crescents formation. [64].

Therefore, blocking the Ang II/AT1 receptor pathways is a highly prospective treatment in patients with proliferative CGN.

3.6. Other Pathways Leading to PEC Activation. Several other signaling pathways have also been described for PEC activation, such as the PDGF/PDGFR, LAT/mTORC1, and SSeCKS/cyclin D1 signaling pathways.

In the glomerulus, there is a frequent presence of the PDGF-receptor on the apical and lateral surface of PECs. Van Roeyen et al. [77] found that overexpression of PDGF-D in podocytes induced progressive crescentic glomerulonephritis and also glomerular sclerosis. Thus it is reasonable to speculate that proliferation of PECs and cellular crescents formation result from activation of PDGF-receptor by podocyte originated PDGF-D.

Recently Kurayama et al. [78] showed that amino acid transporter 2 (LAT2) played a critical role in the pathogenesis of CGN by stimulating the mTORC1 signaling pathway in PECs. Treatment with an mTORC1 inhibitor, everolimus, prevented cellular proliferation and maintained the integrity of glomeruli; however, early treatment with everolimus resulted in more fibrinoid necrosis which may be linked to the disruption of protein synthesis through the mTORC1 pathway. Thus, it is speculated that PEC activation may be one of the protective phenomena to overcome glomerular insult. Further investigations are needed to elucidate the time and dose-dependent effects of the mTORC1 inhibitor, a potential candidate for CGN therapy, on PEC activation and crescent development.

In normal glomeruli, Src-suppressed protein kinase C substrate (SSeCKS) is exclusively expressed in PECs and mesangial cells, but not in podocyte [79]. It can sequester cyclin D1 in the cytoplasm in an inactivated status. Once SSeCKS is phosphorylated by aPKC it releases its scaffolding function and consequently induces cyclin D1 translocation from the cytoplasm to nucleus [80, 81]. SSeCKS knockout mice showed hyperplasia of PECs and increased nuclear cyclin D1 expression. These observations suggest that SSeCKS/cyclin D1 pathway affects the mitotic and proliferative properties of PECs [82], and regulating SSeCKS/cyclin D1 signaling may effectively correct the abnormal PECs activation.

4. Perspectives

PEC activation is manifested in both CGN and noninflammatory glomerular diseases; nonetheless, the underlying etiology and pathogenesis are variable. This is also true for the signaling pathways involved in PEC activation under different circumstances.

Although, currently, more and more evidences of PEC activation and its underlying signaling events are emerging, what we should pay attention to is that the majority of the related data come from experimental overexpression or knockdown studies, which do not exactly mirror physiological conditions in humans. Also in some experimental settings, PECs are only indirectly affected or associated with the hemodynamic or the paracrine effects. Further studies and understanding of the specific correlation between various signaling pathways and the diverse causes of PEC activation are highly required.

Conflict of Interests

The authors have no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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Review Article

Involvement of the Gut Chemosensory System in the Regulation of Colonic Anion Secretion

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The primary function of the gastrointestinal (GI) tract is the extraction of nutrients from the diet. Therefore, the GI tract must possess an efficient surveillance system that continuously monitors the luminal content for beneficial or harmful compounds. Recent studies have shown that specialized cells in the intestinal lining can sense changes in this content. These changes directly influence fundamental GI processes such as secretion, motility, and local blood flow via hormonal and/or neuronal pathways. Until recently, most studies examining the control of ion transport in the colon have focused on neural and hormonal regulation. However, study of the regulation of gut function by the gut chemosensory system has become increasingly important, as failure of this system causes dysfunctions in host homeostasis, as well as functional GI disorders. Furthermore, regulation of ion transport in the colon is critical for host defense and for electrolytes balance. This review discusses the role of the gut chemosensory system in epithelial transport, with a particular emphasis on the colon.

1. Introduction

The primary function of the gastrointestinal (GI) tract is obtaining energy sources from the diet as nutrients. Under physiological conditions, approximately 8 L of fluid is secreted into the small intestine per day. However, the majority of the secreted fluid (~6-7 L/day in humans) is reabsorbed in the small intestine, with approximately 1.5 L of chyme normally passed through the ileocecal valve into the large intestine daily [1]. Less than 100 mL of fluid is excreted outside the body with the feces. The main functions of the colon are the salvage of the remaining fluid and electrolytes entering from the small intestine and the dehydration and storage of feces. However, colonic epithelia are also able to secrete fluid as a host defense mechanism.

In the colon, approximately 100 trillion bacteria, termed the gut microbiota, are present in the lumen. Gut microbiota continuously produces large quantities of bioactive chemicals that can be beneficial or harmful to the host, as confirmed by analysis of the microbiota genome. Such analysis estimated that gut microbiota contains 150-fold more genes than does the host genome [2]. These bioactive chemicals have a profound influence on many aspects of human health, as gut microbiota are able to produce harmful substances, in addition to those that are beneficial. For example, bacterial fermentation of indigestible carbohydrates produces short-chain fatty acids (SCFAs), which can affect various GI functions including ion transport and motility. These SCFAs can be absorbed by the colonic mucosa as energy sources [3]. On the other hand, bile acids entering the colon can be metabolized by microbiota; thus, primary bile acids are converted to secondary bile acids [4]. Secondary bile acids can subsequently promote the development of GI malignancies [5]. Therefore, the colon must be able to discriminate between beneficial and deleterious substances.

One of the more important host-defense mechanisms of the colon is the ability to flush out harmful substances via fluid secretion, mainly through Cl^- secretion. Indeed, the colon is able to adapt to extreme changes in electrolyte flow such as those during severe secretory or reabsorptive states [1, 6]. Under physiological conditions, there is net colonic absorption of Na^+ and Cl^- in the colon. The mechanism underlying colonic Cl^- secretion is well understood and includes the following components: the luminal cystic fibrosis

transmembrane conductance regulator (CFTR), which is a Cl^- channel that is activated by cAMP; basolateral Cl^- uptake via a Na^+-K^+-$2Cl^-$ cotransporter (NKCC1); and Ca^{2+}-activated basolateral K^+ channels that recycle K^+ and provide the driving force for Cl^- secretion. The secretion of Cl^- into the colonic lumen, followed by a paracellular flux of Na^+, induces fluid secretion via an osmotic gradient. The energy for active Cl^- transport is provided by the action of Na^+-K^+ ATPase. Physiological or pathophysiological stimuli of Cl^- secretion act on one or more of these components. In essence, switching between absorption and secretion is controlled by the enteric nervous system (ENS) and by a large number of hormones that usually bind to their respective receptors on the basolateral membrane. In addition to these control systems, recent studies have shown that luminal bioactive substances produced by gut microbiota, including SCFAs, affect epithelial ion transport through the gut chemosensory system [7–9].

The presence of a gut chemosensory system is evident. The same taste transduction molecules that are found in the taste buds of lingual papillae, such as α-gustducin, are present in the human and rodent intestinal mucosa [10, 11]. Accordingly, mRNA expression of taste receptor type 1 (T1R) and type 2 (T2R) families in human and rodent GI tracts has been reported [12]. Therefore, the gut chemosensory system has important roles in controlling GI functions including ion transport. However, the contribution of the gut chemosensory system to the regulation of colonic ion transport is not well understood.

This review aims to provide an overview of the involvement of the gut chemosensory system in colonic ion transport and its mechanisms.

2. SCFA Receptors

SCFAs are the predominant anions in the content of the large intestine, existing at concentration of ~100 mM and mainly consisting of acetate, propionate, and butyrate. They are produced by bacterial fermentation of specific indigestible dietary fibers and oligosaccharides that are not absorbed in the upper GI tract. SCFAs produced in the large intestine are known to affect a variety of physiological and pathophysiological functions; luminal SCFAs are not only absorbed as nutrients across the intestinal epithelia, but also utilized as chemical signals that influence epithelial proliferation [13], mesenteric blood flow [14], colonic motility [15], and colonic ion transport [16, 17]. For example, luminal application of propionate and butyrate, but not acetate, in the colon has been reported to induce epithelial Cl^-/HCO_3^- secretion via both neural and nonneural mechanisms [16, 17]. On the other hand, serosal application of propionate and other SCFAs did not elicit Cl^- secretion [17]. These results suggest that luminal SCFAs are detected by certain chemosensory systems located in the colonic epithelial layer. Until recently, the mechanism by which luminal SCFAs are detected in the intestinal mucosa and the implications of the SCFA-induced secretory response were unclear.

In 2003, two orphan G-protein coupled receptors, FFA2 (GPR43) and FFA3 (GPR41), were discovered to be receptors for SCFA [18–20]. These two receptors share ~40% amino acid sequence similarity and remain conserved across several mammalian species. They differ in their affinity for SCFAs, their tissue distribution, and their physiological roles. FFA2 has similar affinities for acetate, propionate, and butyrate, whereas FFA3 differs in affinities according to the sequence propionate > butyrate $\gg$ acetate. Thus, acetate preferentially activates FFA2, propionate primarily activates FFA3, and butyrate activates both FFA2 and FFA3 equally.

FFR2 and FFR3 have distinct G-protein-couples in their intracellular signaling cascades, FFA2 couples to both pertussis toxin-sensitive ($G_{i/o}$) and -insensitive (G_q) G protein whereas FFA3 only couples to $G_{i/o}$ protein. We have recently demonstrated that colonic epithelia, particularly, peptide YY and glucagon-like peptide 1- (GLP-1-) containing L-type enteroendocrine cells in humans [21], guinea-pigs [16], and rats [22], express two SCFA receptors, FFA2 and FFA3. Our morphological data suggest that SCFA receptors located on colonic epithelial cells can detect luminal SCFA, thus eliciting secretory responses through neural and nonneural mechanisms.

Segmental heterogeneity of electrolyte transport in the colon has also been previously observed in humans and other species [16, 17, 23]. In the case of SCFAs, propionate and butyrate, but not acetate, induce Cl^-/HCO_3^- secretion in the rectum, as well as in the distal and middle colon. On the other hand, propionate and butyrate do not stimulate Cl^-/HCO_3^- secretion in the proximal colon. This regional difference in the secretory responses to luminal propionate can be explained by the regional difference in the acetylcholine (ACh) content and its release in the proximal and distal colon [24].

Luminal application of propionate in the distal colon induces Cl^-/HCO_3^- secretion, and pretreatment of the mucosal surface with procaine or superficial mucosal damage with hypertonic sodium sulfate or xylose inhibits the propionate-induced secretion by 90% [17, 24]. Therefore, propionate-induced Cl^-/HCO_3^- secretion is caused by the activation of SCFA receptors located on mucosal epithelial cells.

Neural blockade with tetrodotoxin (TTX) inhibits the propionate-induced Cl^-/HCO_3^- secretion by 40% compared with the control, whereas atropine and local anesthesia remarkably reduce propionate-induced responses by 81–90% and 76–82%, respectively [17, 24]. Furthermore, propionate-induced Cl^-/HCO_3^- secretion is not affected by tachyphylaxis, calcitonin gene-related peptide, 5-hydroxytryptamine (5-HT), histamine, neurotensin, or substance P [25]. The GI tract is densely innervated by cholinergic neurons, and Cl^-/HCO_3^- secretion is induced by activation of muscarinic receptors located on colonic epithelial cells [26, 27]. These observations suggest that SCFA-induced Cl^-/HCO_3^- secretion is linked to ENS, with involvement of cholinergic secretomotor neurons and nonneural release of ACh.

Recently, Yajima et al. showed that ACh is released from the basolateral side of the distal colon by luminal chemical stimulation with SCFA concomitant with propionate-induced Cl^-/HCO_3^- secretion [24]. Therefore, the remaining 50% of propionate-induced Cl^-/HCO_3^- secretion may be

due to the release of ACh from the epithelial cells into the basolateral side. In the same study, Yajima et al., showed that prior addition of luminal 3-Cl^- propionate completely blocked the short-circuit current (I_{sc}) response and abolished ACh release in response to luminal propionate. They concluded from the results that ACh-storing epithelial cells have a receptor for propionate although further studies are necessary to identify specific cell that store ACh. Therefore, ACh release stimulated by FFAs may affect Cl^-/HCO_3^- secretion by autocrine fashion (Figure 1).

With respect to the involvement of SCFA receptors, FFA3 may be involved in the secretory process since acetate, the preferred ligand of FFA2, has no effect on mucosal Cl^-/HCO_3^- secretion in distal colon of rats [17]. Unfortunately, the intracellular molecular pathways underlying the effects of SCFAs on colonic Cl^-/HCO_3^- secretion are still not fully understood. Therefore, further study is needed to identify the molecular pathways of FFA-stimulated ion transport in the colon.

Indigestible dietary fibers are fermented in the cecum and in the proximal colon by anaerobic microbiota, as mentioned previously. Most bacterial activity occurs in the cecum and in the proximal colon, where substrate availability is highest, with the availability of substrates declining toward the distal colon [28]. Therefore, the proximal colon is continuously exposed to high concentrations of SCFAs, which decrease from the proximal colon to distal colon. However, the proximal colon does not secrete Cl^-/HCO_3^- in response to SCFAs, as mentioned above. On the other hand, the distal colonic mucosa is exposed to SCFAs when semisolid contents containing SCFAs are transported to the distal colon. Therefore, detection of SCFAs is important in the distal colon as it has ability to secrete Cl^-/HCO_3^- after SCFA stimulation.

In combination with the contractile response, the secretory response to luminal SCFAs in the distal colon seems to function as a lubricant for the movement of luminal contents in the colon. Furthermore, the distal colon and rectum are a boundary between the host and external environment; thus, the high secretory ability of the distal colon is physiologically important for host defense, as it needs to flush out harmful agents, in addition to finalizing electrolyte tuning.

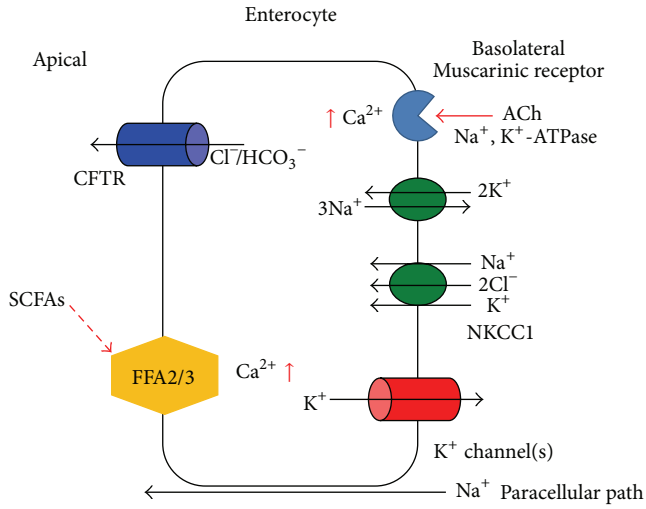

Figure 1: Schematic diagram of Cl^- secretion stimulated by short-chain fatty acids in colonic epithelial cells. At the basolateral membrane, Cl^- enters the cell from the basolateral space across the Na^+, K^+-$2Cl^-$ cotransporter (NKCC1). Na^+, K^+-ATPase causes drainage of Na^+, and K^+ leaves via the K^+ channel. Na^+ also moves to the apical side paracellularly. The cystic fibrosis transmembrane conductance regulator (CFTR), which is located on the apical membrane, allows Cl^- to exit the cell. The colonic epithelium CFTR Cl^- conductance is constitutively active [1]. Luminal SCFAs stimulate the FFA2/3 receptors located on the apical membrane, which in turn activate second messenger pathways to induce ACh release from epithelial cells basolaterally. The released ACh activates muscarinic receptors located on the basolateral membrane of epithelial cells, inducing Cl^- secretion.

3. Bitter Taste Receptors

In recent years, numerous studies have suggested the presence of taste receptors and taste-associated signaling components in the GI tract, in addition to their presence in the gustatory system [29]. The discovery of taste-associated molecules in the GI tract has led to the hypothesis that taste receptors are a part of the gut chemosensory system that recognizes nutrients and chemicals, which enter the GI tract (e.g., FFA2 and FFA3), and trigger various physiological processes [30]. The bitter taste, one of the five basic tastes, is mediated by bitter taste receptors (T2Rs). The bitter taste signal is a "notifier" of toxic substances, allowing the host to avoid harm [31]. Genomic sequencing analysis has identified the T2R family as a receptor family specific to bitter tastants, consisting of ~30 members in humans and rodents [32–35].

The α-subunit of the taste-specific G protein gustducin is expressed in the GI mucosa of humans and rodents [10, 11, 36, 37]. Unlike FFA2- and FFA3-expressing cells, cells expressing the bitter receptor have not been identified. Several studies in model cell lines [38, 39] and a histochemical study using an antibody raised against mouse T2R138 suggested that enteroendocrine cells express putative bitter taste receptor [40]. However, another study has recently shown that a subset of mouse colonic goblet cells also express the bitter taste receptor T2R131 [41]. Since goblet cells produce mucus to protect intestinal epithelia [42, 43], T2R-expressing cells may contribute defense-related functions involving the recognition of harmful bioactive chemicals.

Recently, we have shown that, in mucosa-submucosa preparations mounted in Ussing chambers, the mucosal application of 6-*n*-propyl-2-thiouracil (6-PTU) at concentrations greater than 10^{-4} M increased I_{sc} in both human and rat colons in a concentration-dependent manner [7]. Multiple T2R family members (at least T2R-1, -4, and -38) in humans are known to detect 6-PTU [34, 44, 45], and these genes are the most conserved between humans and rodents [12]. Previous human taste-test studies and brief-access mouse studies have also shown that the minimal effective concentration of 6-PTU is ~10^{-4} M [31, 46, 47].

Therefore, the threshold for 6-PTU in T2R-expressing cells in the colon in both humans and rats is similar to that in gustatory senses. After the addition of 6-PTU, the base line I_{sc} gradually increased and reached a plateau over 10–15 min, which continued for >20 min [7]. The increase in I_{sc} induced by 6-PTU was reduced by bumetanide (10^{-4} M), an inhibitor of NKCC1, to 69% of the control, whereas NPPB (10^{-4} M), an inhibitor of the CFTR, almost completely abolished the 6-PTU-induced increase in I_{sc}. NPPB-sensitive Cl^- channel, for example, CFTRs located at the apical membrane, also secretes HCO_3^- [1]. Thus, the 6-PTU-induced increase in I_{sc} is due to secretion of Cl^- and HCO_3^-. This is further supported by the observation that the 6-PTU-induced I_{sc} response is almost completely abolished in Cl^-/HCO_3^--free solution [7].

The 6-PTU response elicited is reduced by piroxicam, a nonselective COX inhibitor, and NS-398, a COX-2 inhibitor, but is not affected by TTX. Therefore, 6-PTU-stimulated anion secretion is thought to be involved in prostaglandin (PG) synthesis [7]. Furthermore, exogenous addition of prostaglandin E_2 (PGE_2) enhances 6-PTU-induced I_{sc} response in the presence of piroxicam in a concentration-dependent manner [7], indicating that the 6-PTU-induced increase in I_{sc} may be amplified when the concentration of extracellular PGE_2 in the colon is elevated, for example, during inflammation. The PGE_2 concentration in the intestine can be increased by mechanical stimulation [48] or inflammation [49, 50], with PGE_2 concentrations over 10^{-7} M considered pathophysiological [51]. Therefore, the 6-PTU-induced fluid secretion in the presence of high PGE_2 concentrations is considered to be a host defense mechanism to flush out noxious substances from the colonic lumen, during, for instance, inflammation.

PGE_2 is known to increase the concentration of intracellular cAMP in colonic epithelial cells [52]. It has also been reported that STC-1, a mouse enteroendocrine cell line, expresses T2R mRNA and that 10^{-3} M 6-PTU increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [12]. These results raise the possibility that bitter tastants (including 6-PTU) that induce an increase in $[Ca^{2+}]_i$ in colonic epithelia and that elicit Cl^-/HCO_3^- secretion do so via interactions with PGs (Figure 2).

The mRNA expression of human T2R-1, T2R-4, and T2R-38,as well as orthologous rat T2R-1, T2R-16, and T2R-26, is detected in the colonic mucosa by real-time PCR (RT-PCR) [7]. Although which specific receptor responds to 6-PTU has not been determined, many members of the T2R family that can detect 6-PTU are expressed in the colon [11]. These results suggest that 6-PTU may be detected by colonic epithelial T2R in both humans and rats, although the precise cellular localization of T2R is currently unknown.

With respect to its physiological significance, bitter tastant-induced anion secretion in the colon is considered an important mechanism to flush out noxious agents from the colonic lumen. For example, bitter compounds that enter the large intestine under normal conditions are most frequently bile acids and their bacterial metabolites. As secondary bile acids promote tumors [53], bitter sensing in the large intestine may be a necessary mechanism for host defense.

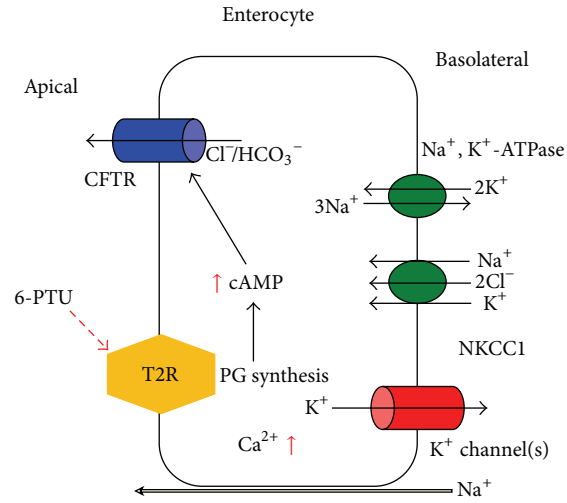

Figure 2: Schematic diagram of Cl^- secretion stimulated by a bitter tastant (6-PTU) in colonic epithelial cells. Activation of apical T2R by luminal bitter tastant results in the synthesis of prostaglandin. This prostaglandin then induces an increase in intracellular cAMP concentration ($[cAMP]_i$). Elevated $[cAMP]_i$ activates the CFTR Cl^- channels to mediate Cl^-/HCO_3^- secretion. Activation of T2R simultaneously causes an increase in $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ modulates the Ca^{2+}-activated basolateral K^+ channels, providing a driving force for the exit of Cl^-/HCO_3^-.

4. Odorant Receptor (OR)

The colonic mucosa of both humans and rats express OR mRNA, and luminal odorants induce 5-HT secretion in isolated duodenal enterochromaffin (EC) cells and in EC cell lines [54, 55]. Thymol, a major odor constituent of edible herbs that is used in oral care products, activates certain types of the apical odorant receptor (OR1G1). Specifically, it activates class II (the terrestrial-type OR group) but not class I (the fish-like OR group) OR1G1 [56]. Since bacteria have the capacity to synthesize isoprene units and terpenoid biosynthesis enzymes [57, 58], active odor molecules may be produced in the mammalian colon. Indeed, a great variety of volatile compounds (including acids, alcohols, aldehydes, and terpenoids) are detected in human feces [59]. It has been reported that the concentration range of fecal indole is 0.5–1 mM in healthy men [60, 61]. Therefore, the monitoring of volatile compounds in the colonic lumen is critical for host defense.

Recently, we showed that mucosal addition of thymol (10^{-3} M) induces Cl^- and HCO_3^- secretion in a concentration-dependent manner in both the human and rat colon [8]. Addition of TTX (10^{-6} M) or piroxicam (10^{-5} M) did not affect this response, suggesting that thymol-induced anion secretion is independent of the neural and PG synthesis pathways. This differs from stimulation of the bitter taste receptor; thus, there are distinct mechanisms for detecting tastants in the colonic mucosa. It has been reported that odorant stimulation leads to an increase in $[Ca^{2+}]_i$ in olfactory neurons and in other OR-expressing cells, depending on extracellular Ca^{2+} [54, 62, 63]. Thymol-induced electrogenic

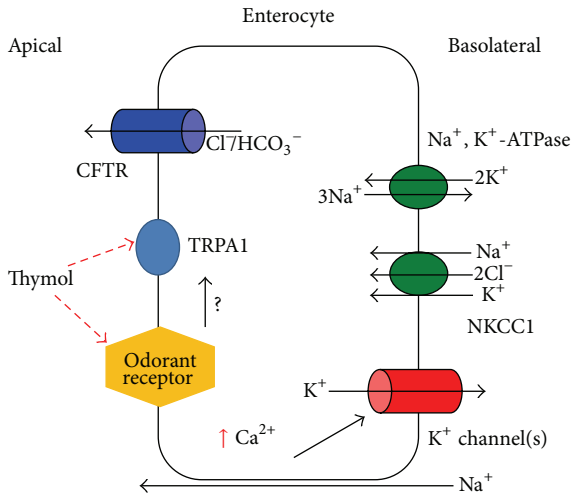

FIGURE 3: Schematic diagram of Cl^- secretion stimulated by an odor tastant (thymol) in colonic epithelial cells. Activation of OR1G1 by luminal thymol increases $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ may modulate Ca^{2+}-activated basolateral K^+ channels, providing a driving force for the exit of Cl^-/HCO_3^-. Activation of OR1G1 may also activate TRPA1 although it is still unclear whether ORs are linked to TRPA1.

anion secretion is also abolished under Ca^{2+}-free conditions [8]. These results suggest that extracellular Ca^{2+} is required to elicit thymol-induced anion secretion in the large intestine (Figure 3).

Thymol-induced anion secretion in the distal colon is reduced by HC-030031, a transient receptor potential A1 (TRPA1) blocker. Furthermore, TRPA1 mRNA is detected in the isolated mucosa of humans and rats [8, 64]. Several odor molecules, particularly those present in spices, are known ligands of not only GPCRs, but also the transient receptor potential (TRP) channel. Thymol activates transient receptor potential vanilloid 3 (TRPV3) and TRPA1 in a cell-expression system [65, 66]. In the GI tract, it has been reported that TRPA1 activity is involved in the motility of the small intestine [67]. Together, these results suggest that thymol-induced electrogenic anion secretion is mediated via the TRPA1 channel. In addition, thymol has been reported to affect luminal SCFA-induced ion secretion. Propionate-induced increases in I_{sc} are almost completely abolished by pretreatment of tissues with thymol [8]. Therefore, the physiological effects of luminal SCFAs in the large intestine are likely to be modulated by luminal odorant chemicals.

Pretreatment of tissues with bumetanide or Cl^- free solution attenuated the thymol-induced increase in I_{sc}. Consistent with other studies, the absence of HCO_3^- and Cl^- completely suppressed the I_{sc} response to thymol. Together, these results indicate that the thymol-induced increase in I_{sc} involves electrogenic Cl^- and HCO_3^- secretion in a NKCC1-dependent manner.

It has been reported that thymol-induced electrogenic anion secretion is mediated by the cholinergic neural pathway in the porcine small intestine [68]. However, the mechanisms of thymol-induced secretion are likely different in the small and large intestine as thymol-induced anion secretion is not blocked by TTX in the rat or human large intestine [8]. This discrepancy may be attributed to the following reasons. First, thymol-induced anion secretion in the small intestine is involved in the release of 5-HT because duodenal EC cells release 5-HT after OR stimulation [54, 55]. Second, thymol-induced anion secretion is not blocked by 5-HT_3 and 5-HT_4 receptor antagonists in the large intestine [8]. Therefore, luminal thymol-induced anion secretion in the large intestine is mediated by nonneural and nonserotonergic pathways in rats and in humans.

As bacteria can synthesize isoprene units [57], production of active odor molecules similar to thymol may be possible in the mammalian colon. Thus, colonic mucosa may be exposed to high concentrations of various volatile odorants. Because irritant odors, similar to bitter tastants, are danger signals for animals, ORs can play an important role in the luminal surface of the colon in host defense. Although a RT-PCR experiment showed that OR1G1 and TRPA1 are present in both human and rat colonic mucosa [8], colonic epithelia consist of many different cell types, including absorptive, goblet, enteroendocrine, and caveolated cells. Therefore, studies should be done to identify the specific sensor cells expressing ORs and TRPA1. At present, it is still unclear whether OR1G1 is directly involved in thymol-induced anion secretion and whether ORs are linked to TRPA1.

5. TRP Channels

The TRP channel member, TRPA1 (also known as ANKTM1), was first identified as a cold-sensitive cation channel in murine sensory neurons and is thought to have a role in nociception [69]. Since multiple environmental irritants can activate TRPA1, TRPA1 functions as a chemosensor in nociceptive neurons [70], in the rat urinary bladder [71], and in human keratinocytes [72]. Endogenous inflammatory mediators can also activate TRPA1 [73–75].

To date, 28 mammalian TRP channels have been cloned and characterized. They are grouped into six subfamilies on the basis of their amino acid sequence homology, namely, TRP ankyrin (TRPA), TRP canonical (TRPC), TRP melastatin (TRPM), TRP mucolipin (TRPML), TRP polycystin (TRPP), and TRP vanilloid (TRPV). TRPA1 expression in the colon has been demonstrated in humans, mice, rats, and dogs by northern blot analysis and by RT-PCR [64, 76–78]. As described in the section Odorant Receptor, luminal thymol-induced anion secretion involves TRPA1. The function of TRPA1 in the transepithelial ion transport system was examined using a potent TRPA1 agonist allyl isothiocyanate (AITC) [9].

In the human and rat distal colon, the addition of AITC (10^{-6}–10^{-3} M) to the luminal side induced an increase in I_{sc} [9]. On the other hand, serosal application of AITC did not elicit an increase in I_{sc}. AITC-induced increases in I_{sc} are significantly decreased in the absence of Cl^- and are abolished in the absence of both Cl^- and HCO_3^-. Further, NPPB (10^{-4} M) and bumetanide (10^{-4} M) significantly reduced AITC-induced I_{sc} increases. These results indicate that transepithelial anion secretion induced by the

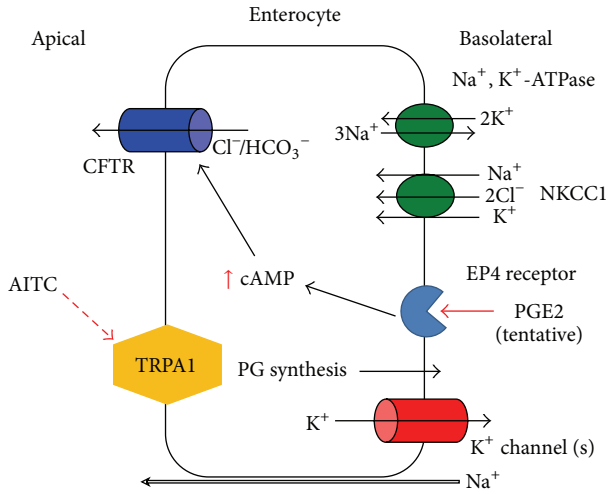

Figure 4: Schematic diagram of Cl^- secretion stimulated by TRPA1 in colonic epithelial cells. Activation of apical TRPA1 by luminal AITC causes prostaglandins synthesis. The synthesized PGE_2 may then be released basolaterally. This released PGE_2 may act on EP_4 receptors. Activation of the EP_4 receptor induces an increase in $[cAMP]_i$ which then activates the CFTR Cl^- channels to induce Cl^-/HCO_3^- secretion.

activation of TRPA1 is dependent on Cl^- uptake by NKCC1 and on excretion of Cl^-/HCO_3^- by Cl^- channels at the apical membrane (Figure 4).

In fractions enriched with isolated EC cells from the rat small intestine, 5-HT was released by AITC and cinnamaldehyde [67]. However, in the colon, a 5-HT_3 antagonist, 3-tropanyl-3, 5-dichlorobenzoate, and a 5-HT_4 antagonist SB204070 (10^{-5} M) did not affect the response to AITC [9]. Furthermore, coapplication of these antagonists abolished the response to the serosal addition of 5-HT (10^{-5} M) but did not affect the AITC-induced I_{sc} response. Serosal application of the TRPA1 blocker HC030031 (10^{-4} M) did not affect the response to AITC, whereas mucosal treatment significantly inhibited the AITC-induced increases in I_{sc}. These observations support the hypothesis that TRPA1 likely functions on the apical side of colonic epithelia.

COX products are involved in the colonic ion transport system. In particular, PGE_2 induces Cl^- secretion in the rat colon [79] and enhances the effects of other secretagogues [7]. Mucosal treatment with a COX inhibitor, piroxicam (10^{-5} M), decreases the AITC-induced response in the rat and human colon. However, exogenous PGE_2 does not enhance AITC-induced anion secretion, even in the absence of the COX inhibitor, suggesting that AITC stimulates PGE_2 synthesis [9].

Although the mechanisms by which PGE_2 contributes to 6-PTU- and AITC-induced anion secretion differ, PGE_2 likely plays important roles in the luminal chemosensory system. The function of PGE_2 in the GI tract has been well studied, especially in relation to its receptors, EP_1, EP_2, EP_3, and EP_4. Only EP_2 and EP_4, which are coupled to the cAMP pathway, mediate PGE_2-induced colonic secretion, but EP_1 and EP_3 do not, as they are coupled to an intracellular Ca^{2+} pathway instead [80]. An EP_4 selective antagonist (ONO-AE3-208) significantly reduced AITC-induced anion secretion, whereas the $EP_{1/2}$ antagonist AH6809 did not affect the response to AITC, indicating that EP_4, a PGE_2 receptor subtype, is involved in AITC-induced anion secretion in both human and rat colons [9]. As application of AITC to the mucosal bathing solution did not result in the release of PGE_2 into the bathing solution, PGE_2 produced by epithelial cells might be rapidly used as an autocrine transmitter [9].

Recently, the EP_4 receptor has been found on the apical membrane of human and rat colonic epithelia via immunohistochemical techniques [81]. Since both serosal and mucosal pretreatment with ONO-AE3-208 abolished the response to AITC, the precise localization of the EP_4 receptor is still unknown. Overall, the evidence clearly indicates that a TRPA1-PGE_2-EP_4 secretory pathway that is independent of the neural reflex exists in both human and rat colonic epithelial cells.

TRPA1-dependent thymol-induced anion secretion requires extracellular Ca^{2+}, as described in the section Odorant Receptor. However, the response to AITC was not altered by the removal of extracellular Ca^{2+} [9]. Therefore, AITC-induced anion secretion is mediated by PG synthesis via a Ca^{2+} independent process. These conclusions were further confirmed after observations that AITC does not increase $[Ca^{2+}]_i$ in mouse colonic epithelia [78] and that AITC-induced TRPA1 current does not require extracellular Ca^{2+} in HEK cells [82]. Overall, TRPA1 activation appears to induce PGE_2 synthesis independently of extracellular Ca^{2+} and can cause anion secretion through the EP_4 receptor in colonic epithelia.

TRPA1 mRNA is detected in isolated crypts of the rat colon [9]. Furthermore, tyramide-based *in situ hybridization* and immunohistochemistry for TRPA1 demonstrated that TRPA1-expressing cells are localized to the surface epithelium of the rat colon [9]. Recently, it has been reported that activation of TRPA1 inhibits spontaneous contractions and transit by direct activation of myenteric neurons [83]. Therefore, TRPA1 agonist-induced colonic Cl^- secretion with inhibition of colonic transient seems to physiologically regulate the movement of luminal content in the colon. In addition, TRPA1 may also play a role in flushing out noxious chemicals via massive fluid secretion.

6. Conclusion

As colonic mucosa is continuously exposed to noxious chemicals, including toxic compounds such as bacterial metabolites and the products of oxidative stress, in addition to nutrients, the chemosensory system in the gut is critical for distinguishing the nutrients from the other luminal contents. Therefore, proper fluid secretion in the colon is crucial to flush away noxious chemicals, while maintaining host homeostasis.

Although neural and hormonal involvement in fluid secretion in the colon are well documented, the involvement of the gut chemosensory system in the regulation of colonic ion transport is much less understood. Activation of luminal chemosensory receptors is a primary signal eliciting

colonic fluid secretion. Gut luminal chemosensing involving FFA2, FFA3, T2R, OR, and TRPA1 may act as a line of defense against noxious agents, preventing the large intestine from being exposed to these agents. Therefore, the gut chemosensory system is important for maintaining luminal homeostasis. A variety of sensory receptors expressed in the colonic mucosa serve important functions, at least in the anion secretory system, which stimulates fluid secretion. However, the specific mechanisms involved in anion secretion induced by the gut chemosensory system are largely unknown. Therefore, more studies are required to define the involvement of the gut chemosensory system in colonic ion transport.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Ouabain-Induced Cytoplasmic Vesicles and Their Role in Cell Volume Maintenance

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Cellular swelling is controlled by an active mechanism of cell volume regulation driven by a Na^+/K^+-dependent ATPase and by aquaporins which translocate water along the osmotic gradient. Na^+/K^+-pump may be blocked by ouabain, a digitalic derivative, by inhibition of ATP, or by drastic ion alterations of extracellular fluid. However, it has been observed that some tissues are still able to control their volume despite the presence of ouabain, suggesting the existence of other mechanisms of cell volume control. In 1977, by correlating electron microscopy observation with ion and water composition of liver slices incubated in different metabolic conditions in the presence or absence of ouabain, we observed that hepatocytes were able to control their volume extruding water and recovering ion composition in the presence of ouabain. In particular, hepatocytes were able to sequester ions and water in intracellular vesicles and then secrete them at the bile canaliculus pole. We named this "vesicular mechanism of cell volume control." Afterward, this mechanism has been confirmed by us and other laboratories in several mammalian tissues. This review summarizes evidences regarding this mechanism, problems that are still pending, and questions that need to be answered. Finally, we shortly review the importance of cell volume control in some human pathological conditions.

1. Introduction: "Cloudy Swelling"

"Cloudy swelling" is an early and frequent morphological cell alteration first identified by Virchow in his "Die Cellular Pathologie" [1]. Swelling is an increase of cell volume due to accumulation of water and ions in subcellular compartments. It occurs under two rather common conditions in human pathology: (a) reduction of metabolic activity leading to a decrease of energy charge (hypoxia, ischemic hypoxia [2], or uncoupling agents) and (b) damage of cell membranes with loss of physiological ionic gradients (plasma membrane rupture or pore formation, Na^+/K^+-ATPase malfunction, etc.). Extreme "cloudy swelling" is irreversible, leading to cell necrosis. Therefore, a clear picture of the underlying pathogenetic mechanisms of cell swelling is important to understand many aspects of human diseases.

Living cells regulate their water content even under apparently isosmotic conditions to compensate the osmotic forces due to higher intracellular concentration of charged macromolecules [3]. Regulation of cell water is necessary to maintain (a) an appropriate physical size of cells and organelles; (b) the optimal concentrations of soluble metabolic components (ions, substrates, and soluble enzymes); (c) the functional subcellular architecture and relational distances necessary for metabolism and vectorial movements into the cell. The latter includes intracellular transport (secretion, endocytosis, axonal transport, and organelles movements) and sarcomeric contraction.

In vitro cell swelling is rapidly induced by reducing metabolic activity (cold incubation and inhibition of energy metabolism and ATP production), by inhibiting ion transport (i.e., ouabain, abnormal external ionic concentrations), or by damaging the membrane(s) (i.e., lipoperoxidation, high temperature, ionophores, etc.). By using different *in vitro* models, Wilson [4], Leaf [5], and Macknight and Leaf [3] demonstrated the role of active transport of ions across the

plasma membrane in preventing swelling under isosmotic condition. They showed that the distribution of Na^+ was crucial and that its physiologic concentrations were maintained by a coupled transport of Na^+ and K^+, operated by a Na^+/K^+-ATPase, a pump apparently totally inhibited by ouabain both in *in vitro* and in soluble systems.

However, in 1976 and 1977, it was strongly evident that inhibition of Na/K-transport by ouabain in a model of tissue slices does not totally abolish regulation of cell volume under isosmotic conditions. To account for the ouabain-resistant fraction of volume regulation, Russo et al., for the first time, by strictly correlating ion and water distribution with ultrastructural observations, proposed that vesicles of exocytosis, highly induced in the presence of ouabain, could be responsible for water extrusion, after swelling by cold incubation [6–9].

A previous account of this proposal has been published long time ago [8]. This review aims to summarize evidences that we have at present about cellular swelling, problems that are still pending, and questions that need an answer.

2. Volume Regulation in Mammalian Tissues

The property of cell volume control is a fundamental feature of all cells and, in particular, of mammalian cells. Cell volume results from the content of water and ions, osmotically active soluble molecules, and macromolecules and supramolecular structures, such as organelles, filaments, polymers, and metabolites. These components are confined in intracellular space delimited by the plasma membrane. Ions and osmotically active molecules play a crucial role in steady state conditions, determining the amount of the intracellular water, which represents the major component of cytosol and intraorganellar space [10]. Changes in cell volume, indeed, may occur through *swelling*, that is, increase in water and ion content, or *hypertrophy*, which is an increase in cell constituents, such as sarcomeric filaments (muscle hypertrophy), lipid droplets (obesity), endoplasmic reticulum (drug metabolism), mitochondria, and other cell structures, following a functional adaptation. In the case of swelling, three major components determine the final volume: the amount of intracellular water, differently distributed in the various subcellular compartments, the amount of osmotically active solutes (ions and macromolecules), and the plasma membrane properties for controlling intracellular concentrations of osmotically active solutes (ions and free amino-acids) and for excluding other extracellular osmotically active molecules.

In mammalian cells, under normal conditions, the intracellular composition and the extracellular environment are almost constant, obeying to three simple principles: (1) cell osmolarity and water content must be equal to extracellular osmolarity; (2) inorganic ion distribution (Na^+, K^+, and Cl^-) on two sides of plasma membrane is controlled by active/passive transport mechanisms, satisfying the basic requirement of electroneutrality; (3) water travels through aquaporins as determined by osmotic equilibrium. The localization and the isoforms of aquaporins may influence the distribution among subcellular compartments of intracellular water and an excess of water extrusion (cell shrinkage) in the presence of extracellular hyperosmolarity [11–13].

Under isosmotic conditions, cell volume regulation in mammalian is, to a large extent, explicable by passage of water directly through aquaporins of plasma membrane in response to ionic balance maintained by a Na^+/K^+-ATPase transport system [10], which is apparently completely inhibited by ouabain [4, 5]. However, observations on a number of mammalian tissues suggest that this ability to regulate cell volume is at least partially resistant to oubain or to the absence of K^+ from the medium [6, 9, 14–18]. Several hypotheses have been proposed to account for this ouabain-resistant extrusion of water: (a) a "cryptic pump" [19] inaccessible to oubain, being hidden in different subcellular sites of plasma membrane, due to impermeable submembranaceous cytoskeletal network (which regulates the secretion in many endocrine and epithelial cells); (b) a second Na^+ pump [20] insensitive to ouabain and uncoupled from K^+ movements. Marín et al. [21] have partially characterized a Na^+-dependent ATPase, inhibited by furosemide, in the basolateral plasma membrane of epithelial cells in rat kidney proximal tubule. (c) Kleinzeller [22], Rorive et al. [23], and others have provided several evidences that a contractile system associated to the cell periphery (submembranaceous cytoskeleton) could be responsible for the extrusion of water and ions accumulated during a period of previous swelling, thus controlling cell volume. This mechanism appears to be typically dependent on Ca^{2+} and ATP.

The work cited above was done with no published morphological and ultrastructural control. A few years later, Garfield and Daniel [15] using uterine smooth muscle and Russo et al. using liver [6, 24] and kidney slices [18, 25] have described that the presence of ouabain is associated with the formation of cytoplasmic vesicles as well with continued extrusion of water and ions and that this could be the basis of ouabain-resistant cell volume control.

The idea that intracellular vesicles (mostly round and electron-clear at TEM) can be involved in volume and osmotic regulation was not completely new. In fact, contractile vacuoles of unicellular organisms, tonoplasts of plant cells, and large vacuoles of mammalian tissues during vacuolar degeneration (hypoxia) are all different types of vesicles with similar morphology and function in sequestering water, maintaining cell volume or turgor, and avoiding cytosolic dilution. Vesicles in excess can be expelled (exocytosis), extruding water. However, ultrastructural observations of different mammalian tissues suggest that in normal conditions vesicles play a minor role in water and ion movement. In contrast, in a number of metabolic and toxic emergencies (including ouabain treatment), cytosolic water rapidly is compartmentalized in different membrane-bound structures of endoplasmic reticulum and Golgi apparatus (vesicles), mitochondria, and other organelles. Indeed, water is extruded from cytosol, nucleoplasm, and mitochondrial matrix (transition from *swelling* → *orthodox* → *condensed forms*), accumulating in vesicles derived from ER cisternae and Golgi apparatus. We will discuss below the different

Table 1: Time-course of (a) water and (b) ionic content of rat liver slices during incubation at 38°C, in the presence or absence of ouabain [6], after 90 min incubation at 1°C. Points at zero time represent the tissue composition at the end of cold incubation at 1°C [8].

Treatment	Time at 38°C (min)	Water (kg/kg dry weight)			Ions (mmol/kg dry weight)		
		Total	Intracellular	Extracellular	Na^+	Cl^-	K^+
Control	0	3.5 ± 0.1	2.7 ± 0.2	1.0 ± 0.1	350 ± 30	320 ± 20	75 ± 10
	15	2.7 ± 0.2	1.6 ± 0.3	1.2 ± 0.1	180 ± 20	175 ± 35	90 ± 12
	60	2.7 ± 0.2	1.5 ± 0.2	1.3 ± 0.1	100 ± 15	180 ± 25	150 ± 15
Ouabain 2 mM	0	3.4 ± 0.2	2.7 ± 0.2	0.9 ± 0.1	360 ± 25	335 ± 20	70 + 10
	15	3.1 ± 0.5	2.2 ± 0.2	1.0 ± 0.2	375 ± 30	350 ± 15	62 + 15
	60	3.1 ± 0.6	2.2 ± 0.4	1.0 ± 0.2	420 ± 10	380 ± 30	50 + 10

morphology and origin of at least two types of vesicles: one that we call "secretory" seen in the presence of ouabain and another observed in the presence of oligomycin (or oligomycin + ouabain or low doses of amytal) for cell water compartmentation [6, 26].

3. The Ouabain-Resistant Na^+/K^+-ATPase Independent Cell Volume Regulation

The experimental model adopted for the work by Russo et al. namely, preincubation of tissue slices at 1°C, followed by recovery at 37°C in the presence of various agents, has been well characterized previously [6, 8, 27, 28]. The qualitative characteristics and quantitative balance of the ionic and water exchanges taking place with and without ouabain are shown in Table 1 and in Figure 2 and are similar to the previous results obtained in many experiments and published in previous papers. Ouabain (2 mM) completely inhibited the net reuptake of K^+ at 37°C suggesting that this concentration effectively inhibited the Na-K ATPase. Thus, increasing ouabain to 5 mM caused no further effect, while omitting K^+ from the medium produced effects closely similar to those of ouabain [7, 29]. Further, the unidirectional influx of ^{86}Rb (an analog of K^+) was maximally inhibited by 2 mM ouabain [30]. It was concluded that the expulsion of water, Na^+, and Cl^- continuing in the presence of ouabain is driven by an energy-providing mechanism other than the Na^+/K^+-ATPase, clearly associated with the presence of round electron-clear vesicles.

Importantly, this mechanism is highly sensitive to the decrease of ATP and involves the entry of water and ions into cytoplasmic vesicles that expand in the presence of ouabain followed by expulsion of the vesicular contents by exocytosis into the canaliculus [6, 8, 29].

In our model of tissue slices, cold (1°C) incubation for 90 min caused advanced swelling with accumulation of Na^+, loss of K^+, and retention of water distributed in the various subcellular compartments (Figures 1(a), 1(b), and 1(c)) [6].

Restoration of metabolic activity by subsequent incubation for 60 min at 38°C allows cells to extrude Na^+, Cl^-, and water and reaccumulate K^+ (Table 1). Additionally, structural recovery started after 5 min of warm incubation and was complete after 60 min (Figure 2(a)).

Treatment with ouabain *in vitro* induces characteristic, rounded vesicles in tissue slices (Figure 2(b)) and/or cultured cells of uterus [16], liver [8, 25, 31], hepatoma [9], renal cortex [18], and lung [32]. In each case, the vesicles have been correlated to an ouabain-resistant mechanism of isoosmotic cell volume control. By contrast, the salt gland of salt-adapted water birds was found not to expel water in the presence of ouabain and did not show vesicles [8].

The first systematic hypothesis on the proposed mechanism and on the formation and nature of the ouabain-induced vesicles for ion and water expulsion has been summarised previously although a number of crucial questions were still pending [8].

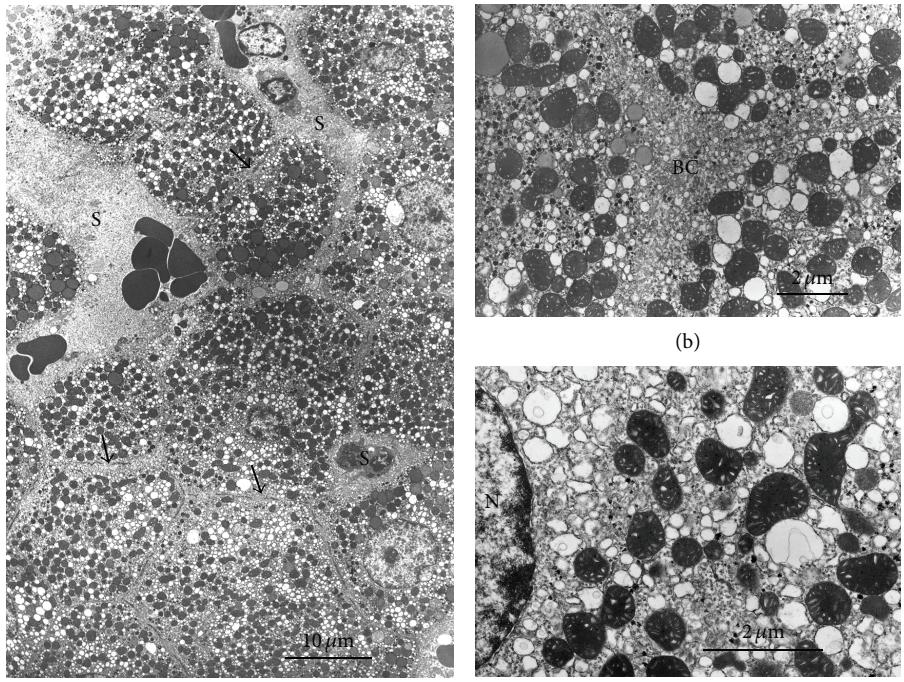
Briefly (see also Figure 9), formation of the vesicles is dependent on the presence of Cl^-. Vesicles appear to originate by expansion of terminal cisternae of the endoplasmic reticulum and of Golgi elements and they have an acidic content. In liver, the vesicles accumulate and secrete at the canalicular pole and in renal cortex near the basolateral infolding of the plasma membrane. In each case and most clearly in the kidney, there is evidence of their fusion with the plasma membrane suggesting expulsion of the content [18, 24].

Accordingly, it has been suggested that accumulation of water in the vesicles is driven by the Cl^- dependent, H^+-transporting vacuolar adenosine triphosphatase (V-ATPase) of the vacuolar membranes [30, 33]. The vesicles are suggested to move to the cell periphery by a microfilament-dependent and microtubule-independent mechanism and then to expel their contents by exocytosis. Cytochalasins addition or absence of Ca^{2+} greatly increases the number, size, and intracellular distribution of the vesicles, suggesting an inhibition of the cytoskeletal function for their secretion [7, 8].

Several points of this proposed mechanism in Figure 9 require attention and analysis, such as the following.

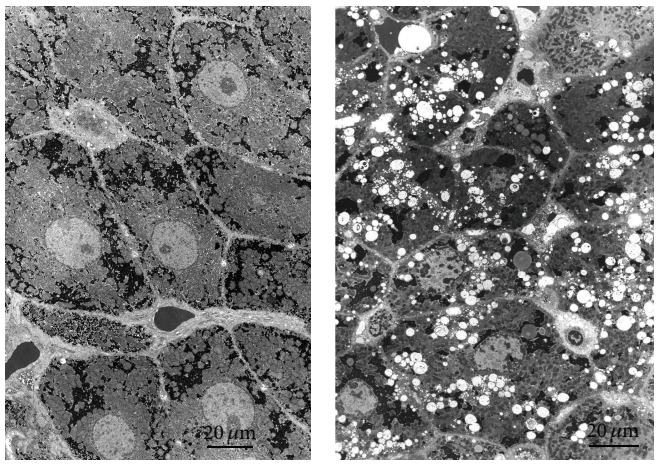
(1) The origin and fate of the ouabain-induced vesicles: what is the role of chloride and protons? If a V-ATPase does, indeed, provide the driving force for water accumulation in the vesicles, depletion of intracellular ATP should block both vesicular expansion and ouabain-resistant water extrusion.

(2) Published data with liver slices indicated a requirement for ATP derived from oxidative phosphorylation: nevertheless, it is unclear at what extent the decrease of energy charge differently inhibits (a) ouabain-dependent vesicles formation and their transport and secretion to the canalicular pole of the cell, (b) the recovery of intracellular water and



(a) (b) (c)

Figure 1: Liver slices after 90 min incubation at 1°C [6]. (a) Low magnification. Cells are highly swollen with volume increase. Cisternae of the endoplasmic reticulum are dilated; mitochondria are mostly in intermediate-condensed form, sometimes swollen. Cytosol and nucleoplasm are rather electroclear decreased and disorganized extracellular spaces. (b) Detail of dilated endoplasmic reticulum, detachment of ribosomes, and boundaries of cells that became uncertain with disorganized microvilli; increase and disorganization of extracellular spaces (sinusoidal, lateral, and canalicular regions). (c) Additional details: cisternae of the endoplasmic reticulum are dilated; mitochondria mostly in intermediate-condensed form, occasionally swollen; cytosol and nucleoplasm are rather electroclear (diluted). S = sinusoid; BC = bile canaliculus; N = nucleus.



(a) (b)

Figure 2: Comparison at low magnification between slices after 60 min recovery at 38°C in the presence (b) or absence (a) of 2 mM ouabain. (a) Control liver slices after incubation for 90 min at 1°C followed by 60 min at 38°C. The ultrastructure has recovered well from the alterations in the cold preincubation (Figure 1) (see [6]). The cytosol and organelles appear to have a normal electron density. (b) Liver slice after 90 min at 1°C followed by 60 min at 37°C in the presence of 2 mM ouabain. A large number of rounded, clear vesicles are present, mainly localized in the Golgi region towards and around the bile canaliculi. The ground substance and other subcellular components are similar to the control in (a).

Table 2: Effect of various treatments on intracellular water content of liver slices in the presence and absence of ouabain.

Treatment	Intracellular water content (kg water/kg dry wt)[a] Incubation[b]					
	90 min at 1°C	Then, at 38°C for 60 min				
		Control	Treatment	Ouabain	Ouabain plus treatment	(n)
Cytochalasin B (100 μg/mL)	2.42 ± 0.33	1.48 ± 0.10	1.60 ± 0.17	1.72 ± 0.10	2.19 ± 0.11[c]	8
Cytochalasin E (33 μg/mL)	2.77 ± 0.07	1.75 ± 0.13	1.92 ± 0.08	1.96 ± 0.06	2.38 ± 0.13[c,d]	18
Colchicine (1 mM)	2.73 ± 0.10	1.68 ± 0.11	1.61 ± 0.08	1.98 ± 0.17	2.15 ± 0.25	10
Cl^- free medium, with NO_3^- as substitute	2.60 ± 0.09	1.52 ± 0.08	1.70 ± 0.08	2.00 ± 0.06	2.47 ± 0.13[c]	15

[a]Intracellular water was determined from total tissue water, measured gravimetrically, and the volume of distribution of inulin [9]. Values are the mean ± standard error of the mean.
[b]Slices were incubated in a medium containing 146 mM Na^+, 5 mM K^+, 1 mM Mg^{2+}, 1.3 mM Ca^{2+}, 161 mM Cl^-, 2 mM phosphate, 1 mM SO_4^{2-}, and Tris (10 mM pH 7.4). It was gassed with O_2. Cl-free medium contained NO_3^- instead of Cl^-. Ouabain was at 2 mM. Cytochalasins were added from stock solutions in dimethyl sulfoxide; final concentration in medium was 0.1% (v/v) DMSO.
[c]Significantly greater than value with ouabain alone, by t-test; $P < 0.01$.
[d]Significantly less than value after incubation at 1°C, by t-test; $P < 0.01$.

ions, and (c) the early recovery of ultrastructure, suggesting a different sensitivity of these functions to the decrease of ATP.

In the next paragraph, we aim to discuss these points.

4. Formation of Vesicles: Role of Anions, Protons, and Aquaporins

In the model proposed, in Figure 9, water that accumulates when Na^+/K^+-pump is inhibited by ouabain must be delocalized from cytosol and other compartments leading to the formation of vesicles. During a time-course of the recovery at warm incubation, in the presence or absence of ouabain, vesicles appear to originate by expansion of terminal cisternae of the endoplasmic reticulum and of Golgi elements [6] and have acidic contents [32] and their formation is dependent on the presence of Cl^- (Figures 3(a) and 3(b)) and vacuolar H^+-ATPase [33, 34] and, finally, on the integrity of Golgi apparatus, as demonstrated by treatment with Brefeldin A, which prevents vesicles formation (Figures 4(a) and 4(b)), extrusion of water, and recovery of ions [7]. The entry of chloride into vesicles represents the driving force for transport of Na^+ and water. In fact, replacement of Cl^- in the medium with NO^{3-} or SO_4^{2-} efficiently prevented water extrusion in the presence of ouabain (Table 2) and vesicles were almost absent (Figure 3(b)). Transfer of these slices in a medium containing chloride after only 15 min at 38°C leads to water extrusion (Figure 3(a)) and the appearance of vesicles [24, 25, 30]. The diuretic furosemide, which inhibits cotransport of Na^+ and Cl^-, caused effects similar to the absence of chloride [30].

Brefeldin A disrupts the structure and the function of Golgi apparatus and interferes with cell membrane traffic [35, 36]. While Brefeldin A alone had no effects on water and ion content and transport, in the presence of ouabain, 36 μM Brefeldin A partially prevented intracellular water extrusion (Figure 4(a)) and reduced the typical round electron-clear ouabain-dependent vesicles (Figure 4(b)) [7].

Vacuolar proton-dependent ATPase is present in a number of vesicles derived from endoplasmic reticulum and Golgi apparatus [37]. Schisselbauer and van Rossum [33] and van Rossum et al. [8] have explored the transport of protons into vesicles suggesting that this could provide the energy-dependent driving force for chloride transfer from cytosol into the vesicles. Isolated Golgi-derived vesicles accumulate chloride by a mechanism that requires ATP and can be saturated by external (or cytosolic) Cl^- [33]. The accumulation is prevented by the anion ionophore tributyltin, suggesting that the uptake of chloride occurs across the membrane against an electrochemical gradient. Its dependence on proton movements is demonstrated by the effect of DCCD (N,N′-dicyclohexylcarbodiimide), a proton ATPase inhibitor, and by the effects of CCCP (carbonyl cyanide m-chlorophenylhydrazone), a specific proton ionophore [34]. In conclusion, protons and a subsequent exchange of H^+ for cytosolic Na^+ or K^+ result in a net uptake of NaCl or KCl which induces water accumulation, confirming that the movements of protons against gradient and the chloride conductance represent the driving force for water movement into the vesicles.

A final question regards the water movements from cytosol (and nucleoplasm) into the vesicles. In the last few years, increasing evidences have demonstrated that water channels aquaporins (AQPs) are the effectors of transmembrane water movements in different mammalian tissues, including liver, kidney, lungs, and various epithelia [38]. The family of AQPs includes 13 members plus a number of isoforms, all involved in water transport and, occasionally, in glycerol transport. Water movements may be transcellular, paracellular, or intracellular: *transcellular* transport occurs at the level of basal or apical pole of a cell to extrude or reabsorb water, *paracellular* transport or exchange occurs at lateral surface of cells through junctional complexes, especially tight junctions, and *intracellular* water movements are responsible for water distribution among various subcellular compartments (i.e., from cytosol and nucleoplasm to various cisternae (endoplasmic reticulum, Golgi apparatus, lysosomes, phagosomes, peroxisomes) or to mitochondrial matrix), implying the presence of APQs in each subcompartment of cell membranes involved in water transport.

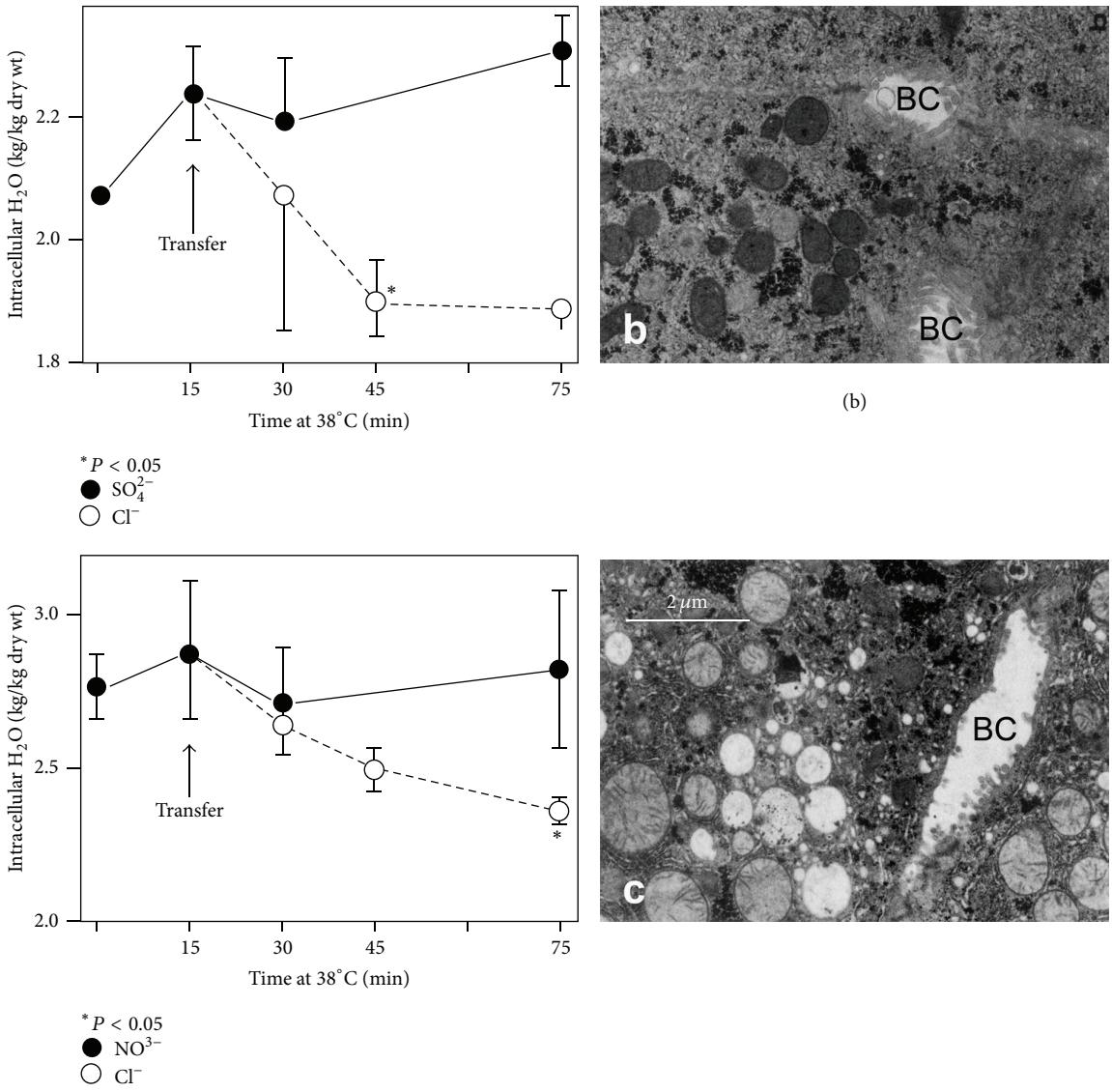


Figure 3: Liver slices were incubated at 1°C for 90 min and then at 38°C for 15 min in a Cl^- free medium (SO_4^{2-} or NO_3^{3-}) and, finally, were transferred in a Cl^- containing medium at 38°C for 60 min. (a) Water transport in the presence of ouabain was efficiently prevented by replacement of Cl^- in the medium with SO_4^{2-} (upper panel) or with NO_3^{3-} (lower panel). (b) At the end of incubation in Cl^- free medium in the presence of ouabain, the almost complete absence of typical vesicles was evident. (c) Transferring slices from Cl^- free medium to normal medium produces the appearance of ouabain-induced vesicles. BC = bile canaliculus.

This occurs in response of osmotic changes and is controlled by AQPs, through posttranslational modifications such as phosphorylation by a AMP/protein kinase which is effective in AQP8 activation and in its trafficking between membranes [39].

Three AQPs have been described in the liver: AQP1 in cholangiocytes, AQP8 in hepatocytes, and AQP9 confined to the sinusoidal (basal pole) membrane of hepatocytes [38]. However, this is a static conception for AQPs. In fact, it is clear that they can rapidly translocate in other compartments in response to osmotic or hormonal stimuli. AQP8 has a role in water movements in/out hepatocyte and among subcellular compartments and in bile formation, before secretion into the bile canaliculus (apical pole of the hepatocyte). Several treatments aimed to increase cAMP (forskolin) rapidly induced redistribution of AQP8 to the plasma membrane, augmenting water permeability [38]. García et al. [39] observed that colchicine, a microtubular inhibitor, blocked the effects of cAMP-dependent translocation, indicating that intracellular AQP8 traffic is microtubule dependent. We have detected AQP8 in the cytoplasm and intracellular vesicles of rat hepatocytes in liver slices by immunoelectron microscopy (Figure 5) (Russo, unpublished results).

In our model of rat liver slices, AQP8, at the end of 90 min cold + 70 min warm incubation in the absence of ouabain, was present in membranes of bile canaliculus

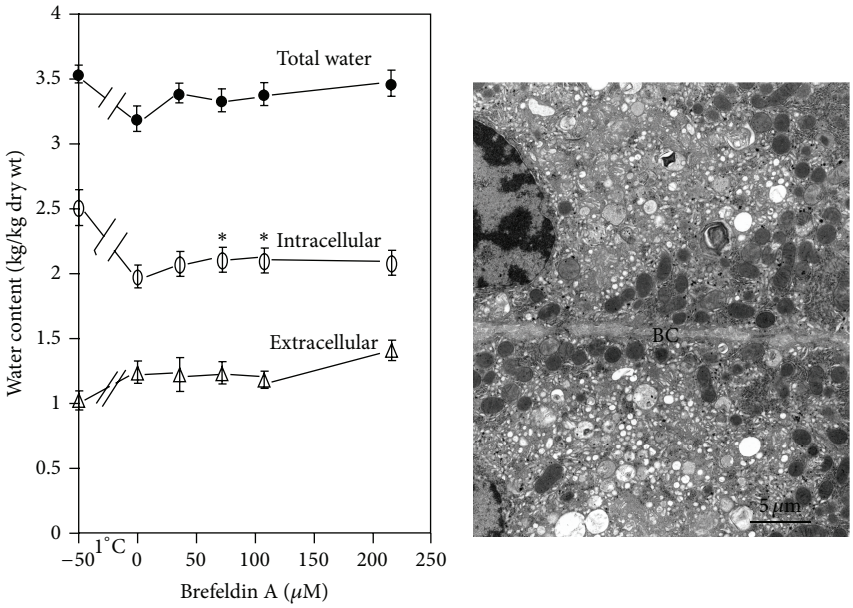


Figure 4: Effects of Brefeldin A on water transport and ultrastructure in the presence of 2 mM ouabain. Liver slices were incubated as described before (see also [7]). (a) Water transport inhibition. (b) Detail of Golgi region of two adjacent hepatocytes: evident Golgi disruption, with only small vesicles left. BC = bile canaliculus.

(Figures 5(a) and 5(b)) and, in small clusters, into the cytosol or associated with membranes (Figure 5(c)). In the presence of 2 mM ouabain, AQ8 was mostly associated with the typical ouabain-dependent vesicles (Figures 5(e) and 5(f)), even when these vesicles were fusing with plasma membrane (Figure 5(d), arrow), or in cytosolic small clusters (Figure 5(f), arrow). This suggests that the inhibition of Na/K-pump and the increase in intracellular water also stimulate, through an unknown pathway, the redistribution of AQ8. It is also unknown if a de novo synthesis of AQPs may occur.

The growth and enlargement of vesicles in the presence of ouabain is partially dependent on time of incubation and on concentration of ouabain (0.5 to 5.0 mM, reaching the maximal effect at 2 mM) [6].

5. Role of Exocytosis: Transport of Vesicles to the Secretory Pole

Ouabain-dependent vesicles, once formed, must be transported and secreted at the apical pole of the hepatocyte, that is, at the bile canaliculus (Figure 9). This requires an intact cytoskeletal system to generate force for polarized transport and a docking system between vesicle membrane and F-actin filaments. In addition, this system should be Ca dependent and ATP consuming, suggesting that the mechanism could be sensitive to the absence of Ca^{2+} and to the decrease of energy charge.

In fact, this step is dependent on the activity of actin components of the cytoskeleton [8]. Russo et al. [7] further explored this aspect for the first time giving evidence that a cytoskeleton-dependent component can contribute to volume regulation even when the Na-K ATPase is inactive.

Cytoskeleton involved in the hepatocyte exocytosis includes two main structures, microtubules [40] and actin microfilaments [41]. In a previous work, the effects of colchicine and other antimicrotubular agents on the ouabain-resistant cell volume control have been explored [24]. At concentrations normally used to disorganize microtubules (1 mM), colchicine was apparently ineffective, while showing some inhibition of vesicle-dependent mechanism when higher concentrations (10 mM) were used. However, these latter concentrations depress ATP synthesis [42] and appeared to be quite toxic as evidenced by large areas of necrosis in particular in the presence of ouabain [24].

The role of actin filaments have been explored by using phalloidin and cytochalasins A, B, D, and E which inhibit actin-dependent processes by, respectively, stabilizing actin filaments (phalloidin) or preventing their elongation (cytochalasins), in both cases resulting in a malfunctioning contractile force generating system.

Each of these agents reduced the extrusion of water, Na^+, and Cl^- in the presence and, to a lesser extent, in the absence of ouabain without affecting the reaccumulation of K^+ (Table 2). Furthermore, these agents alone also induced the type of vesiculation characteristic of the presence of ouabain. These findings provide a strong evidence in favor of a volume-regulating mechanism which is independent of the Na/K ATPase and also suggest that the vesicular mechanism, in the presence of ouabain, is potentially active also in its absence.

In the presence of ouabain, results with phalloidin and cytochalasins A, D, and E were all rather similar to the observations previously obtained with cytochalasin B alone [24].

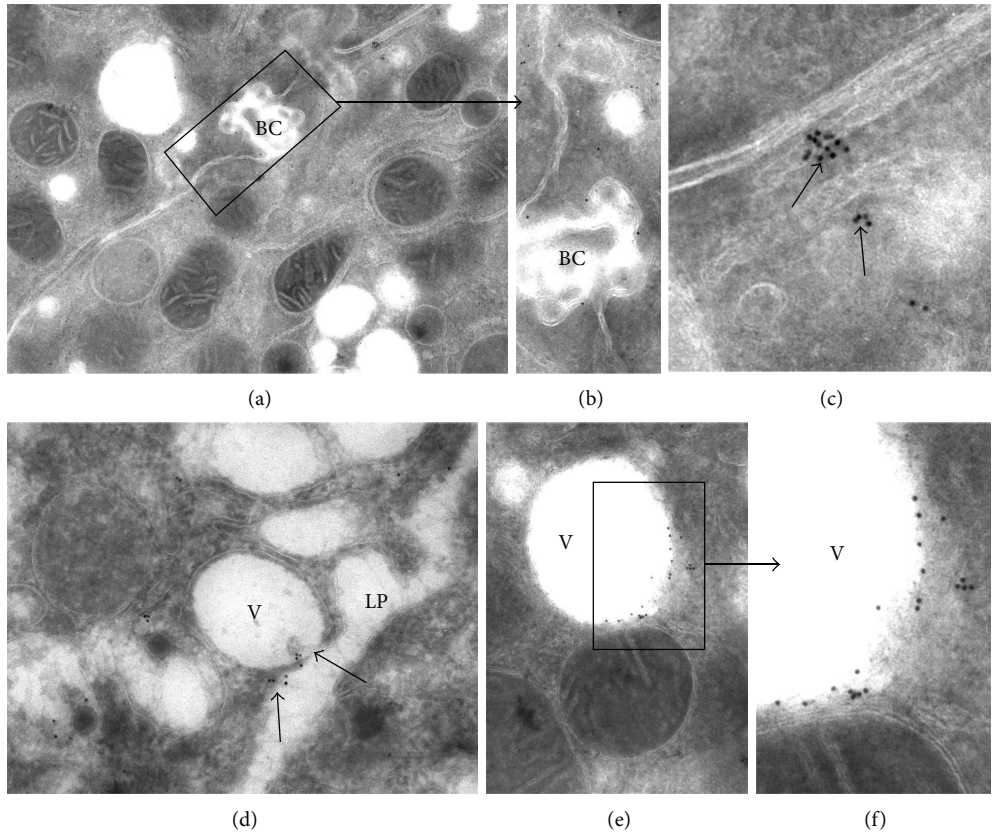


Figure 5: Aquaporin-8 localization in ouabain-induced vesicles and plasma membrane. Liver slices were incubated at 1°C for 90 min and then for 70 min at 38°C in the absence ((a), (b), and (c)) or presence ((d), (e), and (f)) of 2 mM ouabain. At the end of treatments, samples were slightly fixed (5 min in 4% paraformaldehyde) and cryofixed in liquid nitrogen. Ultrathin cryosections were obtained at the cryoultramicrotome, treated with a monoclonal antibody for AQ8 and then with protein A conjugated with 20 nm gold particles. Sections were postfixed and contrasted with osmium tetroxide vapors. (a) Control warm incubation. No gold particles are visible on the vesicle membranes. (b) Control warm incubation. Detail of (a). Few particles are visible on bile canalicular (BC) plasma membrane. (c) Control warm incubation. Occasional clusters of gold particles on the lateral plasma membrane. (d) Control warm incubation in the presence of 2 mM ouabain. A cluster of gold particles is present on the membranes where a typical electron-clear vesicle (V) is fusing with lateral plasma membrane (LP) (arrow). (e) Control warm incubation in the presence of 2 mM ouabain. A number of gold particles are visible on the membrane of an electron-clear vesicle. (f) Control warm incubation in the presence of 2 mM ouabain. Detail of (e) showing the close association between gold particles and vesicular membrane.

All these agents inhibited the ouabain-resistant extrusion of total tissue water to varying degrees and caused an increase in the number, size, and area of distribution beyond that seen with ouabain alone (Figures 6, 7, and 8) [7]. Furthermore, the vesicles typically lacked the orientation towards the Golgi and excretory pole as seen with ouabain alone (Figures 6(a) and 8(a)). This distribution suggests that the vesicles are formed but are unable to move on their normal path and to extrude their content at the canalicular pole. The reduced extrusion of total and intracellular water was, in each case, associated with an approximately equimolar reduction of the extrusions of Na^+ and Cl^-.

Incubation in the Ca^{2+}-free medium caused a partial inhibition of volume recovery (i.e., extrusion of water). However, complete absence of recovery in Ca^{2+}-free medium was observed in the presence of ouabain. The reduction of water extrusion seen in the presence of ouabain or absence of Ca^{2+} was due to mainly a slower initial loss. However, when the Ca^{2+}-free medium contained ouabain, there was total inhibition which was evident during the first 10 min at 37°C [7]. Thus, these two conditions appear to have an additive effect on the mechanism of water extrusion. Slices incubated in the Ca^{2+}-free medium in the absence or presence of ouabain showed a large number of rounded vesicles which were very similar to those seen with ouabain alone, except that they were now more widely distributed throughout the cell without a polarized (Golgi to canaliculus) distribution [7]. At higher magnification, the shape and content of the vesicles and the appearance of bile canaliculi were all very similar with and without Ca^{2+} despite the reduced water extrusion in its absence (Table 2). These observations suggest that (1) Ca^{2+} is not required for the increased formation and expansion of the ouabain-induced vesicles and (2) Ca^{2+} is needed for the vesicles to follow their normal path of

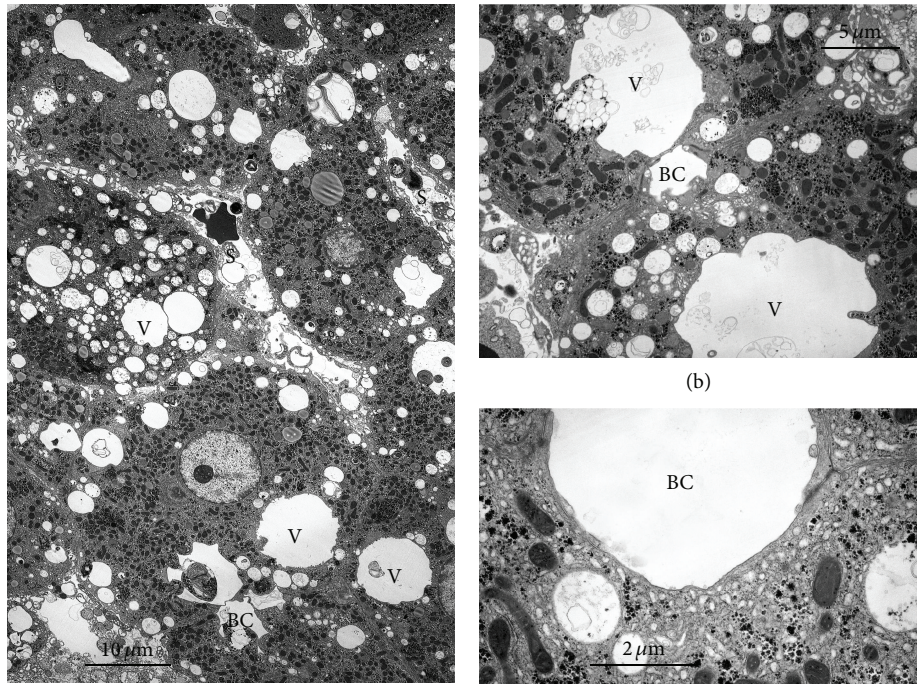


Figure 6: Liver slice incubated as indicated before in the presence of ouabain and cytochalasin D [7]. (a) Numerous vesicles of differing sizes are widely scattered throughout the cells and not particularly oriented to the canalicular region. The large vesicles tend to be more irregular in shape than the small ones probably due to their formation by fusion, and both have rather clear contents. The bile canaliculi appear partially disorganized showing a thin submembranous layer of cortical actin and a loss of microvilli. The perimeter of the canaliculi is round with a smooth surface. (b) Detail of large vesicles. Two cells form an irregular bile canaliculus (BC). Large cytoplasmic vesicles appear to fuse with each other and with the canaliculus. (c) Detail of a bile canaliculus almost devoid of microvilli. The layer of cortical actin is almost unrecognizable because of its thinness. BC = bile canaliculus; V = vesicles; S = sinusoid.

distribution in the Golgi and canalicular region and/or to undergo exocytosis, likely regulating the actin/myosin interaction in the molecular motor moving vesicles [43].

The exocytosis of vesicular content in the presence of ouabain has been less convincingly demonstrated in liver than in slices of renal cortex, with few "Ω-shaped" vesicles of fusion between vesicular and plasma. Electron microscopy of liver slices showed that, in the presence of ouabain, the electron-dense markers, OsO_4 and ferritin, were taken up by basolateral endosomes that fused with the rounded vesicles characteristic of the presence of ouabain. In such slices, electron-dense particles were also present in the bile canaliculi, suggesting exocytotic expulsion of the vesicular contents [24]. In subsequent experiments on fluid-phase endo- and exocytosis, with Lucifer yellow as marker, ouabain somewhat delayed release of the dye. This was consistent with the fusion of Lucifer yellow-containing endosomes with the ouabain-induced vesicles derived from intracytoplasmic membranes, thereby diluting the dye in intravesicular water [31]. It remains to be explored what molecules are specifically involved in docking vesicles to plasma membrane, in the process of fusion, and in opening to the extracellular space.

6. Ouabain-Resistant Volume Regulation in Various Mammalian Tissues

This mechanism has been explored in a number of other cell and tissue models with results that are consistent with our proposal. Results have been already published and/or reviewed before [8] in isolated hepatocytes [31], in hepatoma 3924A, a rat liver tumor of the Morris series which retain many morphological and differentiative features of the rat liver [9], kidney cortex [18], in myometrium [15], in lung [32, 44], and in avian salt gland [8]. Interestingly, in all these different models, results were in agreement with our proposed mechanism.

7. Corollaries in Human Diseases

The mechanisms of cell volume control and in particular the homeostasis of water are central in mammalian physiology and their alterations are crucial in many human pathological conditions.

Cloudy swelling was recognized by Virchow as cell volume alteration following ischemia, hypoxia, and a number of toxic biological (bacterial toxins) and chemical agents and

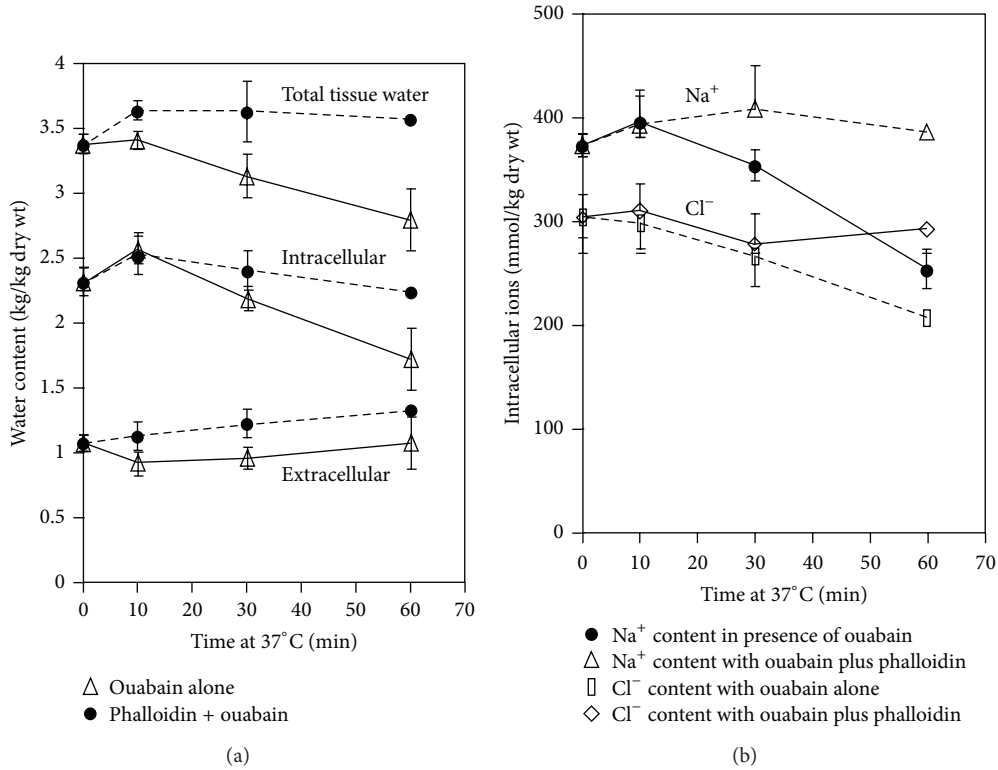


Figure 7: Effect of phalloidin (12 μM) in the presence of ouabain (2 mM) on water (a) and ion (b) content of liver slices incubated for 90 min at 1°C followed by 60 min at 37°C. Water content was paralleled to the retention of Na^+ and Cl^- [7].

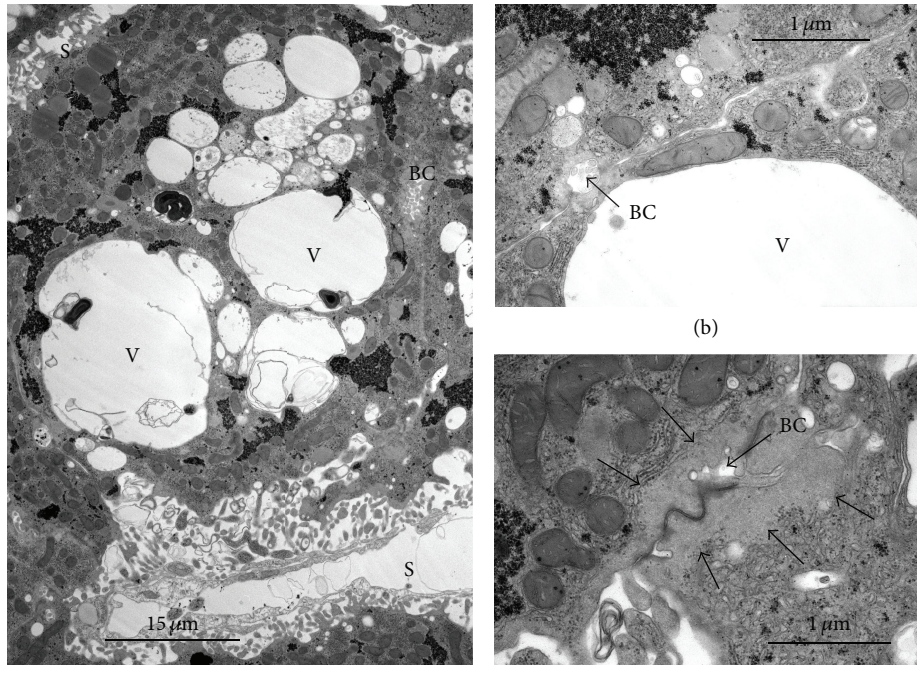


Figure 8: Liver slices were incubated for 90 min at 1°C followed by 60 min at 38°C, with 10 μM phalloidin plus 2 mM ouabain [7]. (a) This micrograph shows an increase in the size and number of vesicles. These show a rather irregular outline probably resulting from the fusion of smaller vesicles. The vesicular contents are clear and similar to that seen with ouabain alone. (b) Detail of a very large vesicle close to a small bile canaliculus. (c) Detail of homogeneous submembrane zone typical of the stabilization effect of phalloidin on cortical actin. BC = bile canaliculus; V = vesicles; S = sinusoid.

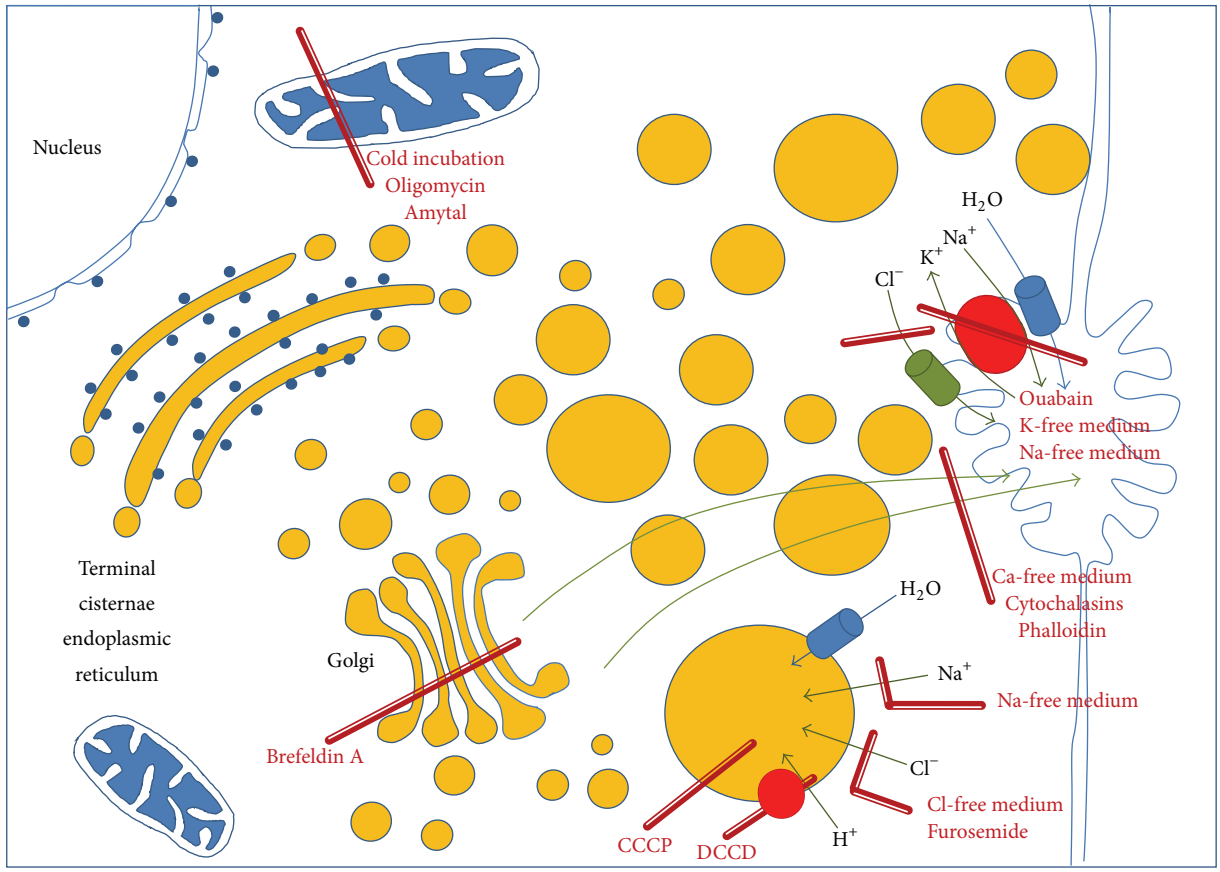


FIGURE 9: Schematic representation of the ouabain-resistant mechanism of cell volume control, the origin of vesicles, their secretion, and the effects of metabolic inhibitors. CCCP = carbonyl cyanide m-chlorophenylhydrazone; DCCD = N,N′-dicyclohexylcarbodiimide.

he clearly stated that extreme cloudy swelling preceded cell necrosis.

Water homeostasis is impaired in edema formation, when intracellular water progressively accumulates in extracellular space of diseased (i.e., inflamed) tissues for vessel permeability alterations.

Hypothermia may cause metabolic inhibition by cold with serious and irreversible damages especially in more susceptible tissues [45]. The lesson learned on water and ion movements and its recovery in the presence of warm exposure may be useful to determine the therapeutic conduct and treatment in case of freezing.

Organ storage for transplant uses hypothermic perfusion to preserve structure and function of organ tissues. Today, it is known that reperfusion at warm is the major responsible reason for cell damage leading to necrosis. Necrotic cells release alarmins which may activate a robust inflammatory response (hyperacute rejection) or trigger the mechanism to activate the chronic rejection. Additionally, the half-life of the transplanted organ, which, for example, in the case of the kidney is about 15 years, can be drastically shortened by the amount of necrosis during recovery from cold perfusion, reducing the number of functionally active cells and increasing the tissue fibrosis.

In conclusion, a better understanding of the mechanisms preserving water and ion homeostasis at cellular level could suggest new molecular targets and strategies to control cell volume, ion composition and structure of cell, and subcellular compartments, modifying the outcome of a number of important pathological conditions or medical situations.

8. Conclusions: What Remains to Do?

Vesicular transport of water and ion transport to control cell volume is a multistep mechanism that has been initially hypothesized on the ultrastructural (morphological) basis and then described in detail analyzing and correlating ion composition and ultrastructure in different experimental conditions affecting the various steps of the sequence.

Despite the fact that an amount of data coherent with this hypothesis has been accumulated, there is still a need of direct evidences to address several important points.

(1) The *ion composition of vesicles* should be explored by TEM microanalysis (combining X-ray microanalysis and EELA (electron energy loss analysis) spectrometry) on cryoultrathin sections of high pressure cryofixed samples.

(2) The *role of aquaporins* in the formation and growth of vesicles needs to be clarified. Different aquaporin isoforms can be constitutively present or rapidly

induced in different subcellular compartments allowing an optimal transport and traffic of water to avoid cytosolic and mitochondrial swelling. We still do not know which aquaporin isoforms are involved in different tissues and if they are synthesized *de novo* and/or are translocated from one compartment to another resulting in water redistribution and vesicle formation.

(3) The precise mechanism of transport of *ouabain-associated vesicles* to the canalicular pole should be investigated. It seems likely that, considering the central role of actin filaments, a molecular motor F-actin + myosin (myosin V or I) could be the best candidate for vesicles translocation [43]. Additionally, the cargo protein (possibly belonging to *rab* family) attaching vesicle to the myosin should be identified.

(4) Several types of *cell stress in human pathology* may show transient or persistent increase of cellular water and changes in ion distribution. It could be extremely interesting for clinical medicine and for better describing this mechanism to understand how cells adapt their gene expression to control this homeostatic mechanism and what are the master transcription factors involved in this adaptation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Placing Ion Channels into a Signaling Network of T Cells: From Maturing Thymocytes to Healthy T Lymphocytes or Leukemic T Lymphoblasts

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T leukemogenesis is a multistep process, where the genetic errors during T cell maturation cause the healthy progenitor to convert into the leukemic precursor that lost its ability to differentiate but possesses high potential for proliferation, self-renewal, and migration. A new misdirecting "leukemogenic" signaling network appears, composed by three types of participants which are encoded by (1) genes implicated in determined stages of T cell development but deregulated by translocations or mutations, (2) genes which normally do not participate in T cell development but are upregulated, and (3) nondifferentially expressed genes which become highly interconnected with genes expressed differentially. It appears that each of three groups may contain genes coding ion channels. In T cells, ion channels are implicated in regulation of cell cycle progression, differentiation, activation, migration, and cell death. In the present review we are going to reveal a relationship between different genetic defects, which drive the T cell neoplasias, with calcium signaling and ion channels. We suggest that changes in regulation of various ion channels in different types of the T leukemias may provide the intracellular ion microenvironment favorable to maintain self-renewal capacity, arrest differentiation, induce proliferation, and enhance motility.

1. Introduction

T cell acute lymphoblastic leukemias (T-ALL) are aggressive neoplastic disorders of the lymphoblasts committed to the T lineage. T-ALL accounts for 15% of pediatric and 25% of adult ALL cases [1]. It is widely accepted that the T cell leukemogenesis is tightly related to the normal T cell development. Various genetic errors during T cell maturation may cause the healthy progenitor to convert into a leukemic precursor cell that lost its ability to differentiate but possesses high potential for proliferation and self-renewal. Accordingly, leukemogenesis is a multistep process, where the genes encoding proteins implicated in the normal T cell development are deregulated. Among them there are transcriptional factors and tumor suppressors, receptors and signal transduction molecules, secreted molecules and growth factors, ion channels, and transporters. Specific genetic alterations define distinct groups of T-ALL with different profiles and levels of gene expression denominated as a gene expression signature. Moreover, gene expression signatures may vary in every special clinical case. Although numerous experimental and clinical reports and detailed reviews dealing with T-ALL are available, the relationships between various components of transcriptional and signaling regulatory networks are very complex and many issues are still to be addressed.

In the present review we are going to reveal a relationship between different abnormalities that drive the T cell neoplasias, with special accent on those occurring in the

expression of ion channels in this type of lymphoproliferative disorders. We suggest that changes in regulation of various ion channels in different types of the T-ALL may provide an intracellular ion microenvironment favorable to maintain self-renewal capacity, arrest differentiation, induce proliferation in T cell precursors, and enhance their motility.

We first review normal T cell maturation and recurrent cytogenetic abnormalities reported in the T-ALL, with their relation to main signaling pathways that contributed to leukemogenesis. Next, we address the question how Ca^{2+} signals may be involved in the T-ALL signaling network. Then we provide an overview of the current knowledge on the abnormal expression of ion channels in leukemias, from the point of view of their possible contribution to shaping and maintenance of Ca^{2+} signal, and other mechanisms where ion channels may be involved. And finally, we will discuss the possibility of targeting ion channels to improve the existing protocols of the T-ALL treatment.

2. T Cell Maturation in the Thymus

It is widely accepted that T leukemogenesis is a multistep process where several genetic lesions drastically mislead the normal thymocyte maturation [2]. A short overview of key events in early thymocyte development and their links to the leukemogenesis is presented at Figure 1.

T cells can be distinguished from other lymphoid lineages by the presence of the unique antigen-specific T cell receptor (TCR) on the cell surface. TCR is a transmembrane heterodimer composed of two chains, either $\alpha\beta$ or $\gamma\delta$. T cells of TCR$\alpha\beta$ lineage constitute the bulk of T cell populations in lymphoid organs and recognize antigen-derived peptides bound to the molecules of a major histocompatibility complex, of classes I or II (MHC-I or MHC-II), on the surface of antigen-presenting cells. T cells of TCR$\gamma\delta$ lineage are generally not MHC-restricted and particularly play an important role in protection of the mucosal tissues from the external infection ([3, 4]; revised in [5, 6]). Intracellular signaling through TCR depends on its association with a multimeric complex of membrane proteins referred to as CD3 and composed of four distinct polypeptide chains that assemble and function as three pairs of dimers ($\varepsilon\gamma$, $\varepsilon\delta$, and $\zeta\zeta$). Accordingly, TCR/CD3 protein complexes are defining features of T lineage and therefore are used as T cell markers. In addition, mature TCR$\alpha\beta$ lymphocytes bear CD4 or CD8 transmembrane proteins that serve as coreceptors for TCR in two subpopulations: T helpers ($CD4^+$) and cytotoxic T cells ($CD8^+$). The extracellular domains of CD4 and CD8 bind to conserved regions of MHC class II and MHC class I molecules, respectively. The coengagement of MHC molecule by both TCR and CD4 or CD8 enhances the avidity of T cell binding to its target and helps to initiate the cascade of intracellular signaling events.

Each of the several millions of T cells circulating in the organism possesses a unique TCR capable of recognizing its own MHC molecules, which present specific antigenic structure, distinct for every T cell clone and without cross-reactivity to self-antigens. Maturation of self-tolerant T cells, which differ in specificity of their TCR receptors and which are restricted to self-MHC, takes place in the thymus. The broad repertoire of TCR is generated by strictly ordered gene rearrangements in TCR loci encoding α, δ, β, and γ chains. The genomic locus coding every TCR chain contains gene clusters corresponding to the variable (V), the diversity (D), the join (J), and the constant (C) regions. Functional TCR genes are produced by the recombination process that assembles V, D, J, and C segments dispersed along a large genetic locus into a single transcriptable gene. Recombination activating genes RAG1 and RAG2 and terminal deoxynucleotidyl transferase (TdT) play a central role in the TCR rearrangement. At this phase, only those thymocytes survive, in which genetic rearrangements were productive and resulted in the appearance of a final unique lineal coding sequence of TCR chains. The apoptotic program is triggered in the rest of cells, which managed the rearrangement task poorly [5, 6]. The earliest T cells, lacking detectable CD4 and CD8 ($CD4^-CD8^-$), are, therefore, referred to as double-negative (DN) cells. Later on, they start to express both CD4 and CD8 ($CD4^+CD8^+$) and are denominated as double-positive (DP) cells. Finally, DP differentiates into single positive (SP) cells, either $CD4^+CD8^-$ or $CD4^-CD8^+$, which leave to periphery. DN T cells are subdivided into four subsets (DN1-4), based on the presence or absence of other cell surface molecules, including CD117, the receptor for stem cell growth factor c-kit; CD44, an adhesion molecule; CD25, the α chain of the IL-2 receptor (IL-2R), determining the IL-2R affinity [5, 7]. In every DN stage, characteristic events of TCR rearrangements take place. DN1 thymocytes express only c-kit and CD44 (c-kit^{++} $CD44^+$ $CD25^-$), but once they encounter the thymic environment and become resident in the cortex, they express CD25 and proliferate, becoming DN2 thymocytes (c-kit^{++} $CD44^+$ $CD25^+$). During this stage, rearrangement and transcription of germ line Dβ and Jβ segments belonging to TCRγ and TCRδ gene locus begin. However, the TCRα locus does not rearrange, because the regions of DNA encoding TCRα genes are not yet accessible to the recombinase machinery. At the late DN2 stage, T cell precursors are fully committed to the T cell lineage and reduce expression of both c-kit and CD44. Cells in transition from the DN2 to DN3 (c-kit^+ $CD44^-$ $CD25^+$) stages continue rearrangement of the TCRγ, TCRδ, and TCRβ chains, start to express CD3, and make the first major decision in T cell development: whether to join the TCR$\gamma\delta$ or TCR$\alpha\beta$ lineage [4, 5, 8, 9]. The choice to become a $\alpha\beta$ or $\gamma\delta$ T cell is dictated by when and how fast the genes, coding for each of the four receptor chains, successfully rearrange. Rearrangement of the β, γ, and δ loci begins during the DN2 stage. To become a TCR$\alpha\beta$, T cell must generate a TCRβ chain—an event that depends on successful VDJ rearrangement. To become a TCR$\gamma\delta$, however, a thymocyte must generate two functional proteins that depend on two separate in-frame rearrangement events [5]. Germline Vβ transcription and rearrangement to assembled DJβ complex occur in DN3 cells. Those DN3 cells that successfully rearrange their β chain and therefore commit to the TCR$\alpha\beta$ lineage lose expression of CD25 halt proliferation and enter the final

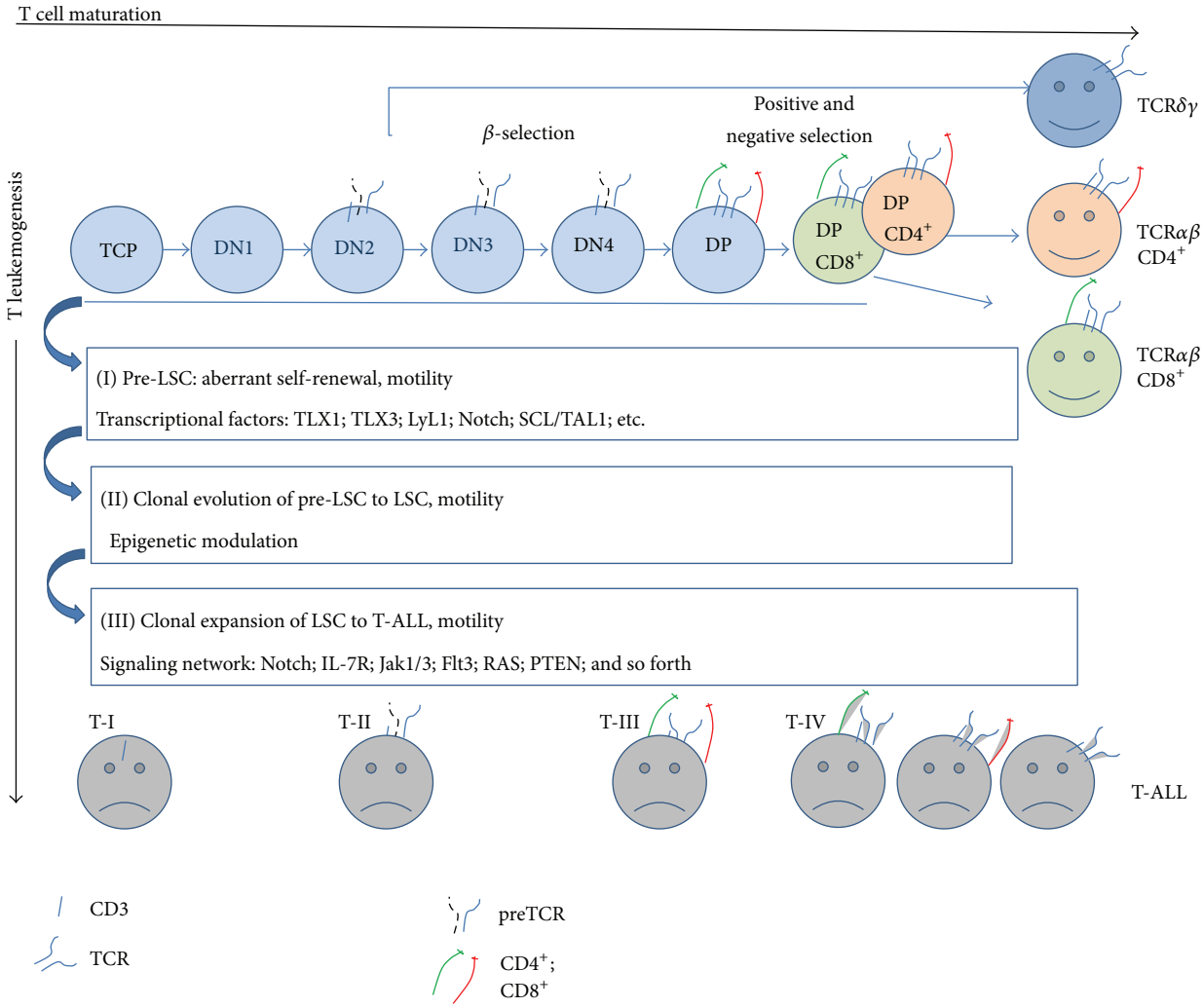


Figure 1: Hierarchical mutagenesis during T cell maturation causes different types of T-ALL (see text for details).

phase of their DN stage of development, DN4 (c-kit$^{low/-}$ CD44^{-} CD25^{-}), which maturate directly into DP thymocytes [5, 8]. Cellular differentiation involves epigenetic changes that regulate the transcription of genes encoding lineage-specific proteins and pluripotency factors. Developmental stage-specific regulation of transcriptional accessibility helps control V(D)J or VJ recombination. For example, Vβ segments on nonrearranged TCRβ alleles are accessible in DN thymocytes, when they recombine, and inaccessible in DP thymocytes, when they do not rearrange [8]. Assembly and expression of functional TCRγ and TCRδ chains that can pair to form TCR$\gamma\delta$ complexes in DN2/3 thymocytes drive cellular proliferation and promote differentiation into $\gamma\delta$T cells. In contrast, DN thymocytes that have successfully rearranged their TCRβ chains are valuable and are identified and expanded via a process known as β-selection. At this stage of development, DN3 thymocytes express the unique pre-Tα chain. It acts as a surrogate for the real TCRα chain, which has yet to rearrange, and assembles with a successfully rearranged and translated TCRβ chain, as well as CD3 complex. This precursor TCR/CD3 complex, known as the pre-TCR, is an important player in the next stages of thymocyte maturation. It initiates a signal transduction pathway resulting in maturation to the DN4 stage (c-kit^{-} CD44^{-} CD25^{-}), rapid proliferation in the subcapsular cortex, and suppression of further rearrangement of TCRβ chain genes, resulting in allelic exclusion of the β-chain locus, and induces development to the DP stage. After entering into the DP stage, cessation of proliferation and initiation of TCRα chain rearrangement occurs. Once a TCRα chain has successfully rearranged, it will dimerize with the TCRβ, replacing the pre-Tα chain. The mature TCR$\alpha\beta$ generates signals that lead to the next stages of positive and negative selection. During positive selection, only T cells are able to recognize host MHC survives, ensuing MHC-restriction. T cells that recognize self-MHC molecules and peptides with high affinity are deleted from the repertoire of cells during negative selection, providing self-tolerance [7]. The vast majority of DP thymocytes (more than 95%) never meets the selection criteria and dies by apoptosis within thymus. Upon its positive selection, TCR$\alpha\beta$ activates intracellular signals that stop TCRα rearrangements and promote differentiation

of DP cells into SP thymocytes, which leave the thymus as $CD4^+$ or $CD8^+$ TCR$\alpha\beta$ cells [4, 5, 7, 8].

Key steps in T cell maturation are controlled by several transcriptional regulators. The most important players in T cell ontogeny that are afterward deregulated in T leukemogenesis are Notch receptors proteins, and transcriptional factors of helix-loop-helix (HLH, the E2A, and HEB) and Homeobox (HOX) families [2, 6, 10].

Notch signaling pathway is evolutionarily conserved and operates in many cell types of different tissues at various developmental stages [11]. It is an important coordinator of different stages of the T cell maturation prior to the DP stage, including self-renewal of common lymphoid progenitor (CLP), commitment decision of the CLP toward T cell versus B-cell fate choice, and assembly of pre-TCR in immature thymocytes [2, 12, 13]. Mammals possess four Notch receptors (Notch 1–4) and five corresponding ligands (Delta-like 1, 3, 4, and Jagged 1 and 2). Mature Notch (1–4) receptor is a heterodimer that consists of Notch extracellular (NEC) and Notch transmembrane (NTM) domains associated noncovalently with the heterodimerization domain (HD). Ligand binding initiates the chain of proteolytic cleavages in NEC, culminating in the formation of intracellular Notch (ICN). Subsequently ICN translocates to the nucleus, to form a part of the large transcription activator complex. Notch signaling is regulated at multiple levels. Primarily, expression of Notch receptors and their ligands is restricted to a certain cell population within certain context. Another level of regulation is to insure that ICN is a short-lived protein, due to ubiquitylation within its degradation PEST domain rich in proline (P), glutamic acid (E), serine (S), and threonine (T) [11].

Notch1 activation in maturing thymocytes occurs upon engagement with its ligand, expressed on the thymic stromal cells [14, 15]. Among multiple Notch target genes, identified in T cells, there are transcriptional factors hes1 (and hes-related genes), Myc, NFAT, and NFκB [13].

Myc exhibits a steady expression at early stages and an abrupt drop in DP thymocytes [16]. A burst of proliferation, triggered by β-selection, requires preTCR, Notch, and Myc signaling [17] and is augmented by IL-7 [10]. The preTCR expression shuts down Notch signaling and therefore negatively regulates these mitogenic pathways [10]. Transcriptional factors NFAT and NFκB were shown to be upregulated in thymus at the transition of DN cells to the DP stage governed by pre-TCR signaling [18, 19]. Both NF-κB and NFAT regulate the transcription of genes, encoding cytokines, antiapoptotic proteins, and cell cycle regulators [20], and their activation is related to mitogen-activated protein kinase (Raf-MEK-Erk) pathway during positive selection [21].

E2A and HEB proteins bind DNA at specific E-box sites in the enhancers of many T cell specific regulatory genes like CD4 and preTα [2]. Members of the HOX family contribute, in some phases of early development, to coordinating the differentiation block, expression of the IL7 receptor, and choice of the $\alpha\beta$ versus $\gamma\delta$ lineage in DN2 [2].

Other important regulators of T cell maturation are numerous cytokines, produced by thymocytes themselves or by thymus stromal cells [22]. Among them, IL-2 and IL-7 are of special importance. IL-2 is an autocrine factor coming into the play as early as at DN2 (see above) and regulating the TCR-dependent clone expansion from this moment over the entire life of a T cell. In contrast, IL-7 is paracrine thymic cytokine produced by stromal cells in subcapsular zone, where DN cells are located. IL-7 participates in a coordination of the basic processes of early thymocyte development, namely, survival (through Bcl-2 upregulation), proliferation, and TCR rearrangement.

3. T-ALL as a False Mirror of the T Cell Maturation

As far as neoplastic transformation may occur at different stages of T cell differentiation (Figure 1), T-ALLs represent a very heterogeneous group of tumors with regard to their immunophenotype, cytogenetic, and clinical features and response to treatment. Arrest of differentiation program at specific stage of normal thymocyte development is a priming event in the T leukemogenesis. Simultaneously, uncontrolled cell growth and clonal expansion occur as a result of several mutations in the genes, involved in regulation of cellular metabolism, cell cycle control, and self-renewal of stem cells. The most of T-ALL oncogenes are downregulated at early stages of the thymocyte development or are not at all expressed in the normal thymus. Different mechanisms of genetic structural rearrangements are implicated in the T-ALL leukemogenesis: (1) translocations involving TCR loci, (2) gene fusion encoding chimeric proteins, and (3) deletions of tumor suppressive genes. As a result, corresponding gene is upregulated (activating mutation) or downregulated (suppressing mutation) (for detailed review, see [2]).

A hierarchical model of mutations, which contributed to the T leukemogenesis, was recently proposed [23]. In accordance with this model, the leukemogenesis occurs in several consequent steps (Figure 1). At the first stage, genetic alterations of transcription factors, leading to the activation of self-renewal program, occur in the immature T cell progenitors and generate the preleukemic stem cells (pre-LSCs). Self-renewal phenotype is essential for acquisition and accumulation of subsequent mutations. Activating mutations of signaling pathways important for T cell maturation allow expansion of pre-LSC, independent of the thymic microenvironment (niche). At the next stage, acquisition of mutations in epigenetic regulators results in transformation of pre-LSCs in LSCs. Finally, apparent T-ALL is generated by a clonal expansion of LSCs, retaining activating mutations in the cytokine signaling pathways.

Considering a rearrangement of gene loci, encoding variable regions of the TCR chains as a key event during the T cell maturation, it is not surprising that, in more than 30% of T-ALL patients, oncogenes are activated while being translocated and juxtaposed to one of the TCR loci [24, 25]. Partner oncogenes, involved in the TCR gene translocation, encode developmentally regulated transcription factors and signaling molecules. They are transcribed simultaneously at early stages of the thymocytes maturation and possess open chromatin configuration, which is vulnerable to the action

of recombinase enzymes RAG1 and RAG2. As a result, target genes are put adjacent to strong promoter or enhancer elements of the TCR genes.

The data about frequency of cytogenetic and molecular changes in T-ALL clinical cases are reviewed in detail elsewhere [2, 26]. Here we present summarizing remarks of the most frequent genetic lesions. Microarray-based gene expression analysis revealed that T-ALL patients cluster into four major groups based on the aberrant, subtype-specific expression of transcriptional factors TLX1 or TLX3 (HOX family), LYL1 (HLH family), and TAL1 oncogenes [27].

TLX1 and TLX3 are normally involved in the early embryogenesis being implicated in the organogenesis and differentiation of specific cell types. They are not expressed in developing T cells but seem to be involved in spleen development. In leukemogenesis, they are the most frequent aberrantly expressed genes becoming active due to the translocation involving the TCR loci [1, 28]. TLX1 is expressed in 7% of children and in about 30% of adults with T-ALL, displaying an early cortical phenotype. TLX3 is detected in 20% of children and in 13% of adults. Specific mechanisms of T cell transformation downstream of TLX1 involve the repression of the TCRα enhanceosome activity, the blockage of TCRα rearrangement, and downregulation of mitotic control genes, which induces the loss of the mitotic checkpoint in nontransformed preleukemic thymocytes [29, 30].

The LYL1 gene is not normally expressed in T lineage. Its upregulation in the T-ALL is due to chromosomal translocation, which juxtaposes it with the T cell receptor β gene locus. LYL1 reflects an early arrest in the T cell differentiation. Accordingly, related T-ALLs universally express the early hematopoietic marker CD34 and for the most part lack the expression of both CD4 and CD8 [31]. LYL was shown to be also important for the angiogenesis [27]. Overexpression of TLX3 and LYL1 in leukemic patients correlates with a worse prognosis [27].

TAL1 is a homologue of LYL1. It is involved in the embryonic and adult hematopoiesis and in the angiogenesis but is not normally expressed in maturating T cells. It has been shown that TAL1 and its partners LMO1/2 are coexpressed in the most primitive thymocytes [2]. Genetic evidences that the TAL-1 aberrant overexpression may involve t(1;14) (p32:q11) translocation or submicroscopic interstitial 1p32 deletion with resulting fusion of TAL1 with SIL promoter were provided. TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB and interfering with several E47/HEB target genes critical for the thymocyte differentiation [32]. TAL1-positive leukemias show the transcriptional upregulation of CD3 and TCR genes, developmental arrest in DP stage, and an overexpression of antiapoptotic gene Bcl2 [1, 33]. Recently, Sanda and coworkers (2012) have identified a set of transcriptional regulators that collaborate with TAL1 to generate a "core" regulatory circuit that contributes to the initiation and maintenance of human T-ALL [34]. Among TAL1 regulatory partners other transcriptional factors like LMO1/2, HEB, E2A, GATA3, and RUNX1 were found among others. An elevated expression of the TAL1/SCL is detected in over 60% of children and adults [35]. Patients with TAL1/SCL activation respond poorly to existing therapy, ensuing that no more than 50% of patients survive 5 years after diagnostics [1].

Cryptic deletion of the INK4/ARF locus is another frequent anomaly, detected in about 65% T-ALL, which results in cell cycle control defects [2].

Notch mutations are very frequent genetic alterations found in over 50% of T-ALL clinical cases, irrespective to their stage of the differentiation arrest [27, 36]. In contrast to T-ALL, Notch1 mutations are not found in B-ALL [36] and are seen only rarely in acute myeloid leukemia (AML) [37]. Since Notch coordinates self-renewal program of early lymphoid progenitors, activating Notch1 mutations increase the self-renewal capacity of the LSC, resulting in their susceptibility to acquire and accumulate additional genetic abnormalities. Thus, Notch1 signaling deregulation is considered to be crucial for the T cell leukemogenesis. Most common causes in Notch1 signaling deregulation are activating mutations clustered in regions coding HD and PEST domains [38], whereas HD mutations seem to enable the ligand-independent Notch cleavage resulting in the constitutive activation of the Notch protein [39], PEST domain mutations are thought to stabilize the structure and prolong the half-life of the active Notch 1 [40]. In rare cases (<1% of T-ALL), the expression of truncated ligand-independent and constitutively active Notch1 receptor is caused by rearrangement translocation, which juxtaposes the C-terminal region of human Notch1 gene to the TCR-β enhancer [41]. Additionally, the half-life of Notch protein may be also increased due to loss-of-function mutations in the FBXW7 gene, coding for a component of ubiquitin ligase complex. In addition to the Notch1, it degrades various proteins, important for the T-ALL pathogenesis such as Myc and cyclin E. The FBXW7 mutations result in inability to bind to its target proteins (Notch1) or bind its targets but fail to tag them for degradation (Myc), in both cases prolonging their half-life. FBXW7 gene lesion was identified in various T-ALL cell lines and in significant number (20%) of T-ALL patients, most of those undergoing disease relapse and resistance to treatment [1, 28].

Aberrant Notch1 activation in T-ALL is suggested to promote deregulated proliferation and prevent apoptosis. Molecular mechanism of Notch-mediated cell-cycle progression was shown to involve activation of c-myc [36, 42], NFAT [43, 44], and AKT/PI3K pathway and inhibition of PTEN expression [45] (Figure 2). Among the targets, activated by the Notch1 in the T-ALL, transcriptional factors HES1, HERPI 1&2, and EGF-containing fibulin—like extracellular matrix protein 1 (EFEMP1), vascular endothelial growth factor VEGF, inhibitor of DNA binding 1 (ID1), and SnoRNAs of the box H/ACA quantitative accumulation (SHQ1), immune associated nucleotide 4 like 1 IAN4L1/GIMAP5, and coreceptor CD28 were also reported [46, 47]. Notch1 upregulation enhances the G1/S transition through the induction of Skp2 expression; Skp2 is the component of E-3 ligase complex that degrades p27Kip1 and p21Cip1, inhibitors of cyclin-CDK2/4 complexes [48]. NF-κB cascade seems also to be activated by the Notch1 upregulation, and attenuation of NF-κB resulted in T-ALL suppression, both *in vivo* and *in vitro* [49]. Inhibition of apoptosis may occur through Notch1

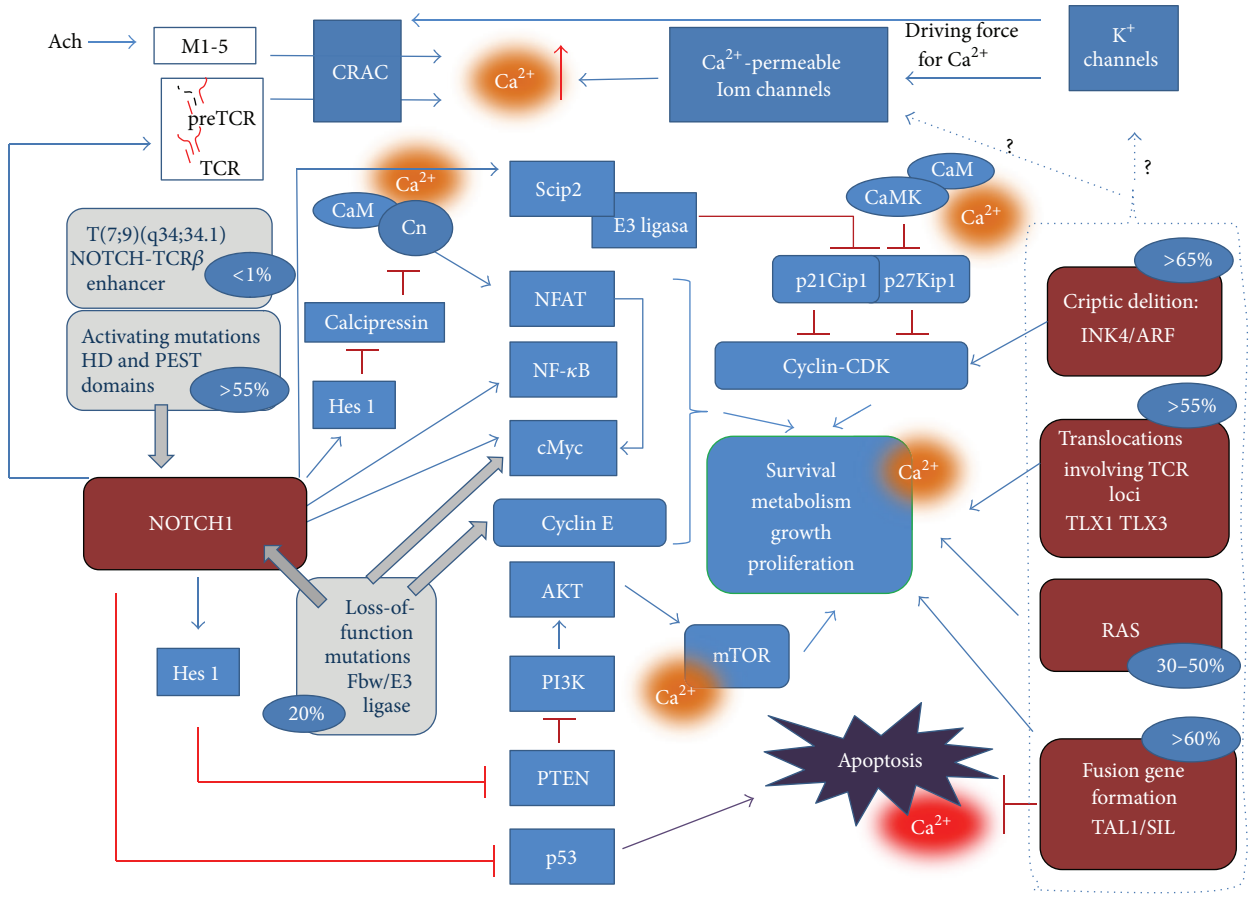


FIGURE 2: Ca^{2+}-dependent signaling pathways in T-ALL. Ca^{2+} influx may occur through CRAC channels, activated in receptor-dependent manner: (a) muscarinic receptors (M1–M5) are stimulated by Ach produced by leukemic cells themselves, but mechanisms of elevated Ach production are not studied yet; (b) TCR or preTCR receptors are activated through mechanisms which may engage Notch upregulation. Another mechanism for Ca^{2+} influx involves activation of nonselective Ca^{2+}-permeable channels, activated by different mechanisms. Driving force for sustained Ca^{2+} influx is generated by K^+ efflux through selective K^+ channels. Most important genetic lesions and signaling pathways are indicated, together with percentages for each type of lesion recognized in clinical cases. For more details, see the text.

activation of different pathways, including the NF-κB or the PKB/AKT/mTOR ones, leading to the p53 inhibition [25].

The question whether Notch aberrant expression is sufficient to induce T-ALL on its own was addressed. For this, different human gain-of-function Notch1 alleles were tested for their ability to drive an ectopic T cell development and to induce leukemia, when expressed in murine bone marrow progenitors [40, 50]. It was shown that the induction of the T cell leukemia is dependent on the Notch1 signal strength. Only rare Notch1 mutations with strong downstream signaling were able to drive the T cell leukemia *per se*, whereas common weak gain-of-function alleles were effective only in combination with a constitutively active Ras oncogene; nonetheless they gave rise to tumors sensible to inhibition of Notch1 signaling. Thus, Notch1 mutations, being indispensable for the majority of clinical T-ALL, require additional mutations in order to drive the leukemogenic process.

Ras proteins play a critical role in the transmission of survival signals from the cell membrane receptors to the intracellular transduction pathways. Mutations of RAS genes are common and have been described in various malignancies including acute leukemias [2]. They lead to the constitutive activation of the RAS-MAPK signaling cascade. Indeed, RAS lesions and activation of the tyrosine kinase genes, as a result of FLT3 and Janus kinase JAK1 mutation, or due to the ABL1/JAK2 gene fusion, have been identified, according to different studies, in 30 to 50% of clinical T-ALL cases [2]. The interest to identify these aberrations resides in the fact that they involve tyrosine kinases, for which specific inhibitors are known. These pathways are attractive candidates for a targeted therapy [51]. The AKT pathway plays a key role in the cell cycle progression and differentiation. Activation of AKT, either through PTEN loss-of-function mutations, activating mutations in the phosphatidyl inositol-3 kinase (PI3K) subunits, or AKT1 itself seem to be relatively common and occurs in nearly half of the (adult) T-ALL cases [52].

Genetic abnormalities that cause leukemia should meet a special favorable microenvironment to be realized. In pathways upregulated in the T-ALL, many functional elements depend directly or indirectly on the Ca^{2+} signaling (Figure 2).

4. TCR and Ca^{2+} Signaling in the T Cell Physiology

In resting healthy T cells, the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) is kept about 100–200 nm [53]. Changes in concentration of $[Ca^{2+}]_i$ represent ubiquitous signaling mechanism that regulates different phases of T cell physiology, including the proliferation, lineage decision in the maturation, and antigen activation [53–55]. To be able to maintain low $[Ca^{2+}]_i$ in resting cell or generate Ca^{2+} signal of a particular frequency and amplitude, time course, and intracellular location, every cell possesses the unique set of components, involved in this regulation, a so-called Ca^{2+} toolkit [56]. Ca^{2+} toolkit includes external sensors (plasma membrane receptors), signal transducers (such as G proteins and phospholipase C isoforms), signal-generating Ca^{2+}-permeable ion channels, selective K^+ channels that hyperpolarize plasma membrane and generate a driving force for Ca^{2+}, ER-localized Ca^{2+} storage proteins (such as calreticulin and calsequestrin), signal terminators that serve to return intracellular Ca^{2+} levels to prestimulation levels, such as the ER- and plasma membrane-localized Ca^{2+} pumps (SERCA and PMCA, resp.), plasma membrane exchangers, mitochondria and cytosolic buffer proteins, and Ca^{2+} sensors and effectors such as calmodulin (CaM) and its downstream targets, including CaM kinase (CaMK) and Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin (Cn) and protein kinase C (PKC). The Ca^{2+} toolkit may be modified during every developmental stage according to the changes in signaling events or may be remodeled in tumor cells to sustain the proliferation and avoid the cell death [56].

4.1. Mature T Cells. Antigen recognition through TCR receptor is a key event in the T lymphocyte physiology, leading to cell activation, clone expansion, and differentiation to effector cells (reviewed in [57]). Binding of the TCR to the MHC-Ag results in the assembly of the TCR/CD3 (TCR$\alpha\beta$/CD3$\gamma\varepsilon\delta\varepsilon\zeta$2) signaling complex, which is formed by membrane-resident molecules that are physically segregated in resting T cells. The TCR recognition module governs MHC-Ag recognition and the association of the protein tyrosine kinase (PTK) zeta-chain-associated protein kinase of 70 kDa (ZAP70) with the intracellular immunoreceptor-tyrosine-based activation motifs (ITAMs) of CD3. ITAMs are phosphorylated by the Src family PTK lymphocyte-specific protein tyrosine kinase (Lck). The Src kinase module is in charge of regulating the activity of the PTKs Lck and Fyn and ensures the TCR activation threshold. CD45 dephosphorylates inhibitory tyrosine of membrane-localized Src family kinases Fyn and Lck, previously recruited and activated by CD4 or CD8 co-receptors. Activated Fyn and Lck phosphorylates ITAMs on the CD3 ζ chains. This allows binding ZAP-70 to the ITAM, and activated ZAP-70 phosphorylates tyrosines on the adaptor protein LAT, which then attracts the phospholipase C (PLC-γ). Phosphatidylinositol-4,5-bisphosphate (PIP_2) is the substrate for PLC-γ. PLC-γ hydrolyses the PIP_2, generating two second messengers, inositol(1,4,5)trisphosphate ($InsP_3$) and diacylglycerol (DAG). Whereas DAG activates the RAS/PKC pathway, binding of $InsP_3$ to the InsP3R Ca^{2+}-permeable channel, located on the endoplasmic reticulum (ER) membrane, mediates the release of Ca^{2+} from this store. Depletion of ER Ca^{2+} stores is sensed by the stromal interaction molecule 1 (STIM1) protein integrated in ER membrane, which in turn induces the opening of ORAI1 calcium release-operated calcium channels (CRAC) located in plasma membrane and Ca^{2+} influx [58, 59].

The process of conversion the Ca^{2+} signal into a biological response is assured by Ca^{2+}-binding regulatory proteins, which together form an intricate network of feedback loops to control the location, amount, and effect of calcium influx. In T cells, CaM is considered as a major sensor and transducer of Ca^{2+} signals, and among CaM-binding signal traducers are Cn and Ca^{2+}/CaM-stimulated protein kinases II and IV (CaMKII and CsaMKIV) [60].

Cn activation is foremost dependent on the intracellular Ca^{2+} concentration (reviewed in [61]). The Cn complex is composed of a catalytic subunit A (CnA) and a regulatory subunit B (CnB), tightly associated at resting conditions. CnB possesses four Ca^{2+}-binding sites, two of which are of low and another two of high affinity. High-affinity sites are often referred to as structural ones and bind Ca^{2+} at nanomolar range, stabilizing the heterodimeric Cn structure. Little or no phosphatase activity is observed at $[Ca^{2+}]_i$ about 100 nM in resting cells. The low affinity sites with K_ds in the micromolar range are considered as Ca^{2+} sensors. During signaling events causing a Ca^{2+} rise, binding of Ca^{2+} ions to these sites results in sequential conformational changes (partially active form), dissociation of the CaM-binding region, binding of CaM, and displacement of the autoinhibitory inhibitory peptide from the active site (fully active form). Additionally, Cn activity is regulated by Ca^{2+}-independent endogenous inhibitor calcipressin [62].

In T cells, NFAT is considered as a major substrate for the Cn [63]. NFAT activity is regulated by its phosphorylation status. Under resting conditions, NFAT is highly phosphorylated. During activating events, dephosphorylation of multiple sites by Cn causes a conformational switch of NFAT protein that allows its translocation to the nucleus. Once inside the nucleolus, NFAT cooperates with multiple transcriptional partners and binds to specific DNA response elements to regulate the transcriptional program, which is specific for every cell type and for the stimulation pattern [64, 65]. Ca^{2+}/Cn/NFAT signaling was initially described in mature T cells as a critical regulator of the TCR-induced IL2 gene transcription [66–68]. Later it was shown that this pathway regulates the expression of numerous genes, including cytokines, as well as genes, encoding proteins involved in the regulation of survival and proliferation, apoptosis, and cell cycle [64, 65].

It was estimated that 75% of all activation-regulated genes in T cells demonstrate dependence on the Ca^{2+} influx [53]. The changes in $[Ca^{2+}]_i$ have been detected as the cell grows and passes through G1, G1/S, and mitosis. Ca^{2+} not only operates upstream of the cell-cycle machinery by regulating

the expression, activity, and/or location of the transcription factors that control expression of the G1 cyclins (FOS, JUN, MYC, CREB-ATF1 (activating transcription factor 1), and NFAT), but also acts more directly on the cyclins, CDKs, and/or their small protein inhibitors to regulate the assembly and activation of CDK complexes (reviewed in [56]).

Whereas initial T cell activation is related to short-term local $[Ca^{2+}]_i$ raise, subsequent events related to a new gene expression all require a sustained Ca^{2+} influx to maintain $[Ca^{2+}]_i$ at level higher than basal one in resting cells. In addition to CRAC channels, other Ca^{2+}-permeable channels have been found in T lymphocytes and proposed as Ca^{2+} influx pathways during different physiological events (as discussed below).

4.2. Maturing Thymocytes. TCR signaling coordinates thymocytes maturation as well [6]. The survival of "correctly" developed thymocytes in the thymus depends crucially on the signaling trough pre-TCR [6]. The process of β selection is the first critical checkpoint related to the pre-TCR signaling [69]. Only DN3 cells that have productively rearranged a TCRβ-chain, which can assemble with the invariant pre-Tα and CD3 molecules to form the pre-TCR complex, are selected for further differentiation [70]. Expression of the functional pre-TCR in the DN3 promotes survival and proliferation (DN4) and following differentiation to the DP stage. Remarkably, transition of the DN to the DP demonstrates apparent independence of the pre-TCR on the ligand. The ligand-independent nature of pre-TCR signaling has been attributed to its localization closely to other signaling molecules in lipid rafts and to the relatively low signaling threshold of pre-T cells [71, 72]. Notch1 is considered as a probable candidate for this signaling molecule, because the interaction between Notch and its Delta-like ligand expressed at stromal cells indeed plays an essential role in enabling the autonomous signaling capacity of the pre-TCR complex [17, 73]. These findings provided a functional basis for the observed pattern of the Notch receptor expression and activation in developing thymocytes, since several reports have showed that levels of Notch1 and Notch3 expression and activity are significantly higher in the DN than in the DP thymocytes [10, 74–76].

T cell development from immature DP thymocytes to the mature $CD4^+$ or $CD8^+$ SP stage is also coordinated by the TCR signaling. The current working model suggests that the interaction strength between the TCR and the self-MHC complex determinates the destiny of maturated thymocyte. Little-or-none signal means that this thymocyte is unable to recognize self-MHC and will undergo death by neglect. A too strong signal will lead to a negative selection to avoid a generalized T cell aggression toward self-tissues. Only signals of intermediate range will culminate in survival (positive selection) [6].

TCR engagement activates the Cn/NFAT signaling not only during the activation of mature T lymphocytes, but also at the specific steps of thymocytes development (revised in [77]). By Cn inactivating during haematopoietic development, it was demonstrated that this signaling pathway plays an important, nonredundant role in the regulation of lymphocyte developmental checkpoints, in contrast to development of myeloid lineages [78]. More specifically, absolute requirement for Cn in positive but not in negative selection was demonstrated [79]. Early studies using a Cn inhibitor cyclosporine A (CsA) showed an impaired development of the DP thymocytes into SP [80, 81]. NFAT is upregulated during the T cells maturation [18, 19]. Disruption of NFAT in the DP thymocytes results in fewer SP cells and is associated with defects in the expression of the antiapoptosis protein Bcl-2 by DP cells [82]. Since NFAT activation is strongly dependent on the Ca^{2+} rise, involvement of transport systems in regulation of thymocyte maturation should be expected. Nevertheless, there are still few studies on this subject.

In experiments with mouse models, it was shown that the constitutive pre-TCR signaling induces the NFAT and NF-κB activation, associated with an increased rate of the Ca^{2+} influx through the CRAC channels. Herewith, the biphasic nature of the cytosolic Ca^{2+} rise was observed, which differentially modulated the activities of the transcription factors NF-κB and NFAT in developing T cells [28].

Recently, Lo and colleagues have investigated the Ca^{2+} signaling during positive and negative selection in the $CD4^+$ MHCII-restricted T cells [83]. They demonstrated that negative selection induced a strong Ca^{2+} flux, and such a high Ca^{2+} peak might play a key role in inhibiting channel activity and decreasing transcript expression. On the other hand, a weaker yet more sustained Ca^{2+} flux was observed during positive selection. It was suggested that it may activate the Cn- and Erk-dependent pathways, leading to the survival and maturation. The authors consider that a sustained character rather than the magnitude of Ca^{2+} flux is the key function to support the positive selection. They identified a voltage-gated Na^+ channel (VGSC), essential for a positive selection of $CD4^+$ T cells. Pharmacological inhibition of the VGSC activity inhibited sustained Ca^{2+} influx induced by positive-selecting ligands and *in vitro* positive selection of $CD4^+$ but not $CD8^+$ T cells.

Interestingly, there are indications that the positive selection of $CD4^+$ T cells may involve somewhat more intense and long-lasting signals that are required for the positive selection of $CD8^+$ T cells [84]. Melichar and colleagues reported a distinct temporal pattern of the T cell receptor signals during positive versus negative selection in $CD8^+$ cells *in situ* [85]. However, in contrast with studies carried out on $CD4^+$ cells [84], they found that brief serial signaling events, which were separated by migratory periods and low cytosolic Ca^{2+}, correlated with the positive selection of MHCI-restricted thymocytes, whereas sustained signaling and arrest of thymocytes were associated with negative selection [85].

It is widely accepted that Ca^{2+} entry through CRAC channels is the main pathway to increase intracellular Ca^{2+} concentration in the peripheral blood T cells [58]. But CRAC seems not to play a central role in Ca^{2+} signaling during T cell maturation because T cell positive selection is normal in multiple separate knockouts of STIM and ORAI [86]. Loss-of-function STIM1 mutations were also reported in human patients [87]. Clinically, they demonstrated severe immunodeficiency with susceptibility to viral and bacterial

infections, but practically normal T cell repertoire. The latter clearly indicates that the T cell maturation was not greatly affected. But, as expected, their T cells were unable to generate Ca^{2+} rise in response to antigenic stimuli.

Thus, regulation of multiple decision steps in thymocyte development is coupled to a complex modulation of Ca^{2+} fluxes, but CRAC channels do not seem to necessarily play the central role. It was proposed that nonstore operated Ca^{2+} channel(s) which might operate independently of STIM and ORAI may be involved, or alternatively, CRAC channels play an important but redundant role [83, 88]. There are some evidences in favor of this hypothesis. For example, deletion of TRPM7 Ca^{2+}/Mg^{2+}-permeable channel with an intrinsic kinase activity results in a block in thymocyte development at the DN stage [89]. Thereby the presence of "unusual" ion channels reported in leukemic cell lines may reflect both normal developmental thymic events and leukemogenesis.

4.3. T-ALL. It was the subject of long time debate, whether the contribution of Ca^{2+} signaling is imperative for the tumor growth progression. Conventional view supported the idea that malignant cells are much less dependent on Ca^{2+} during the proliferation than healthy cells, and even loss of proliferative dependency on Ca^{2+} was considered as a hallmark of malignant transformation (reviewed in [56]). Although relative independence on Ca^{2+} may occur in some type of cancers, the situation in general is much more complex. The question rather should be discussed in terms of the Ca^{2+} signaling deregulation, where some elements of the Ca^{2+} toolkit in transformed cells are downregulated whereas others are upregulated. Monteith and colleagues have undertaken a thorough analysis of available data, concerning variations in the expression and activity of some Ca^{2+} channels and pumps in tumors and cancer cell lines. They did not reveal any uniform profile characteristic for cancerous cells [90]. Rather, they point out to some potentially predictable consequences of the trends. They also provided available data on the aberrant location of Ca^{2+} channels in many types of tumors, which could change the nature of the Ca^{2+} signal and a subsequent biological response.

Ca^{2+} homeostasis controls various cellular processes, which are relevant to the tumorigenesis, such as proliferation, apoptosis, gene transcription, and angiogenesis (see for review [90]). Ca^{2+} is a key regulator of proteins, implicated in the cell cycle regulation: Ras, immediate early genes in G0/G1 transition, retinoblastoma (Rb) protein in G2. In addition to cell cycle regulation, Ca^{2+} is implicated in cellular motility, which in turn contributes to the tumor invasion and metastasis. Ca^{2+} was shown to be an important regulator of genomic stability and transcription, critic events in leukemogenesis. There are limited studies that specifically address possible alterations in different elements of Ca^{2+} toolkit and Ca^{2+} signaling in tumorigenesis. Therefore it looks that question about these kinds of alterations should be addressed specifically for every type of tumors.

Taking into account that the TCR signaling related to the Ca^{2+} rise and Cn activation is a central coordinator of T cell physiology, we will analyze the possibility of contribution of this pathway to T cell malignancy.

Proliferation of leukemic cells is obviously antigen-independent. But being derived from the T-lymphoid precursors, arrested at different early stages of the development, T-ALLs demonstrate an astonishing heterogeneity in their TCR/CD3 phenotypes [91, 92]. Apropos, T-ALL classification by EGIL (European Group for the Immunological Classification of Leukemias), is based on the presence of CD3 and TCR chains [93]. The cytoplasmic and then membrane expression of CD3 is an early event in the T cell ontogeny [94]. Then the presence of CD3, either at the surface (sCD3) or in the cytoplasm (cCD3), is a determinative feature of these malignances [94]. TCR genes rearrangements are found in a majority of the T-ALL; the presence of TCR chains at the surface or in cytoplasm is mostly characteristic for mature stages [95]. Accordantly, T-ALLs are distributed in five immunophenotypic subtypes: pro-T-ALL (TI), pre-T-ALL (TII), cortical-T-ALL (TIII), and mature $\alpha\beta$ or mature $\gamma\delta$ (TIV) [93, Figure 2].

TI leukemias do not possess the TCR chains [2]. In some animal models like E2A-deficient mice, defects that prevent the pre-T cell antigen receptor expression even tend to accelerate the Notch-dependent lymphomagenesis [96]. Then the question arises, whether the pre-TCR or TCR is involved in the neoplastic transformation in other more mature T progenitors. It seems logical that the pre-TCR/TCR requirement for leukemogenesis greatly depends on the context and other signaling pathways involved in process. Accordingly, the role for the pre-TCR/TCR was studied in diverse mouse models, where leukemogenesis was provoked by abnormalities in different signaling pathways. It has been observed that the pre-TCR assists leukemogenesis, driven by the Notch activation [97–99], c-Myc overexpression [100], or Ikaros deficiency [101]. In contrast, other T cell leukemia mouse models, such as eg *Trp53*- or *ATM*-deficient mice, do not show such pre-TCR dependency [102, 103]. Some of these studies are described in more detail below.

Bellavia and colleagues used transgenic mice with upregulated constitutively active intracellular domain of the Notch3, which is ordinarily downregulated as thymocytes maturate [97]. The mice developed early and aggressive T cell neoplasias with features of immature thymocytes, including expression of the pTα, a defining component of the pre-T cell receptor, known to be a potent signaling complex provoking thymocyte survival, proliferation, and activation. Deletion of the pTα in Notch3 transgenic mice abrogates tumor development, indicating a crucial role for the pTα in the T cell leukemogenesis. In addition, the analysis of 30 samples, derived from children with T-ALL, demonstrated expressions of Notch3 and its target gene HES-1, as well as of pTα transcripts. Remarkably, the expression of all these genes was dramatically reduced or absent in the remission. In another clinical report, SCL overexpression was invariably associated with a high TCR expression in childhood T-ALL [1].

Similarly, pre-TCR expression was demonstrated to cooperate with TEL-Jak2 to transform thymocytes and induce rapid T-ALL [99]. However, in the pre-TCR-deficient TEL-JAK2 mice, the T cell leukemogenesis was only delayed but not canceled [104]. In Notch-dependent T-ALL, pre-TCR

signaling was required to condition mice for the Notch-dependent transformation but it was not required to sustain the malignant growth of the T-ALL [98]. Since the pre-TCR signaling is associated with a proliferative burst of thymocytes, accompanying differentiation stages [28], it was suggested that the pre-TCR-assisted proliferation in preleukemic cells increases the probability to acquire secondary oncogenic events, ultimately leading to a clonal disease [99]. Importantly, TCR expression induces the leukemic cell expansion in secondary lymphoid organs indicating the importance of the TCR-related signaling for the motility of leukemic cells [99].

Further studies of the pre-TCR signaling in leukemogenesis were designed to reveal the importance of the associated CD3 molecules for the leukemogenesis. The experiments were based on the fact that the pre-TCR and TCR require association with the Cd3ε for signaling [105]. Therefore, the Cd3ε-deficient cells have nonfunctional pre-TCR/TCRs. As it was shown, the absence of the pTα chain only slightly delays the appearance of the TAL1/LMO1-induced T-ALL in mice, while *CD3*ε-deficient mice do not develop the TAL1/LMO1-induced T-ALL [106]. Then it was concluded that pTα chain seems to play a minor role, but the *CD3*ε-mediated signal transduction pathway is essential for the transformation process. Similar results were obtained with another, namely, SCL/LMO1-induced T-ALL, using transgenic mice as a model [33]. They show that mice with SCL/LMO1 upregulation developed the Notch1 activation and T-ALL with a 100% penetrance, whereas, in strain with an additional Cd3ε deficiency, the penetrance of the disease was decreased by 48% and the median survival significantly increased. It was suggested that SCL, LMO1, and Notch1 together with an active pre-TCR/CD3 might represent the minimum set of complementing events for the transformation of susceptible thymocytes [33].

Immunophenotyping of frequently used T cell leukemia cell lines revealed high heterogeneity in the TCR expression [91]. It was suggested to consider the particular differentiation stage of each individual cell line, while using the T-cell leukemia lines as models for malignant or normal T cells.

Altogether the data available to the moment indicate that the pre-TCR/CD3 signaling accelerates the T cell leukemogenesis, being involved in the proliferation and in the expansion of leukemic cells to secondary lymphoid organs.

Cn is sustainably activated in T lymphoid malignances, both in animal models and biopsies from human lymphomas [43, 107–109]. The role of Cn activity in the pathogenesis of T-ALL was demonstrated in well-designed experiments with the usage of two different mouse models, related to human T leukemias [43]. In one model, bone marrow cells were retrovirally transduced with a construct, encoding the activating intracellular Notch1 domain. In a second model, transgenic mice expressed the TEJ/JAK2 fusion protein. In both models, mice developed the T-ALL, with a constitutive dephosphorylation of the NFAT in leukemic cells, indicating an aberrant Cn activation. Mice treatment with the Cn inhibitors CsA and FK506 resulted in disease remission with the hematopoiesis restoration [43].

Increased Cn/NFAT activity by the Notch signaling was shown to involve a downregulation of calcipressin through the Hes1-dependent mechanism [110]. As far as the Hes-1 is upregulated in T leukemias through the Notch1, this mechanism was proposed to be involved in the leukemogenesis [62, Figure 2]. Few reports have described different mechanisms of a sustained Cn activation due to the gain-of-function mutation in the *CnA* in T or B lymphoma-derived cell lines [111, 112]. In the EL4 murine T lymphoma cells, a missense mutation changed an evolutionary conserved aspartic acid to the asparagine within the autoinhibitory domain of the *CnA*α gene [111]. This substitution leads to the generation of a mutant CnAα, hypersensitive to Ca^{2+} [111]. But still, the elevated $[Ca^{2+}]_i$ is an indispensable factor to maintain the Cn activity, since mutations resulting in a persistent Cn activation, independent of Ca^{2+}, were never reported.

As was already mentioned earlier, the NFAT activation is a hallmark of the T-ALL. While activated mutations of NFAT genes have been never observed in human cancers, the aberrant NFAT signaling in tumors was suggested to involve either its overexpression and/or hyperactivity (reviewed in [113]). NFAT hyperactivity in the T-ALL is likely to be related to the Notch-dependent Cn upregulation (Figure 2). NFATc1 nuclear localization or dephosphorylation of both NFATc1 and NFATc2 were found in primary tumor samples and cell lines, derived from a patient with an aggressive T-cell lymphoma. Moreover treatment of these cell lines with CsA triggered the cell cycle inhibition and induced apoptosis [43].

5. Neglected Pathway in the T Cell Physiology: Lymphoid Cholinergic System Is Upregulated in Leukemias

Calcium mobilization, following the TCR ligation during the T cell activation is essential but is not the only way of the Ca^{2+} rise generation in T lymphocyte. Less considered pathway is related to a nonneuronal lymphoid cholinergic system. Lymphocytes possess all components of independent cholinergic system that include the acetylcholine (ACh), choline acetyltransferase (ChAT), its synthesizing enzyme, and both muscarinic (mAChR, M1–M5) and nicotinic (nAChR) ACh receptors [114–116]. Human T lymphocytes produce a small quantity of Ach and up-regulate the ChAT and mAChR mRNA expression in a response to the TCR activation. Ligand binding to the PLC-coupled M1, M3, and M5 mAChRs induces rapid increases in $[Ca^{2+}]_i$ with Ca^{2+} oscillation via the IP_3-evoked Ca^{2+} rise from the intracellular store. This pathway was suggested as an amplification mechanism to increase the IL2 and IL2R production [117] and the c-fos expression [116] during the TCR stimulation. In addition, M1 is known to play a critical role in the differentiation of $CD8^+$ cells into the CTL [118]. Emerging evidence indicates that mAChRs may be implicated in the regulation of the cell proliferation and cancer progression in leukemogenesis [119]. As for the T cell leukemias, the Ach production was shown to be drastically increased in different T-ALL-derived cell lines, when compared to the resting mononuclear cells [120]. It could be suggested that a sustained Ca^{2+} influx in leukemic cells may be maintained by elevated Ach production and autocrine stimulation through the mAChRs ligation.

6. What Do We Know about the Relationship between T Leukemia-Related Mutations/Pathways and Ion Channel Expression and Activity?

Taking into account the purpose of the present review, we have undertaken the bibliographic search of the data on possible involvement of transcriptional elements, misregulated in the T-ALL, in the ion channel expression and activity.

To identify TAL1 direct target genes, Palomero and colleagues have undertaken the chromatin immunoprecipitation experiments with antibodies raised against the TAL1 in Jurkat $CD4^+$ cell line as a model [121]. It was shown that the TAL1 binds to promoters of 71 target genes, encoding proteins important for many vital cellular processes. The list of target genes includes receptor and surface molecules, intracellular signal transduction elements, transcription factors, and DNA-associated proteins, proteins that participate in the DNA reparation, vesicular trafficking, drug resistance, secreted molecules and growth factors, ion channels, and transporters. Among ion channels and transporters which represent a significant portion of direct targets for the TAL1 the authors specifically indicated the CHRNA5 (subunit alpha-5 of the nicotinic acetylcholine receptor), ACCN2 (Amiloride-sensitive cation channel 2), CACNG4 (gamma-4 subunit of the L-type voltage-dependent calcium channel), KCNJ9 (G protein-activated inward rectifier potassium channel 3), SLC4A11 (sodium bicarbonate transporter-like protein 11), and OKB1 (organic cation transporter). The presence of other transcriptional factors as TAL1 target proteins suggests the existence of a very complex TAL1-dependent transcriptional network in the T-ALL with aberrant expression of a number of proteins implicated in different cell processes. Importantly, it was shown that TAL may act as an activator or as a repressor for target genes. Further experiments with other T leukemic lines (MOLT) and primary T-ALL samples demonstrated high levels of variation in the expression profiles of TAL1 target genes.

Notch 1 activation, being a hallmark of many types of the T-ALL, is also involved in the pathophysiology of other cancers. Then we undertook a search for data on the possible relationship between the Notch1 activation and ion channel expression. For example, aggressive and malignant state of glioblastoma multiforme (GBM), the most frequent and incurable type of the brain tumor of adults, was shown to be related to an increased activation of the Notch1 provoked by hypoxia [122]. Notch1 activation in turn induced the expression of transient receptor potential 6 (TRPC6) Ca^{2+}-permeable channels in primary samples and cell lines derived from GBM. Functionally, TRPC6 caused a sustained elevation of the intracellular Ca^{2+} coupled to the activation of the calcineurin-related NFAT pathway. TRPC6 was shown to be required for the development of the aggressive tumor phenotype, because a knockdown of the TRPC6 inhibited the glioma growth, invasion, and angiogenesis. Notch-dependent transcription of the TRPC6 was reported in pheochromocytoma PC12 cells [123]. Interestingly, TRPC6 mRNA was also found in the T-ALL cell line Jurkat, in contrast to T cells obtained from healthy donors, but the question about its relation to aberrant activation of Notch1 and NFAT was not addressed yet [124].

7. Nondifferentially Expressed Genes in T-ALL Signaling: Ion Channels May Be Involved

Traditionally, the T-ALL diagnostics and corresponding therapeutic strategies are based on the differential expression (DE) of genes, that occurred in the T-ALL patients as compared to healthy persons. However, as it was recently pointed out by Maiorov and colleagues (2013), the expression of some genes might not vary in the T-ALL, but, instead, these genes may be interconnected with highly differentially expressed ones [125]. They have proposed a network-based approach instead of the expression-based one for better understanding and management of the T-ALL and identified 19 significant subnetworks that represent clusters of functionally related, both DE and non-DE, genes. So non-DE genes code the proteins that in pathologic conditions may be involved in signaling networks different from those they normally belong to. It was proposed that non-DE genes could be essential in the interconnection of numerous DE genes and play important roles in malignant transformation of the precursor T cells. Purinergic receptor P2RX7, complement component C9, plasminogen, Ca^{2+}-binding protein CHGA, and peptide hydrolase MEPIA were pointed out among the non-DE genes in T-ALL.

8. Ion Channels in T Cell Physiology

Tumor cells survival and proliferation, activation, differentiation and malignant progression, invasiveness/migration (via volume regulation, polarization, cytoskeleton, and extracellular matrix reorganization), and, at last but not the least, the resistance to anticancer therapies, all these critically depend on the function of ion channels. T lymphocytes and leukemic T cells bear authentic orchestras of K^+-selective, Ca^{2+}-selective, and nonselective cation and anion channels. Wherein, their local combinations one with another and with other signaling components form a highly specific microenvironment, essential for cellular performance. The first purpose of this chapter is to give an overview of ion channels, expressed in T-cells, along with their known functions and giving emphasis to those, which are differentially expressed in healthy and leukemic cells. The second purpose is to revise the channel-to-channel functional communications, especially those related to a formation of specific Ca^{2+} signal. The third is to present, whenever it is possible, the structural and functional view on membrane signaling complexes, involving an ion channel as a core element.

Potassium- (K^+-) selective channels reported up to the date in healthy or neoplastic lymphoid cells belong to several families: voltage-gated (K_v), Ca^{2+}-activated (K_{Ca}), and tandem-pore domain (K2P) channels. K^+ channels may act via their ionotropic function or via noncanonical nonconducting mechanisms, that is, via direct interaction with other membrane or cytosolic proteins. Channels' mediated K^+

transport causes a change of membrane voltage, affects a driving force for Ca^{2+} influx, and, via changes of intracellular K^+, regulates the cell volume (in parallel with the activity of anion channels, see below) and, more specifically, affects the activity of the intracellular machinery, for example, activation of caspases in the course of apoptosis [126–128]. Membrane potential affects the cell cycle progression. Whereas G1/S transition is associated with a hyperpolarization (high K^+ conduction), the G2/M one occurs with a depolarization and low K^+ conductance [129]. Blockers of K^+ channels cause G1 arrest [130]. Also volume changes during cell cycle, for example, volume decrease during the M-phase prior to cytokinesis, requires a K^+ efflux via voltage-gated (e.g., $K_V10.2$) K^+ channels [131]. G1/S progression and G2/M transition also require Ca^{2+} bursts. The former includes activation of CDKs and culminates through phosphorylation of Rb1 in activation of E2F transcription factors. During progression of the G1 phase several processes like expression of AP1 (JUN and FOS) and CREB transcription factors, as well as regulation of cyclins, are calmodulin dependent, whereas transport of the transcription factor NFAT to nucleus requires the calcineurin-mediated dephosphorylation [56]. In T cells the Ca^{2+} signaling is critically dependent on the activity of partner K^+ channels as will be discussed below.

8.1. $K_v1.3$. $K_v1.3$ is the only one of 40 of the K_v family members known in mammals [136], which is expressed in healthy human T cells; in murine T cells, $K_v3.1$, $K_v1.1$, $K_v1.2$, and $K_v1.6$ were found in addition [126]. $K_v1.3$ is steeply activated by a depolarization with a half-activation at about −30 mV and inactivates up to by 95% in even more steeply voltage-dependent process, with a midpoint around −50 mV. At resting membrane potentials (−40−−50 mV) only a tiny fraction of $K_v1.3$ channels remains open at a steady state [137–139]. Thus, lower membrane potentials down to K^+ equilibrium, which are observed at the G1/S transition in the cell cycle, require the activity of some voltage-independent K^+ (e.g., K_{Ca} or K2P) channel(s), open at this voltage range. $K_v1.3$ channel at its C-terminus forms a functional complex with the β1-integrin, the PDZ-domain protein SAP97, linked to the p56Lck kinase and an adapter ZIP protein; the N-terminus in turn could bind a $K_v\beta2$ subunit (redox sensing), which may eventually interact with ZIP protein [140, Figure 3(a)]. Channel opening, provoked by membrane depolarization, stimulates functional and physical interactions between $K_v1.3$ and β1-integrin moieties and activates the integrin function, adhesion, and migration, whereas specific $K_v1.3$ channel blockage prevents the integrin signaling [141]. Functions of the $K_v1.3$ in T cells include but not restricted to (1) control of membrane potential against a depolarization challenge, depending on the T cells subset [142, 143]; (2) regulatory volume decrease (RVD), together with VSOR [144, 145], and apoptotic volume decrease (AVD) [146, 147]; (3) support of a sustained Ca^{2+} influx by CRAC, generation of Ca^{2+} oscillations (together with $K_{Ca}3.1$ and TRPM4). A sustained, lasting over hours, Ca^{2+} increase, which is primordial for new gene expression, is triggered in T cells via the Ca^{2+}/calcineurin/NFAT pathway [55]. A block of both $K_v1.3$ and $K_{Ca}3.1$ tends to abolish Ca^{2+} oscillations, with an impact on T cells proliferation [148]. In Jurkat leukemic T cells, selective inhibition of $K_v1.3$ abolished oscillations of the CRAC-mediated Ca^{2+} entry but not the average Ca^{2+} entry [149], which likely depends more on the K_{Ca} channels activity (see below). Obviously, CRAC and $K_{Ca}3.1$ activities are linked in a feedforward manner, so that the activation of $K_{Ca}3.1$ by Ca^{2+} influx via CRAC will hyperpolarize the membrane, increasing CRAC-mediated Ca^{2+} influx, and so forth. To reverse this Ca^{2+} increase, additional mechanisms are required which may be CRAC inactivation by Ca^{2+} [150] and/or membrane depolarization, caused by unique Ca^{2+}-impermeable member of the TRP family, the TRPM4 (see below). Kv1.3 is a predominant (several hundred copies per cell) K^+ channel in naïve human T cells, which functionally express just few $K_{Ca}3.1$. $K_v1.3$ plays a very essential role in the lymphocyte activation and associated Ca^{2+} signaling and IL-2 production. Upon the activation, differential behavior is observed in diverse T cell subsets. In activated T_{EM} (effector memory) cells $K_v1.3$ is selectively upregulated (high$K_v1.3$: low$K_{Ca}3.1$ phenotype); on the contrary, in T_{CM} (central memory) cells, Kv1.3 only modestly upregulated, whereas $K_{Ca}3.1$ dramatically (by 1.5 order of magnitude increase), producing low$K_v1.3$: high$K_{Ca}3.1$ phenotype [151–154]. Accordingly, specific inhibition or silencing of the $K_v1.3$ decreases the proliferation of T_{EM} without significant effect on T_{CM} and naïve cell population [155]. Immunosuppression can be achieved via the Kv1.3 specific inhibition and resulting depolarization, which attenuates the Ca^{2+} influx via CRAC. Selective $K_v1.3$ suppression was only efficient upon Ca^{2+}-dependent lymphocyte activation and not in the cases of CD28 or IL-2-induced activation, which is independent on the intracellular Ca^{2+} rise [156]. Curiously, when expressed in heterologous system, $K_v1.3$-mediated cells proliferation was unaffected in a poreless mutant (albeit sensitive to supposedly sole open-pore blockers, MgTx and PAP-1) but abolished in a mutant with altered voltage gating [157]. It appears then that the conformational change of $K_v1.3$ protein upon the channel opening may be sufficient for an efficient signaling, without involving a K^+ flux or membrane polarization. Such conformational coupling may be mediated by a close association of Kv1.3 channels with β1-integrin [141, 158].

Comparison of the $K_v1.3$ channels density in human peripheral blood resting T cells [137, 138, 141, 153, 159–163] and Jurkat lymphoblasts [139, 147, 149, 164–167] gives mean values of 380 and 215 active $K_v1.3$ copies per cell, respectively. In activated T cells, depending on subpopulation, the number of $K_v1.3$ channels could increase, modestly or very significantly, up to 1800 copies per cell [153]. Taking an approximately 3-fold larger membrane surface in Jurkat lymphoblasts into account, the $K_v1.3$ density, expressed as number of channels per unit area, is substantially lower in Jurkat cells. Yet, bearing in mind a complex Kv1.3 regulation within a signalosome, with all or some of interacting proteins involved [140] one may wonder, should such regulation be equally or more important than just a variation of the $K_v1.3$ copies numbers. Signaling complex, presented in Figure 3(a), exists within an immunological synapse. Thus,

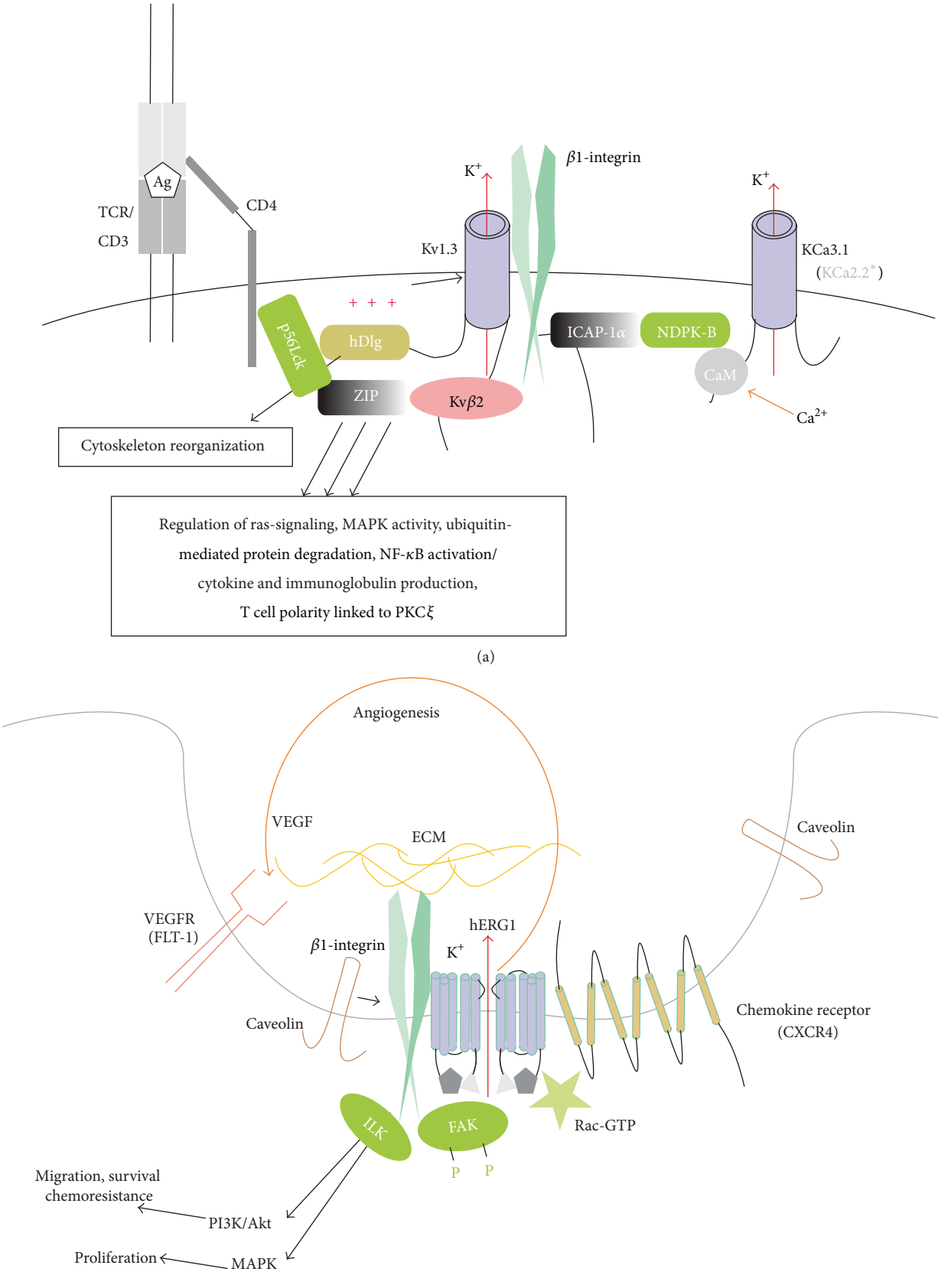
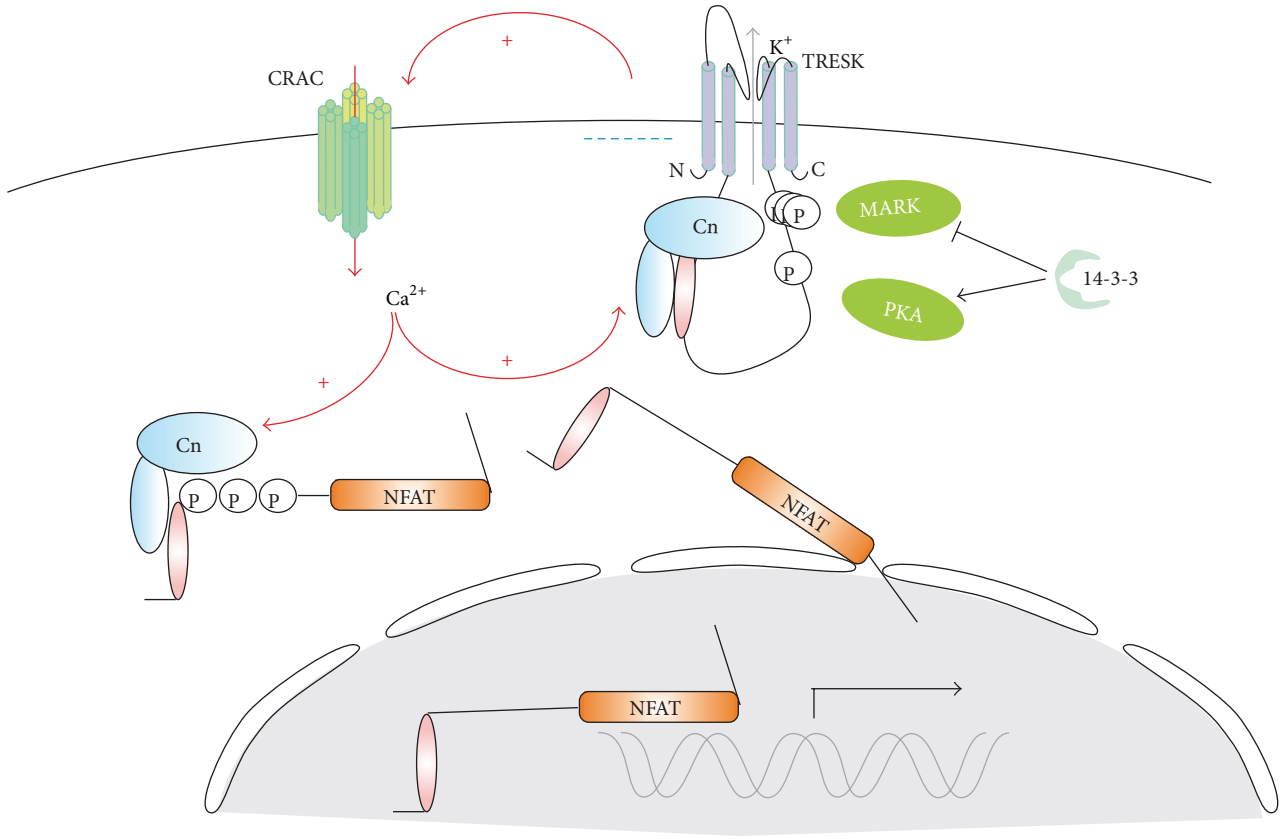


Figure 3: Continued.



(c)

FIGURE 3: K^+ channels and their partners in the plasma membrane of T-cells. (a) Kv1.3 channels are activated and inactivated by the membrane depolarization. Its cytosolic N terminus binds to auxiliary, NADP binding β-subunit Kvβ2, possessing oxidoreductase activity, and sensing cell metabolism and redox state. It also mediates trafficking of members of Kv1 subfamily to plasma membrane. Beta-subunit via multifunctional adapter protein ZIP is linked to p56Lck, phosphotyrosine kinase in a phosphotyrosine-independent way. ZIP regulates a variety of intracellular processes as indicated. In turn, p56Lck is connected via PDZ-domain (hDlg) to the last three residues at the C-terminus; binding of hDlg to a kinesin motor protein (GAKIN) and provoking cortical cytoskeleton reorganization. Open state Kv1.3 conformation favors its binding to β1-integrin and the latter via integrin cytoplasmic domain associated protein (ICAP1-1a) and nucleoside diphosphate kinase NDPK-B (activating KCa3.1 via phosphorylation) communicates to intermediate conductance Ca^{2+}-activated K^+ channel KCa3.1 (substituted for small conductance KCa2.2 in Jurkat cells). Kv1.3 can promote clustering of all aforementioned interacting proteins plus CD4 in the immunological synapse. A colocalization of Kv1.3, KCa3.1, and CRAC channels is essential for synapse stability, via local K^+ (K^+ accumulation in the cleft, membrane depolarization) and Ca^{2+} signaling (for a further discussion, see [127]). Linking of Kv1.3 to integrin allows a bidirectional signaling, when K^+ channel gating transmits to cell adhesion (inside-out signaling) and, vice versa, integrin-mediated external signal (outside-in) may be traduced to intracellular events via Kv1.3 and its interacting proteins. (b) hERG1 channels physically interact with β1-integrin receptors and are concentrated in caveolas, a type of lipid rafts, enriched of caveolins. Binary complexes of hERG1 with β1-integrin are typical for cancer cells. "Outside-in" signaling: hERG1 activation links β1-integrin adhesion to fibronectin (in the extracellular matrix, ECM) to the tyrosine phosphorylation of FAK and activation of small GTPase (Rac); both are coprecipitated with the channel protein. Caveolin association with β1-integrin promotes the channel activation [132]. FAK and ILK are primary targets for integrin-mediated cell adhesion, which transmits to the activation of MAPK, PI3K, and small GTPases. In leukemias trimolecular complexes can be also formed. In AML the third partner may be VEGF receptor, with an autocrine (hERG1-dependent) mechanism via VEGF secretion [133]. In ALL cells, hERG1/β1-integrin complex interacts with 7-TM domain (CXCR4, chemokine) receptors, stimulating signaling via ILK to Akt. Activation of this complex can be achieved via integrin engagement or CXCR4 chemical activation; ILK activity is suppressed by hERG1 specific block [134]. FAK (focal adhesion kinase), PI3K (phosphoinositide-3 kinase), VEGF (vascular endothelial growth factor), ILK (integrin linked kinase), and Akt (protein kinase B). (c) Tandem-pore domain K^+ channel TRESK is activated by dephosphorylation by calcineurin (Cn) and suppressed by a phosphorylation of certain serine residues within the cytosolic loop between transmembrane domains II and III by protein kinase A (PKA) and a second kinase, likewise MARK, or microtubule affinity-regulating kinase [135]. Cn is activated either directly by Ca^{2+}, Ca^{2+}-CaM, or Ca^{2+}-CaM-dependent kinase. Ca^{2+} influx in T-cells is mainly mediated by CRAC. Thus, TRESK and CRAC potentiate the activity of each other: TRESK generates driving force for Ca^{2+} influx by CRAC via membrane hyperpolarization, and CRAC shifts TRESK phosphorylation status to the dephosphorylated active form via Ca^{2+}-Cn. Sustained increase of Ca^{2+} via Cn dephosphorylates NFAT and allows its entrance through a nuclear pore, hence activating genes transcription. Both TRESK and NFAT contain a characteristic Cn-docking site (PQIVID for TRESK and PxIxIT for NFAT), thus coupling the Ca^{2+} signal positive feedback loop to genes expression via Cn activity. 14-3-3 protein docking to the phosphorylated S264 protects it from the dephosphorylation; additionally, 14-3-3 protein inhibits the second kinase.

in case of leukemic cells, another question is whether there are alternative regulatory proteins, which interact with $K_v1.3$ in these cells and what is the outcome of such interaction. Regulation of $K_v1.3$ by p56Lck kinase [164, 165] as well as a colocalization with TCR/CD3 [168] is preserved in Jurkat cells. We are not aware, however, of any evidence pro or contra the functional interaction of $K_v1.3$ with integrins in leukemic T cells. It should be noted that p56Lck kinase mediates oxygen sensing by $K_v1.3$; hypoxia is a common condition within a tumor tissue and tends to suppress the $K_v1.3$ channel activity, thus, T-cell proliferation under conditions of O_2 deprivation [169]. Healthy human leucocytes and Jurkat cells express a second type of β-subunit, KCNE (1 to 5). KCNE 4 but not 2 physically interacts and substantially modifies the Kv1.3 gating, decreases the channel surface expression, and impairs channel's targeting to lipid rafts [170]. Upon Jurkat activation with fetal bovine serum (FBS), KCNEs 2 and 4 were upregulated, but the same KCNEs were downregulated upon Jurkat activation by PHA [171]. Voltage gating of Kv1.3 was differentially modulated by the lipid rafts disintegration in healthy T cells [138] and Jurkat cells [139], suggesting a different microenvironment.

Here we will mainly discuss the roles, played by plasma membrane ion channels. However, several intracellular channels and transporters, expressed in nuclear, ER, and mitochondrial membranes, especially the latter, play important roles in cancer cells proliferation and survival [172]. In particular, some K^+ channels have dual expression, in the plasma and intracellular membranes. $K_v1.3$ channel, discussed above, is expressed both in plasma and inner mitochondrial membranes. Plasma membrane $K_v1.3$ may be selectively blocked by membrane-impermeable toxins, whereas mitochondrial $K_v1.3$ is only blocked by membrane-permeable drugs; blockage in the latter case induced apoptosis in cancer cells, including leukemic ones [172]. In addition, mito$K_v1.3$ interacts directly with Bax in Jurkat cells. This gives rise to a sequence of events, including the hyperpolarization of the inner mitochondrial membrane, ROS generation, and cytochrome c release from mitochondria, thus, promoting the intrinsic apoptotic pathway [173].

8.2. hERG1. hERG1 (human ether-a-go-go-related gene, Kv11.1) channels are normally expressed in excitable cells, primarily, in neurons and heart [174]. In heart it encodes a rapid delayed rectifier (I_{Kr}) K^+ current, activating at membrane depolarization and mediating action potential repolarization. It has very peculiar biophysical properties, possessing a rapid inactivation at positive potentials and a rapid relief of inactivation at negative ones. Therefore, the steady-state I/V relationship for this channel is bell-shaped, with a peak around −10 mV, where also the channel's open probability reached its maximum. Yet, channel's deactivation (a process of the reversal of activation) at negative potentials is slow (hundreds of milliseconds). Thus, at the peak and plateau of the action potential hERG1 conductance is greatly reduced due to the inactivation; it started to increase and reached its maximum at phase 3 repolarization [175]. In conclusion, hERG1 channels need to open first upon the action potential firing (depolarization), but they act (contribute) mainly on the way back, when a rapid relief from the inactivation followed by a slow deactivation allows a large K^+ efflux, promoting further repolarization. However, the midpoint of the hERG1 activation is around −30 mV, so that it possesses a significant activity down to −40 mV, which is close to resting potential in some nonexcitable cells, including lymphocytes. hERG1 is undetectable or expressed at very low levels in healthy immune cells. But it is frequently abnormally overexpressed in many cancer types, in particular, in acute lymphoblastic as well as in chronic lymphocytic (CLL) and both in acute and chronic myeloid (AML and CML) leukemias [134, 176–178]. hERG1 was found in self-renewing population of leukemic stem cells [179] and also in healthy precursor $CD34^+$ cells from peripheral blood, but only after cytokine stimulation [176]. Importantly, unlike in healthy cells, in tumors hERG1 is often found in functionally binary complexes with β1-integrin, or even in triple protein complexes (Figure 3(b)), thus linking external to intracellular signaling and vice versa [127, 180]. hERG1 can increase oncogenic potential in leukemias by affecting one of several ways facilitating leukemogenesis: (1) balance between proliferation and cell death; (2) invasiveness, depending on the fine balance between adhesion and motility, the latter being in turn dependent on polarized volume changes; (3) resistance to chemotherapy [127, 180]; (4) angiogenesis, via secretion of vascular endothelial growth factor (VEGF) and positively feedbacked microvesicles release [181, 182]. hERG1 surface expression depends on the interaction of different isoforms; presence of $hERG_{uso}$, which form heterotetramers with other hERG1 isoforms that resulted in an arrest of hERG1 in endoplasmic reticulum, with a further degradation, thus, substantial decreasing hERG1-mediated currents across the plasma membrane [183]. Cancer cells, including leukemias, often express a truncated hERG1b isoform, lacking the oxygen-sensing domain. It forms heterotetramers with full length isoform hERG1 and the higher is hERG1/hERG1b ratio, the lower is voltage threshold for the channel activation, and more hyperpolarized is the membrane potential. Upregulation of hERG1b during the S-phase consequently resulted in more depolarized membrane potential thus, assisting the cell cycle progression. On the other hand, at hypoxic conditions, which are typical for tumor progression, increase of hERG1/hERG1b ratio could hyperpolarize membrane potential, reducing K^+ loss, thus handicapping AVD and the apoptosis [184]. Although there is specific pharmacology against hERG1, its usage to treat hERG1 in leukemias is handicapped by the fact that cardiac hERG1 may be also affected, producing long QT-syndrome, which can eventually cause a fatal fibrillation. Yet, drugs only affecting the open channel state may be given a preference compared to blockers, acting also in the inactivated state, in case of hERG1 R-roscovitine and E4031, respectively [174]. In heart, hERG1 opens only shortly, at phase 3, whereas most of time hERG1 is either deactivated (at resting potential) or inactivated (at depolarized potentials). However, to properly address anti-hERG1 therapy to T-ALL treatment more studies on this type of leukemia are required. Except a single report on CEM cell line [177], hERG1 function and signaling were investigated on all types of leukemias but not on T-ALL. In particular, we and others were unable to

detect any measurable hERG1-mediated currents in Jurkat cells.

8.3. $K_{Ca}3.1$. $K_{Ca}3.1$ (IKCa1), intermediate conductance K_{Ca} channel, is voltage independent and is activated by Ca^{2+}-CaM (CaM bound to C-terminus) with an apparent K_d = 300 nM [126]. $K_{Ca}3.1$ also requires the phosphatidylinositol-3 (PI(3)P) for its activity, albeit it is not clear how this activation achieved; the effect on the channel is indirect [185]. Downstream to the PI(3)P is a reversible phosphorylation of channel's tyrosine 358; silencing of respective kinase (nucleoside diphosphate kinase B or NDPK-B) or phosphatase (histidine phosphatase PHPT-1) caused a suppression or activation of the $K_{Ca}3.1$ current and efficiently modulates, in opposite way by NDPK-B or PHPT, Ca^{2+} influx and $CD4^+$ T cells proliferation after stimulation [186, 187]. The activation of K_{Ca} provokes a stable hyperpolarization down to equilibrium potential for K^+, E_K [188]. This hyperpolarization is indispensable for T cells, apparently lacking depolarization Ca^{2+} channels (see below), where CRAC (SOCE) is believed to be a central Ca^{2+} influx channel. Although resting T cells express only few copies of $K_{Ca}3.1$, it is rapidly upregulated upon the activation and its expression level rises about 25-fold [189]. $K_{Ca}3.1$ channel is involved in various cellular processes, namely, activation via TCR-engagement and stabilization of the immunological synapse; control of membrane potential, RVD, cell migration, and tumor-related angiogenesis [126, 150, 190]. $K_{Ca}3.1$ interacts functionally with $K_v1.3$ and CRAC [126, 190].

8.4. $K_{Ca}2.2$. $K_{Ca}2.2$ (SKCa2), small conductance K_{Ca} channel, is expressed in a variety of healthy tissues, including brain and muscle [191]. It was also described in Jurkat leukemic cells, where its functional expression channels were high, comparable to the $K_{Ca}3.1$ expression in activated healthy T cells [189, 192, 193]. $K_{Ca}2.2$ is activated by Ca^{2+} with an apparent K_d = 700 nM and is upregulated by p56Lck [192]. It has a higher expression in G_0/G_1 compared to G2/M phase, supporting a sustained Ca^{2+} influx [194]. In Jurkat, $K_{Ca}2.2$ silencing but not $K_v1.3$ inhibition suppresses the Ca^{2+} entry; it can be restored by the ectopic expression of $K_{Ca}3.1$ [193]. This study obviously argues that $K_{Ca}2.2$ and $K_{Ca}3.1$ may be interchangeable, but it is not clear to which extent. It should be noted that in Jurkat the functional expression of $K_{Ca}2.2$ channels is constitutively high and can be compared to the $K_{Ca}3.1$ expression in activated healthy T cells [189, 193]. Remarkably, mitogenic stimulation instead of increase causes a 2-3-fold decrease of the $K_{Ca}2.2$ expression both at mRNA and functional expression in the membrane levels [192].

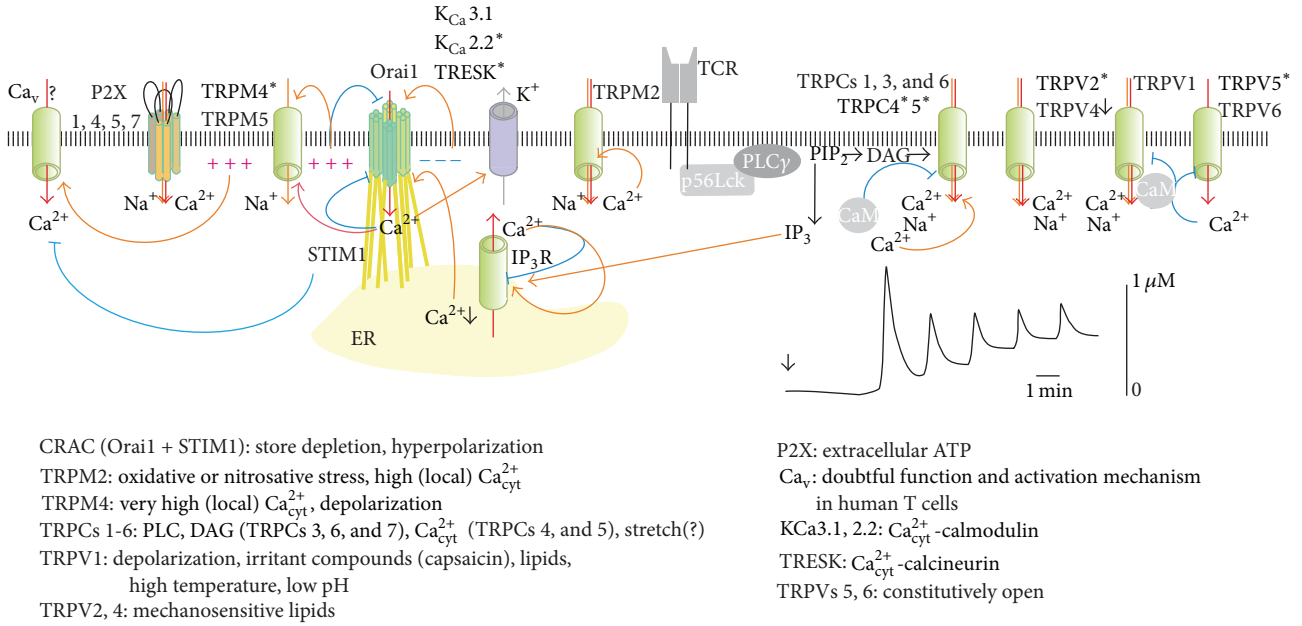
8.5. Tandem-Pore Domain (K2P) Channels. K2P represents K^+ selective channels, genetically unrelated to the former two families (K_{Ca} and K_V). Each channel subunit possesses two pore domains, and a minimal number of transmembrane segments, two per pore, as compared to the 6-TM subunit structure of K_{Ca} and K_V. Consequently, functional K2P channels are dimers; functional heterodimers were reported only for closely related TASK1 and TASK3, and the rest of active K2P channels are homodimers. There are 14 members of K2P family discovered in mammals (see [135] for a review). The activity of K2P channels is practically independent of the membrane voltage; rather, they are regulated by a variety of metabolic and physical factors. First reports on the functional expression of TASK1 and 3 in T cells [195] and of TRESK in Jurkat leukemic T cells [196, 197] in human models came relatively recently. Further on, TASK2 was found to be constitutively expressed in human T cells, but not in B or NK cells. Within T cells the relative expression was dependent on the T cells subset; TASK2 was strongly upregulated in $CD4^+$ and $CD8^+$ cells of patients with multiple sclerosis [198]. Later on it was shown that TASK2 expression in $CD4^+$ T cells strongly correlates with rheumatoid arthritis disease activity [199]. Mechanistic explications of both correlations are still lacking. However, roles of TASK1 and TASK3 in T cells function seem to be clearer. TASK1 and 3 belong to acid-sensitive K2P, which are inhibited by low external pH; for TASK1 and TASK1/3 heterodimer apparent pK is 7.3 and TASK3 homodimer displays pH-sensitivity outside the physiological range; intracellular pH changes were inefficient for both channels [200]. TASK channels are directly inhibited by anandamide and some synthetic cannabinoides. They are also inhibited by the stimulation of G_q-coupled receptors, possibly via the breakdown of PIP_2, with TASK1 and TASK1/3 heterodimer being more sensitive than TASK3 homodimer [135]. According to the data of Meuth and coworkers (2008) TASK channels may account for up to 40% of the outward K^+ current in human T cells; their inhibition caused a significant decrease in production of cytokines and cells proliferation [195]. In addition, the role of TASK2 and TRESK in the RVD is comparable to that of $K_v1.3$, whereas TASK-3 shows a lower contribution, which was somewhat higher in activated T cells [145]. TASK-3 is often amplified in different types of cancer; it is also expressed in mitochondrial inner membrane, which likely underlies its role in apoptosis. Yet its impact on the oncogenesis may be not necessarily negative and in some cases its high expression correlates with a better prognosis [128]. No studies of TASK expression in T-ALL are available to date. With TRESK the situation is different. Normally, it not only is abundantly expressed in neurons of dorsal ganglia, but also is reported in spleen and thymus in murine models [201]. Yet TRESK is strongly upregulated in several leukemic cell lines as well as in patients with T-ALL [197]. *In vivo* real time K^+ flux measurements and concurrent patch-clamp data on Jurkat cells revealed that both TRESK and $K_v1.3$ mediate AVD in the intrinsic apoptosis pathway, yet TRESK is transiently upregulated by apoptotic stimulus (staurosporine) and then completely inactivated in a half of hour, causing a strong membrane depolarization, whereas the $K_v1.3$ contribution to the K^+ efflux was more constant in time [147]. TRESK, which does not have close homologues, is unique among K2P channels, because its principle way of activation is Ca^{2+}-dependent (Figure 3(c)). TRESK possesses a large cytosolic loop between transmembrane segments 2 and 3, which harbors, similar to NFAT, the Cn docking site (the NFAT-like domain) and several phosphorylation sites. Phosphorylation by MARK and PKA in the two distinct sites caused TRESK inhibition. On the contrary, binding of Ca^{2+}-calcineurin to NFAT-like domain and dephosphorylation at

both sites provoked the TRESK activation [202]. This is a very important point, as activation of the NFAT-pathway by calcineurin at prolonged Ca^{2+} increase would cause also the TRESK activation. TRESK activation may be not only the consequence of a prolonged Ca^{2+} increase, but also its cause, due to a feedback support of the Ca^{2+} influx via the TRESK-mediated membrane hyperpolarization (Figure 3(c)). TRESK dependence on Ca^{2+} is different from that of K_{Ca} channels. K_{Ca} are directly activated by Ca^{2+} increase and immediately deactivated after Ca^{2+} removal. TRESK in contrast, once activated via dephosphorylation by calcineurin, could maintain its activity for a longer period after the Ca^{2+} removal. Thus, TRESK could be directly involved in the gene expression in T-ALL and may be considered as a plausible target for the immunomodulation [203, 204]. Yet, it is a general problem with K2P channels, and there are no specific blockers of any of mammal K2P channels, which handicaps studies of their roles *in vivo* as well as K2P-targetted therapies. However, recent advances in studies of low-molecular weight modulators for K2P are promising. In particular, antihistamine loratadine appears to block TRESK with a high affinity and does not display drastic collateral effects [205].

8.6. SOCE/CRAC (Orai1-STIM1). Ca^{2+} signaling in T cells differs greatly from those in excitable cells, which mainly rely on the voltage-dependent (depolarization-activated) Ca^{2+} channels of the plasma membrane. In lymphocytes, contrary to this, Ca^{2+} rise in cytosol is mediated by the store-operated Ca^{2+} entry (SOCE), named also CRAC, for Ca^{2+} release-activated Ca^{2+} current, that is, Ca^{2+}-selective current of the plasma membrane, activated by the Ca^{2+} depletion in the ER. It is extremely (factor > 1000) selective for Ca^{2+} over monovalent cations and has extremely low single channel conductance for Ca^{2+} (30 fS), which is compensated by a very high channels density (ca. 100 channels/μm^2). Due to its intrinsic inward rectification, Ca^{2+} influx via CRAC is strongly potentiated by membrane hyperpolarization, whereas depolarization reduces Ca^{2+} entry in lymphocytes [126]. For a long time it was thought that CRAC is mediated by members of TRPC subfamily (see below). Of course, these relatively weakly selective channels alone may not be responsible for a strongly Ca^{2+}-selective CRAC, but now there is also an ample evidence that TRPCs and CRAC are functionally and physically separated [206]. Crucial for molecular identification of CRAC was a study of severe combined immune deficiency (SCID), which was characterized by nonfunctional CRAC in T cells from some patients. In such a way, Orai1 was discovered as a pore-forming protein of CRAC, as single mutation in Orai1 from SCID patients was responsible for a defective CRAC function [207]. Orai does not relate to any known ion channel. In humans, three different isoforms form very similar CRAC channels, but in lymphocytes only Orai1 seems to be of functional importance [208]. Store depletion is communicated to Orai via STIM (stromal interaction molecule) proteins, located in the ER-membrane. In Ca^{2+} replete stores STIM proteins are randomly distributed at the membrane surface, and store depletion causes oligomerization of STIM in special contact areas with plasma membrane, where cytosolically exposed STIM domain directly interacted with both N- and C termini of Orai1, thus, causing CRAC activation (Figure 4, see also [208] for a recent review). There are two STIM isoforms in T cells, and both are important for CRAC, yet in murine models STIM1 or STIM2 deficiency caused a complete or partial abolishment of CRAC, respectively [209]. CRAC plays a central role in cytokines production, firstly, via Ca^{2+} activation of the NFAT transcription factor; conversely, it does not play a very significant role in the antibodies production by B cells (see [208] and references therein). Orai1 displays Orai1 displays two-time lower current density in Jurkat lymphoblasts as compared to resting T-cells; no significant difference in STIM1 expression was revealed between these two cellular models [210]. Relatively modest changes in the CRAC expression per se may not underlie changes in Ca^{2+} signaling in activated and malignant T cells. More likely, differences in the expression and regulation of "partner" K^+ channels, especially those activated by intracellular Ca^{2+} rise, may be more important for the modulation of the CRAC function (Figure 4). As CRAC-mediated Ca^{2+}-influx is inhibited by inflowing Ca^{2+} [211] and membrane depolarization, its activity may be further modulated by TRPs. TRPs differ greatly not only in the modes of their activation and expression in leukemic T-cells (see below), but also by their Ca^{2+}/Na^+ selectivity, hence differentially affecting membrane depolarization and Ca^{2+} signal. Figure 4 represents possible cross-talks between plasma membrane cation channels, including a feedback, provided by their differential dependence on the cytosolic Ca^{2+}. More scenarios, exploiting TRP and ORAI competition for STIM1, physical interactions, affecting ORAI surface expression and membrane localization, or existence of hybrid SOCE channels are discussed in the recent review by Saul and coworkers (2014) [212].

8.7. TRP (Transient Receptor Potential) Family Channels. TRP channels (currently, 28 described in mammals) are cationic, mostly nonselective and Ca^{2+}-permeable ones; they resemble by transmembrane topology but distantly are related to voltage-gated (e.g., K_v) channels [213, 214]. They are subdivided into TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPML (mucolipin), and TRPP (polycystin) subfamilies. TRPA, TRPP, and TRPML detection in lymphocytes was not addressed. However, TRPA may have a relatively restricted expression, mainly in sensory neurons, whereas TRPML forms intracellular channels [213].

TRPC 1, 3, and 6 were reported in PBL and Jurkat cells, Jurkat expresses additionally TRPC 4 and 5 [124]. All above channels are weakly selective (P_{Ca}/P_{Na} = 1–5), with linear or dual-rectifying I/V relation [215]. TRPC channels at cytosolic C-terminus contain two specific Ca^{2+}-binding sites, a EF-hand and CIRB, a calmodulin/IP3R-binding domain and may be directly regulated by intracellular Ca^{2+} [213]. Mostly, they are inhibited via Ca^{2+}/CaM pathway [216]. According to their properties, TRPC can be subdivided into TRPC1/4/5 and TRPC3/6/7 (*trpc2* is a pseudogene in humans) subgroups. A common property of the latter subgroup is their activation by DAG (or its membrane permeable form OAG), whereas



CRAC (Orai1 + STIM1): store depletion, hyperpolarization
TRPM2: oxidative or nitrosative stress, high (local) Ca^{2+}_{cyt}
TRPM4: very high (local) Ca^{2+}_{cyt}, depolarization
TRPCs 1-6: PLC, DAG (TRPCs 3, 6, and 7), Ca^{2+}_{cyt} (TRPCs 4, and 5), stretch(?)
TRPV1: depolarization, irritant compounds (capsaicin), lipids, high temperature, low pH
TRPV2, 4: mechanosensitive lipids

P2X: extracellular ATP
Ca_v: doubtful function and activation mechanism in human T cells
KCa3.1, 2.2: Ca^{2+}_{cyt}-calmodulin
TRESK: Ca^{2+}_{cyt}-calcineurin
TRPVs 5, 6: constitutively open

Figure 4: Ca^{2+} influx network in T cells. Channels, marked with asterisks, are overexpressed or present exclusively in T-ALL. Central is activation of CRAC- (Orai1+STIM1) mediated Ca^{2+} influx. Activation of PLC (e.g., via the T cell receptor) causes cleavage of PIP_2 with the production of DAG and soluble IP_3; the latter activates IP_3-receptor Ca^{2+} release channels of the endoplasmic reticulum. Ca^{2+} store depletion is sensed by specialized transmembrane sensors (STIM1), which oligomerize and move to special contact zones of ER with the plasma membrane, where they physically interact with the channel-forming proteins (Orai), forcing them to form active Ca^{2+} selective channel (CRAC), which mediates Ca^{2+} influx. Operation of CRAC is further modulated by the activity of other channels, which affects the membrane polarization and intracellular Ca^{2+}. Voltage-independent Ca^{2+}-dependent K^+ channels potentiate CRAC-mediated Ca^{2+} influx, lowering the membrane potential, thus increasing the driving force for Ca^{2+} uptake. Conversely, channels with a predominant Na^+ permeability (TRPM4) cause membrane depolarization and abrogation of the Ca^{2+} influx. Depending on the channel selectivity high Ca^{2+} (e.g., TRPV5) or indiscriminate Ca^{2+}/Na^+ (e.g., TRPC) as well as (when applicable) on the nature of the feedback (positive or negative, see respective loops) via Ca^{2+} and on the context (differential ways for the activation of particular ion channel), overall Ca^{2+} signal can be positively or negatively modulated. An idealized Ca^{2+} response to a mitogen stimulation, which contains both oscillatory and monotonous increase components, evidencing a feedback regulation via Ca^{2+}, is given as an example. Ways of the channels' activation are summarized below. From the left to the right: Ca_v (voltage-dependent Ca^{2+} channels), P2X (purinergic ionotropic receptors), TRP (transient receptor potential channels), Orai-STIM1 (CRAC, Ca^{2+} release-activated Ca^{2+} channel), K_{Ca} (Ca^{2+}-activated K^+ channels), TRESK (TWIK-like spinal cord K^+ channel), and CaM (calmodulin).

TRPC1/4/5, albeit being dependent on the PLC activity, are not responding to DAG [217]. OAG-activated current mediated the influx of Ca^{2+}, Ba^{2+}, and Sr^{2+} into Jurkat and PBLs; on the contrary, in case of SOCE (CRAC) activation cation influx was Ca^{2+}-selective, without measurable Ba^{2+} component [218]. In HPB-ALL, acute T lymphoid leukemia only TRPC1 was expressed at detectable levels among TRPCs 1 to 7, as compared to Jurkat, expressing TRPCs 1, 3, and 6 and more (see below). Ca^{2+} influx, induced in HPB-ALL cells by Δ^9-tetrahydrocannabinol, was less sensitive to SOCE and, based on pharmacological evidence, was mediated by TRPC1 via CBD-2 receptors and DAG [219]. Yet there are some doubts that TRPC1 could form functional homomeric channels, whereas it definitely can form heteromeric channels with all others TRPCs, including 4 and 5 [220] as well as with TRPV4 [221]; in the latter case translocation of TRPC1/TRPV4 complex to plasma membrane requires store depletion and STIM1 involvement. TRPCs 4 and 5, which are not present in PBLs, are robustly expressed in Jurkat cells [124]. Importantly, homomeric TRPC 5 or heteromeric TRPCs 4 and 5 act as a positive loop for the NO production: channel's nitrosylation provokes a formation of disulphide bond between neighboring cysteine residues, locking the channel in the open conformation, thus, promoting Ca^{2+} influx and Ca^{2+}-dependent NO production [222]. It remains to be elucidated whether such mechanism may operate in leukemic T cells. Partial silencing of TRPC3 in $CD4^+$ caused a reduction of intracellular Ca^{2+} only under limiting external Ca^{2+} (<0.1 mM) and slightly (<20%) decreased the proliferation of activated cells [124]. However, specific TRPC3 blocker, Pyr-3, strongly reduced the late phase of Ca^{2+} entry induced by anti-CD3 antibodies in Jurkat cells [223], confirming the TRPC3 role in Ca^{2+} signaling.

TRPM2 form nonrectifying channels, almost equally permeable to K^+, Na^+, and Ca^{2+} [224] and slightly less to Mg^{2+} [225]. TRPM2 is known as "chanzyme" because its dual function of ion channel and C-terminal enzyme domain [226]. (TRPM2) mRNA was highly abundant in $CD34^+$ precursors and in hematologic malignant cell lines of different

lineages, like Jurkat cells (T-ALL), K562 (erythromyeloblastoid leukemia), AML-193 (acute monocytic leukemia), U937-ecoR (monocytic leukemia), and TF-1 (erythroleukemia) [224, 227, 228]. TRPM2 is activated by reactive oxygen species, and this way may be the principle route of TRPM2 activation in Jurkat cells, whereas intracellular ligands, like cADPR, appear to be inefficient for this model system [229]. The combination of channels responsible for Ca^{2+} influx varied depending on the method of stimulation. In Jurkat cells, activation by ConA led to late Ca^{2+} influx via TRPM2 without CRAC involvement, whereas TRPC (TRPC3?) channels were responsible for anti-CD3-activated Ca^{2+} influx [223]. In $CD4^+$ T cells of healthy donors, stimulation by anti-CD3/anti-CD28, caused a transient increase of the TRPM2 expression [124]. TRPM2 mediates apoptosis upon the oxidative stress [227]. Overexpression of TRPM2 increases tumor susceptibility to oxidative stress, favoring the mitochondrial Ca^{2+} overload and triggering the intrinsic pathway of apoptosis, so TRPM2 is considered as a tumor suppressing factor [230].

TRPM4 encodes an intrinsically voltage-dependent outwardly-rectifying [231] monovalent cation channel, equally permeable to Na^+ and K^+ [232]. It requires high (micromolar) cytosolic Ca^{2+} for its activation [231]. Wenning et al. (2011) reported nondetectable levels of TRPM4 mRNA in naïve T cells, but a consistent expression both in Jurkat and effector $CD4^+$ cells; TRPM5 was expressed in stimulated and nonstimulated PBLs and Jurkat. TRPM4 and TRPM5 possess similar biophysical characteristics and are unique among TRP superfamily, which are virtually Ca^{2+} impermeable [124]. Thus, under physiological conditions TRPM4 mediates Na^+ influx, producing membrane depolarization [232]. This tends to reduce the CRAC-mediated Ca^{2+} influx. Indeed, suppression of TRPM4 in Jurkat transforms PHA-induced Ca^{2+} signal from oscillations to a higher sustained Ca^{2+} increase, resulting in a higher IL-2 production [232]. Similarly to TRPM4, TRPM5 is activated by high intracellular Ca^{2+}, but, in contrast to the former, it is strongly suppressed by external acidification [233], which may be relevant physiological situation under hypoxia in lesions. Analysis of polymorphisms in TRPM5 genes revealed correlation of childhood leukemia with certain genotypes [234]. Interestingly, TRPM5 gene is located in the human chromosome 11, which frequently shows aberrations for a number of hematological malignancies, including ALL [235]. In addition, on the protein level TRPM5 is conservatively regulated by residual proteins, formed in the Notch pathway, which in turn is important pathway associated with T-ALL [236]. However, in contrast to TRPM4, direct experimental demonstration of the TRPM5 channels activity in T cells is lacking so far.

TRPM7 is another, in addition to TRPM2, "chanzyme," consisting of ionotropic divalent cation-permeable channel and kinase, linked to cytosolic C-terminus [237]. It is ubiquitously expressed, but normally at relatively low levels [213]. *In vivo* current, generated by TRPM7 channels, is strongly outwardly rectifying, due to a blockage by cytosolic Mg^{2+} and, possibly, some additional internal factor [213]. Mg^{2+} block of TRPM7 channel and a role of a very similar TRPM6 channel in Mg^{2+} homeostasis led to a hypothesis that TRPM7 could equally participate in sensing and control of the intracellular Mg^{2+}. Yet, current experimental evidence is more against that in favor of this hypothesis [208, 213]. TRPM7 expression is higher in Jurkat as compared to healthy T cells [124]. Importantly, cell-specific TRPM7 deletion arrests T cells differentiation at the DN stage [89]. Although TRPM7 is the only channel up to date with an approved role in the lymphocyte development, it is not known, whether this role is mediated by Mg^{2+} or Ca^{2+} influx through its pore, kinase domain activity, or a combination of these factors. Interestingly, TRPM8, a crucial motor element for cell migration in glioblastomas [238] but oppositely acting in other tumor types (see [230] for a review), is expressed neither in healthy PBLs nor in Jurkat lymphoblasts [124].

There are two subgroups in the TRPV family: TRPV1/4 and highly Ca^{2+}-selective TRPV5/6 ones. TRPV1 (vanilloid receptor), the founding member, represents an outward-rectifying voltage-gated channel, which is upregulated by different physical factors (external pH, heat) and a great variety of unrelated chemical compounds (Figure 4) and is modulated by protein phosphorylation by PKA and PKC [239]. Its pore is flexible and the channel selectivity changes upon the activation and it depends also on the activation factor [216, 240]. TRPV1 is modestly selective for Ca^{2+} over Na^+, given values between 10 for capsaicin and 4 for heat [241]. Thus, the fraction of Ca^{2+} current inflowing the cell via TRPV1 counts only a few percent of the total current, mainly carried by Na^+. Yet, this Ca^{2+} influx is sufficient to cause the TRPV1 desensitization, which likely is caused by Cn-mediated dephosphorylation of several sites [216]. TRPV1/4 can be also considered as chemosensors; among the agonists for TRPV1, 2 and 4, cannabinoids should be mentioned [216, 242].

TRPV1 and TRPV2 are the most expressed among TRPV1/4 channels in human peripheral blood and leukocytes, with the TRPV2 mRNA being more abundant than that of TRPV1 by a factor of 150 and 20, respectively [243, 244]. TRPV2 is relatively poor expressed in other tissues (except smooth muscle and lung) but in blood [245]. TRPV2 is expressed in lymphoma and leukemic cell lines, including T-ALL, Jurkat, and MOLT-4 [124, 245, 246]. On note, TRPV2 activation by cannabidiol in some tumors increased their sensitivity to anticancer drugs, by increasing drugs uptake and stimulating cell death [235, 247]. TRPV2 was also detected in the intracellular (endosomal) membrane, where it mediates Ca^{2+} release [248]. There are two splicing variants of TRPV2: full-length and poreless short (s-TRPV2) ones. S-TRPV2 is more typical for tumor cells including leukemic ones; it is localized in cytosol, and it inhibits full TRPV2 translocation to the cell membrane [246]. TRPV2 is probably one of the least understood TRP channels when it comes to its regulation. It has a bipolar current-voltage dependence and similar permeability for Ca^{2+} and Na^+ [249]. In humans, in contrast to rats, TRPV2 appears to be not activated by noxious heat [250]. It is thought that the main way of its activity regulation is the mobilization from the ER to the

plasma membrane, for example, by growth factors [246, 251]. However, in murine aortic myocytes and neurons, TRPV2 is activated by membrane stretch and mediated a Ca^{2+} influx [252, 253]. Activation by mechanical stretch and hypotonicity was demonstrated also for TRPV2-like channels, expressed in human mast-cell line [254]. Our own study on Jurkat cells revealed that the properties of major mechanosensitive channel in their plasma membrane are indistinguishable from TRPV2 by its voltage dependence, cation selectivity, and pharmacological profile (Pottosin et al., unpublished). Properties of mechanosensitive currents in Jurkat were clearly distinct from TRPV1 and 3 but may be confused with those generated by TRPV4 [255], albeit the latter channel shows a somewhat higher Ca^{2+} over Na^{+} selectivity. TRPV4 channel is also proved to be mechano- and osmosensing element in different tissues [256–258]. It is regulated by intracellular Ca^{2+} in a complex way, via CaM binding to C- and N-termini; it activates at moderate intracellular Ca^{2+} increase and inactivates at higher (>800 nM) Ca^{2+} levels [256]. It is still a matter of debate, whether this activation may be direct or mediated by mechanosensitive phospholipase A2 activity, which metabolizes the arachidonic acid and produced epoxyeicosatrienoic acid, which in turn activates the TRPV4 [213]. We give lesser preference to TRPV4 versus TRPV2 due to their very low expression in Jurkat cells [124], but this point requires further exploration. Emerging evidence is accumulated that TRPV2 could colocalize within a network of $K_v1.3$, $K_{Ca}3.1$, and CRAC, thus contributing to a variety of vital T cells functions by modulation of the Ca^{2+} signaling [245]. In Jurkat cells and mouse thymocytes, the RVD in response to hypotonic treatment is a Ca^{2+}-dependent process, unlike mature peripheral lymphocytes, either mouse or human, where hypotonic stress does not provoke any intracellular Ca^{2+} change [150]. About respective Ca^{2+} signal it is known that it is mediated by the plasma membrane nonselective channels (TRPs?), which are 100-time less sensitive to Gd^{3+} as compared to the CRAC. Likewise, in immature T cells and T-ALL the volume regulation is controlled by a mechanosensitive TRP, which, in accordance with our data, could be the TRPV2.

TRPV5 and 6 are inwardly rectifying and the only highly Ca^{2+}-selective ($P_{Ca}/P_{Na} > 100$) members of the TRP family [213, 215]. Both TRPV5 and TRPV6 channels could be found in resting human PBL and Jurkat cells, as transcripts and, functionally, based on the different sensitivity of measured single-channel currents to ruthenium red (RR). However, TRPV6 expression in Jurkat cells and PHA-activated PBLs is much higher as compared to resting cells, implying a stimulating role of TRPV in the proliferation. Indeed, inhibition of TRPV currents by RR arrested the progression of the cell cycle in activated PBLs or Jurkat in G0/G1 and S or G2/M phases, respectively [259]. TRPV6 in Jurkat may physically interact with or even contribute to the CRAC/SOCE [260].

8.8. Purinoreceptors (P2X). P2X forms nonselective Ca^{2+}-permeable channels, activated by external ATP [261]. First evidence that peripheral blood T cells bear P2X, whose activation by ATP produces depolarization and Ca^{2+} influx, was obtained by Baricordi and coworkers (1996). It was demonstrated that P2X may contribute to T cell proliferation, induced by mitogenic stimulation [262]. There are seven P2X subtypes (P2X1-7), of which mainly P2X1, P2X4, and P2X7 were expressed in primary $CD4^{+}$ and Jurkat cells [263, 264]. T cell activation was shown to induce ATP release and considerable augment of P2X1, P2X4 [264], and P2X7 [263] expression. In turn, stimulation by ATP contributes to Ca^{2+} rise and enhances IL-2 production [263, 264]. Silencing or chemical inhibition of P2X receptors strongly impair Ca^{2+} influx, NFAT activation, and interleukin production [263, 264]. Therefore P2X along with STIM1 and Orai1 was suggested to contribute to Ca^{2+} entry, providing an amplification mechanism for TCR signaling [263, 264]. ATP release required for P2X stimulation may be achieved by exocytosis, or from dying cells, but also from intact T cells [265] via some wide-pore channels, like pannexin 1 [266] or, notably, through P2X7 themselves. Importantly, stimulation of T cells or Jurkat causes a rapid (within minutes) translocation and clustering of P2X1 and P2X4, but there are no changes in the P2X7 distribution [264]. The same is true for the immunological synapse, where in addition pannexin 1 is rapidly recruited. It is hypothesized that such a colocalization may produce a strong positive purinergic feedback, with a further amplification due to the concentration of ATP in the synaptic cleft, thus, allowing sensation of just few presented antigens [261]. Additionally, P2X7 seems to interact with apoptotic pathways: prolonged activation of this receptor by extracellular ATP released by neighboring apoptotic cells promotes overall apoptotic process [267].

Remarkably, the evidences emerged, which link P2X7 with leukemogenesis. Bone marrow samples obtained from patients with different types of leukemias, mainly AML and CLL, show a substantially higher level of P2X7 mRNA expression as compared to normal donor group [268]. Quite a few samples from ALL patients were analyzed in this study, and, although they showed increased level of P2X7, it was not indicated if there were B-ALL or T-ALL cells. In contrast, recent study on identification of interconnected markers for T-ALL, which included 173 T-ALL patients, revealed P2X7 as non-DE gene [125]. As was mentioned earlier, non-DE genes may be involved in signaling networks, upregulated during leukemogenesis.

8.9. Cl^{-} Currents. Any osmotic or volume adjustment (e.g., RVD or AVD) requires a massive transport of solute across the plasma membrane. Such a massive transport is possible only when it occurs in electroneutral manner, so that cations (e.g., K^{+}) transport needs to be accompanied by a parallel transport of anions via anion channels. In ALL (T-ALL) the expression of ClC2-5 was detected by PCR; ClC-3 is robustly expressed in healthy PBL and leukemias, whereas ClC-2 is mainly in leukemias [269]. ClC2 and ClC3 play roles in volume regulation, whereas there are ClC2 and ClC4- in pH homeostasis. Activator-induced proliferation of healthy T cells involved DIDS-sensitive NPPB-insensitive Cl^{-} channels, whereas in case of leukemic cells (Daudi, Jurkat, and H-60) it is mediated by DIDS-insensitive NPPB-sensitive ones (but

not ClC-2, as evidenced by silencing experiment) [269, 270]. VSOR (Cl_{swell}) (volume sensitive outward rectifying Cl^- current, activated by swelling) was first discovered in peripheric human T cells [271–273]. Identical biophysical properties (intrinsically outward-rectifying) Cl^- current for CFTR (cystic fibrosis transmembrane conductance regulator) cAMP-activated [274] and volume-regulated [273] currents were reported also for leukemic Jurkat cells. VSOR may also require lck-kinase ($p56^{lck}$) activity: the inhibition of this kinase blocks swelling-induced Cl^- current, whereas addition of $p56^{lck}$ to excised patches caused the current activation [275]. Once activated, VSOR may stay extremely long time in open state (several minutes) without transitions to closed state. Yet more careful inspection revealed close-open events. At physiological ionic conditions and zero membrane voltage unitary conductance of VSOR is about 40 pS [274–276]; single channel current displays a strong outward rectification, which mimics the voltage dependence of the whole cell VSOR. Likewise, VSOR is encoded by CFTR, unique member of ABC-transporter family, encoding Cl^- channel and not a pump. Transfection of the CFTR-defective B cells and lymphoblasts with wild-type CFTR cDNA resulted in a restoration of a cell-cycle-dependent (maximal expression in the G1 phase, low expression in the S-phase) cAMP—activated outward rectifying Cl^- current [277, 278]. It was shown that the outward rectifying Cl^- current can be activated in a triple manner: transiently by cAMP and steadily by either hypotonic stress or intracellular Ca^{2+} increase; only the activation by cAMP was abolished by genetic defect in CFTR [279]. In addition, CFTR deficient mice have shown a higher NFAT to nucleus translocation and higher interleukin and immunoglobulin E production by T cells due to altered (elevated) intracellular Ca^{2+} increase in response to T cell receptor activation. This may be a depolarization, caused by outward-rectifying Cl^- current which normally moderates Ca^{2+} influx via CRAC [280]. Summarizing, all aforementioned Cl^- channels are expressed in normal lymphocytes and lymphoblasts. Whereas in case of ClC channels direct demonstration of respective currents is lacking, in case of VSOR its molecular identity remains to be revealed. Preliminary pharmacological analysis suggests differential dominant Cl^- currents in normal and malignant lymphocytes.

8.10. Other Channels. Nicotinic AchR forms nonselective cation channel, with important roles in neoplastic progression, both via conducting and nonconducting signaling mechanisms. There is circumvent evidence for nAchR expression in lymphocytes and nicotine affects hematopoiesis [281, 282]. Yet functional roles of nAchR and cholinergic signaling in healthy lymphocytes and leukemias remain to be elucidated. Both human T and leukemic Jurkat cells appear to express ionotropic glutamate receptors of AMPA and NMDA types. Their activation by respective ligands promoted cell adhesion and migration [283, 284]. NMDA receptor antagonists decreased proliferation of T and Jurkat cells, but in a different manner. In Jurkat cells this reduction was associated with G1-S transition arrest and increased apoptosis; neither phenomenon was associated with a decrease of proliferation in healthy T cells [284, 285]. In early studies one may find the notion that a few percent of T cells from human peripheric blood express functional voltage-dependent Na^+ channels [159], but their role in nonexcitable cells remains obscure. Expression of α-subunits of voltage-dependent L-type Ca^{2+} channels (Ca_v1) as well as interacting β-subunits is consistently reported in human healthy T and Jurkat cells. However, up to now neither depolarization-activated Ca^{2+} selective currents nor significant Ca^{2+} influx evoked by a depolarization was detected in human T cells [126, 208]. However, on murine models, typical L-type Ca^{2+} currents, which disappeared upon $Ca_v1.4$ silencing, were reported in a single study [286]. Furthermore, mice with mutant β-subunits or Cav1.4-deficient mice, or mice with knockdown $Ca_v1.2$ and/or $Ca_v1.3$ display clear immunopathological phenotypes (see [208, 287] for a recent update). Yet no immunopathological phenotypes were demonstrated in humans. The situation may be even more complicated due to the fact that STIM1, activating CRAC, inhibits the L-type Ca^{2+} channel activity [288], so that CRAC dominance in T cells implies a suppression of the Ca_V activity. In the latter study, authors were able to show a depolarization-activated Ca^{2+} increase in mutant STIM1-deficient Jurkat cells, transfected with $Ca_v1.2$ along with β1b and $\alpha 2\delta 1$ subunits; yet without such a transfection no significant depolarization-activated Ca^{2+} influx was detected, which questions again the functional role of background Ca_v1 channels. It was speculated that instead of activation by large depolarization, hardly attainable in T-cells, Ca_v channels may be activated due to their clustering and interaction with PKC [287].

9. Targeting Ion Channels for the T-ALL Treatment

Although an impressive progress has been made in the treatment of T-ALL, it is still a disease with enormous need for innovation in the therapeutic field. Undoubtedly, the concept of total chemotherapy has been a milestone in the history of the treatment of this disease. The stage of differentiation, at which the proliferative arrest occurs, denotes not only the degree of maturation of the leukemic cell, but also its clinical behavior and response to a particular treatment. Early T leukemias, regarding the mature T cell neoplasias, are more chemoresistant and, therefore, demonstrate lower rates of complete remission and overall survival. The current therapeutic strategy is based on a combination of chemotherapeutic agents targeting DNA and protein synthesis (methotrexate, L-asparaginase, doxorubicin, cyclophosphamide, cytarabine, and nelarabine), or inducing apoptosis (dexamethasone). Allogeneic bone marrow transplantation has been shown to be beneficial in a selected population in this leukemia subtype. Although the leukemia-free survival has significantly improved in the last decades, being reported in some series in children up to 85% at 5 years [289], traditional antileukemic drugs demonstrate numerous short- and long-term toxic side effects, which may lead to significant morbidity. More than 50% of pediatric cancer survivors

will develop long-term complications, including cardiovascular, gonadal, and gastrointestinal/hepatic dysfunction, neurocognitive sequelae, auditory complications, and decline in growth, with approximately 25% of cases being severe [290]. Additionally, long-term toxicities of these antineoplastics include the possibility of a future secondary malignancy.

Among novel molecular targets FLT3, JAK1/JAK3 and Notch were proposed [291]. Some FLT3 inhibitors as lestaurtinib currently have been studied in the phase III clinical trials, showing *in vitro* apoptosis induction capability in LLA cells lines that express high levels of FLT3 and interacting synergistically with multiple chemotherapeutic agents. JAK inhibitors are currently in varying stages of development and they have only been in adult trials. Notch inhibitors are under development. Though, in animal models and in the phase I clinical trials, serious gastrointestinal toxicity has been shown, which can be reversed by concomitant use of glucocorticoids, subsequent additions have been few and mainly restricted to the advent of some new chemotherapeutic drugs (clofarabine, nelarabine), the use of monoclonal antibodies such as alemtuzumab, and more recently drugs able to impact molecular targets (see for review [292]). Sustained activation of Cn/NFAT pathway is a hallmark of the T-ALL [43, 107, 108]. However, this pathway is ubiquitous not only in malignant, but in many healthy tissues as well. As a result, although administration of the Cn inhibitors showed an antileukemic effect [43, 77], it was associated with a number of undesirable offtarget effects [293].

Leukemias show altered expression of a variety of ion channels (for review see [127] and this review). Even if the profile of channel expression is not changed, as was shown for nondifferentially expressed genes, it may be involved in newly upregulated signaling network [125]. However it is unlikely that these alterations by themselves launch the leukemic scenario. Rather, ion channels represent important components, which ensure homeostatic conditions favorable for the migration, proliferation, and leukemic survival, or facilitate the expression of oncogenes, involved in the leukemogenesis. Almost all signaling pathways, upregulated in the T-ALL, show a dependence on the Ca^{2+} signaling. Channels may be involved in the cell motility and tumor metastasis. These phenomena may provide the basis for the targeting ion channel in leukemogenesis.

For example, TRESK channels are abnormally expressed in some types of T leukemias [197]. As was mentioned, this channel is activated through Cn-dependent mechanism and is involved in a positive feedback regulation of the Ca^{2+} entry. Thus, TRESK inhibition could result in a downregulation of the Cn. However, the issue is much more complex than it might seem. First of all, TRESK is normally expressed in some healthy tissues [201], foremost in the central nervous system where it is related to the migraine pathology [294]. Next, no specific blockers for the TRESK are known so far. To resolve a similar problem related to the $K_v10.1$ channel, specific functional antibodies were designed [295]. Although it is possible that an antibody actively crosses the blood-brain barrier, the concentrations efficient for tumors are likely lower than those required to reach the brain parenchyma and to cause massive side effects [295]. Another classical example is $K_v11.1$, aberrantly expressed in many tumor types [127]. The difficulty lies in the fact that $K_v11.1$ normal localization is the cardiac tissue, where it contributes in the repolarization phase of the cardiac action potential [175]. Although specific blockers of this channel are available, its inhibition can retard the repolarization and prolong QT interval leading to the ventricular arrhythmia, with a possible fatal fibrillation [296]. Therefore, $K_v11.1$ is generally considered as an undesired pharmacological target. To overcome this issue, it was proposed to consider different blocking mechanisms and target a particular conformational state of leukemic ion channels, as well as bipolar antibodies, raised against the complex of the channel and one of its auxiliary proteins, characteristic for tumors [127].

Yet another very important point should be taken into consideration, namely, hierarchical tumor development, its genetic instability, and heterogeneity of the T-ALL population. Obviously, LSC and T-ALL clones, representing the same clinical case, differ in their "gene expression signatures" and, accordingly, in their sensitivity/resistance to chemotherapy. Chemotherapeutic agents, used nowadays, successfully eradicate the blast cells in many patients; however, they have very little if any effect at the level of the blast progenitor cell, namely, LSC, which is biologically distinct from most of the cells found in a typical patient [297]. Then LSCs that survived chemotherapeutic attack finally cause the chemoresistance and relapse. Additionally, some therapeutic targets may be present only in a few nondominant clones, which does cause unresponsiveness to the treatment. Targeting the LSC was suggested as the Holy Grail of leukemia therapy [298]. Then, considering ion channels as a possible target, the search for ion channels expressed specifically in LSC would be of a special advantage. We suggest that the experiments with primary blast cells or cell lines derived from patients in relapse may give some important information.

10. Conclusions

Tumor-specific expression of a certain ion channel is a relatively rare phenomenon. However, changes in the expression (including its variation during cell cycle) and subcellular localization, splicing of channels (and relative expression of different variants), role of heteroligomerization, of β-subunits, and of other auxiliary proteins (especially integrins), and modification of the channel protein, as well as every aspect of microenvironment and metabolic regulation and signaling pathways context, affecting the channel's activity, may be of potential importance for the tumor progression. Here, the integration of the channels function and their crosstalks, most apparently via cell membrane polarization and intracellular Ca^{2+} changes, needs to be considered.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content of the paper.

Authors' Contribution

Oxana Dobrovinskaya and Igor Pottosin had an equal contribution.

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Review Article

Blood Brain Barrier: A Challenge for Effectual Therapy of Brain Tumors

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Brain tumors are one of the most formidable diseases of mankind. They have only a fair to poor prognosis and high relapse rate. One of the major causes of extreme difficulty in brain tumor treatment is the presence of blood brain barrier (BBB). BBB comprises different molecular components and transport systems, which in turn create efflux machinery or hindrance for the entry of several drugs in brain. Thus, along with the conventional techniques, successful modification of drug delivery and novel therapeutic strategies are needed to overcome this obstacle for treatment of brain tumors. In this review, we have elucidated some critical insights into the composition and function of BBB and along with it we have discussed the effective methods for delivery of drugs to the brain and therapeutic strategies overcoming the barrier.

1. Introduction

Brain is the most delicate organ of human body. Several diseases like encephalitis, neurological disorders, multiple sclerosis, stroke, and tumor induce deterioration of brain function. The development of new therapeutic approaches for these diseases is a difficult challenge, and there is no effective treatment for almost all the brain diseases. In most of the cases, the major cause of the failure in the development of drugs to treat brain diseases is the presence of BBB. Out of the several brain disorders, brain tumors commonly have poor prognosis, which varies according to the type and grade of the tumor. Due to the presence of BBB, drug delivery to brain tumors has long been a problematic issue. Some group of researchers like Vick et al. and Donelli et al. mentioned BBB as a controversial problem for brain tumor chemotherapy [1, 2]. They indicated that BBB is not the only factor responsible for impeding the success of brain tumor chemotherapy, but later, studies revealed the involvement of BBB in drug restriction to different brain neoplasias [3–6].

Brain tumors can be classified into two major classes, namely, primary brain tumors that start in the brain and secondary brain tumors that are generated by the cancer cells that migrated from tumors developed in other parts of the body. Primary brain tumors can arise from different type of brain cells or even from the membranes around the brain (meninges), nerves, or glands. The most common type of primary tumors in the brain is glioma, which arises from the glial tissue of the brain. Gliomas comprise several types, namely, astrocytoma, oligodendroglioma, and ependymomas. Astrocytomas are further classified as grade I (pilocytic), grade II (fibrillary), grade III (anaplastic), and grade IV (glioblastoma multiforme or GBM). BBB is poorly developed in these types of brain tumors causing an increased vascular permeability [7].

It has been shown earlier that leaky interendothelial tight junction is present in human glioma [8] due to the fact that poorly differentiated neoplastic astrocytes do not release factors essential for BBB function [9–11]. This tight junction opening causes increased chances of cerebral edema occurrence [12]. It is also observed that BBB stability in lower grade gliomas is better than that in GBM. As the degree of BBB disruption differs from the malignancy of the tumor, treatment of low grade brain tumors is still a challenging task, because of the presence of almost intact BBB. On the contrary, recent studies have suggested that

Table 1: Type of common brain cancers and their BBB status.

Type of brain tumors		Origin	Involvement of BBB	Status of BBB
Primary	Astrocytomas			
	Pilocytic astrocytoma (grade I)	Usually from astrocytes of cerebellum	Yes	Not well formed
	Fibrillary/mixed oligo astrocytoma (grade II)	From neoplastic astrocytes	Yes	Mostly intact
	Anaplastic astrocytoma (grade III)	From brain astrocytes which infiltrate through white matter of cerebral hemisphere, dura, and spinal fluid	Yes	Altered or disrupted
	Glioblastoma multiforme (GBM) (grade IV)	From glial cells	Yes	Altered or disrupted
	Oligodendrogliomas	From oligodendrocytes and glial precursor cells	Yes	Mostly intact
	Ependymomas	From ependyma	Yes	Intact
	Meningiomas	From meninges of brain and central nervous system	No	—
	Schwannomas	From Schwann cells	No	—
	Craniopharyngiomas	From pituitary gland embryonic tissue	Yes	Intact or disrupted
	Germinomas	Germ cell tumors from pineal gland	No	—
	Medulloblastomas	From cerebellum, below the tentorium of brain	Yes	Intact
	Pineocytoma	From pineal parenchyma	No	—
	Pineoblastoma	From pineal parenchyma	No	—
Secondary	Different metastatic cancers to brain	From cancers like breast, lung, bowel, kidney, ovary, and skin	Yes	Intact or disrupted

although the BBB may be disrupted at or near the core of the high grade brain tumors, most certainly it seems to be intact near the growing edge of the tumor where the invasive tumor cells may reside. The presence of the intact BBB in such regions of the tumors can considerably impede drug delivery to these regions [13–15]. On the other hand, lack of BBB has been observed in other primary brain tumors like meningiomas, schwannomas, or pineocytomas [16–18]. Disrupted BBB also exists in metastatic secondary brain tumors, but the disruption is negligible in smaller aggregates of metastatic tumor cells. Therefore, the drug delivery to these micrometastatic regions is not optimum; consequently, the tumor keeps growing and ultimately reaches to clinically significant size. Thus, along with the existing therapeutic modalities, new approaches of therapy are needed to combat against the BBB of different brain tumors (see Table 1).

2. BBB

BBB protects neural tissues in the brain and works as a diffusion barrier that impedes the influx of toxins and other compounds from blood to the brain. BBB was discovered in 1880s. It took almost 70 years to successfully prove the existence of BBB by electron microscopic cytochemical studies [19, 20]. Later, in 1981 Stewart and Wiley explained the initial understanding about the uniqueness of BBB tight junction and its physiology [21].

Molecular character of BBB shows the presence of two types of cellular junctions, the intercellular adherens junction and the paracellular tight junction. The functional integrity of BBB is maintained by adherens junction that is composed of vascular endothelium (VE), cadherin, actinin, and catenin [22]. But the major functionality of BBB is maintained by tight junctions, as they are primarily responsible for permeability through BBB [23, 24]. The BBB in adult is comprised of a complex cellular network. The main components of this system are brain endothelial cells, highly specialized basal membrane, a plenty of pericytes embedded in the basal membrane, and astrocytic end-feet (see Figure 1).

Brain Endothelial Cells. These cells are required for proper barrier formation and interaction with the adjacent cells. They are also known as brain microvascular endothelial cells (BMECs). The BMECs differ from the endothelial cells present in the other organs in the following ways: (i) paracellular movement of molecules is prevented by continuous tight junctions present between brain endothelial cells, (ii) BMECs have few cytoplasmic vesicles and more

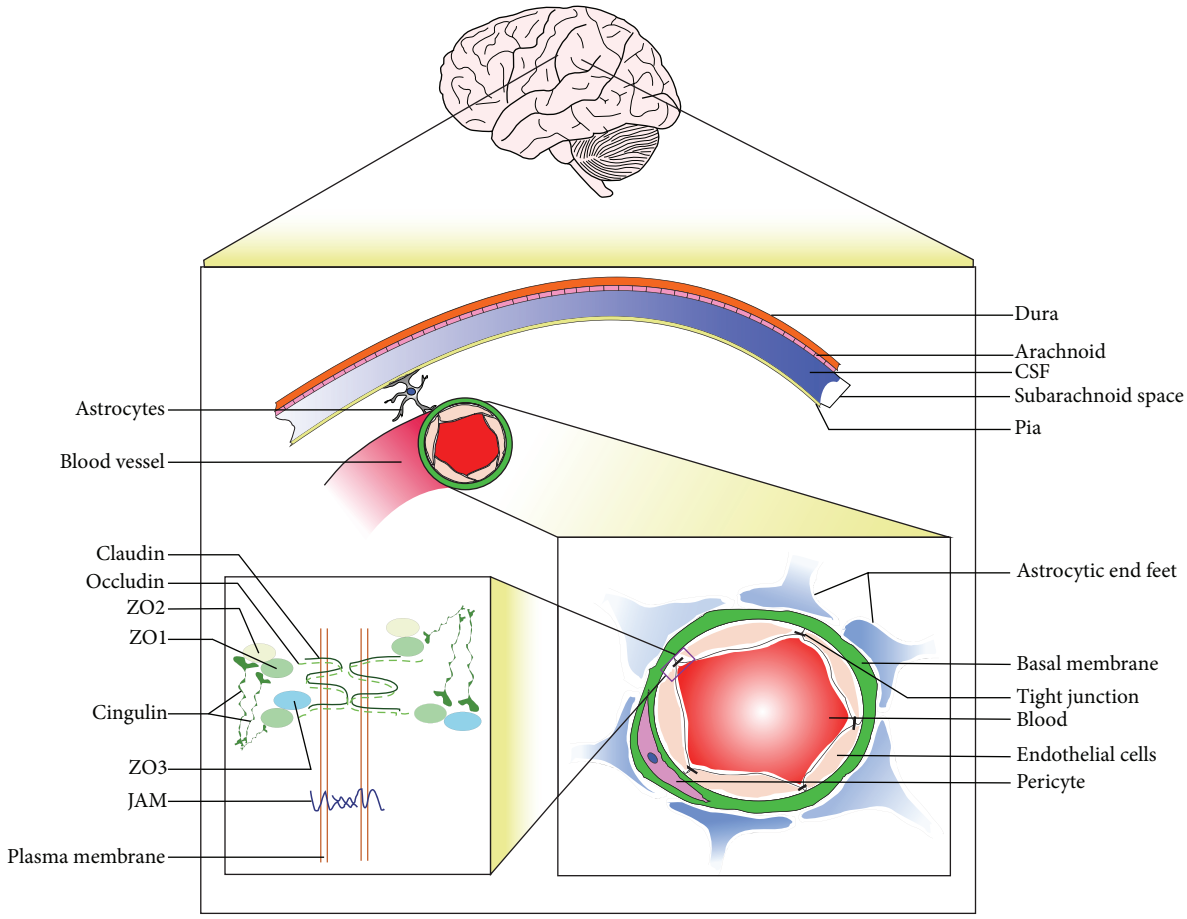

Figure 1: A pictorial representation of the BBB and its tight junction structure. The figure shows an irrigated blood vessel in the brain which forms the BBB. The BBB is constituted by endothelial cells with tight junctions, surrounded by pericytes and astrocytic end-feet. The tight junction is further established by the interaction of proteins like claudins, occludin, junction adhesion molecules, and cytoplasmic accessory proteins (ZO1, ZO2, and ZO3) of adjacent endothelial cells. The details of each component of the BBB are mentioned in the text of this review.

mitochondria, and (iii) detectable transendothelial path like intracellular vesicular transport is not present in BMECs [22, 25]. Complex intercellular tight junctions restrict the passive diffusion of molecules into the brain and therefore the blood vessels showing extremely high transendothelial electrical resistance (TEER) *in vivo* [26]. BMECs are also endowed with the ability to shuttle essential nutrients and metabolites across the BBB, which include molecules, like efflux transporters (p-glycoprotein). These transporters contribute to the BBB properties by efflux of small lipophilic molecules that are able to diffuse into BMECs, back to the blood stream.

Basal Membrane. It consists of type IV collagen, fibronectin, and laminin that completely covers the capillary endothelial cell layers. Pericytes are embedded in this membrane and surrounded by astrocytic end-feet. The potential function of this membrane is to restrict the movement of the solutes [27, 28].

Pericytes. The contractile cells which are wrapped around the endothelial cells are called pericytes. These cells play an essential role in the formation of BBB in several ways such as by regulating the expressions of BBB-specific genes in endothelial cells by inducing polarization of astrocytic end-feet surrounding CNS blood vessels, and also they inhibit CNS immune cells from damaging the proper formation of BBB. Besides, these cells also help in reduction of the expression of molecules that increase vascular permeability [29].

Astrocytic End-Feet. It is assumed earlier that the astrocytic end-feet encircling endothelial cells do not play substantial role in maintenance of BBB [30]. But recent study by Nuriya et al., 2013, indicated the heterogeneity of diffusion patterns around astrocytic end-feet [31]. They proved the existence of some astrocytic end-feet which can form tight networks that are able to block free diffusion of molecules across them. The types of blood vessels and morphological differences in the gliovascular interface like the space between the endothelial cells and astrocytic end-feet determine the heterogeneity of diffusion patterns. Thus, these networks cover the blood vessels tightly which suggests the potential functional roles of astrocytic end-feet [32].

2.1. Molecular Composition of BBB. The tight junction of BBB mainly consists of three main integral membrane proteins, namely, occludin, claudin, and junction adhesion molecules. Other than that, cytoplasmic accessory proteins like zonula occludens (ZO 1, ZO 2, ZO 3, etc.), cingulin, and others are also present in BBB (see Figure 1).

Occludin. It is the first transmembrane protein of the tight junction to be discovered. Occludin was first identified in 1993 by immunogold freeze fracture microscopy in chicken [33] and then in mammals [34]. It is formed by four transmembrane domains: a long carboxy-terminal cytoplasmic domain, a short amino-terminal cytoplasmic domain, and two extracellular loops. The ZO proteins are directly associated with cytoplasmic domain of occludin. Phosphorylation of specific Ser/Thr/Tyr residues of occludin regulates its interaction with ZO proteins which in turn plays a regulatory role in tight junction formation [35].

Claudins. These are a multigene family of at least 24 members. They form tight junctions through homophilic "claudin-claudin" interactions mediated by their extracellular loops [36]. Carboxy terminal of claudins binds to the cytoplasmic proteins including ZO family members [37]. Occludins and claudins can also assemble into heteropolymers to form intramembranous strands. It has been proposed that these strands contain fluctuating channels, which allow the selective diffusion of ions and hydrophilic molecules [38]. Claudins-1, -3, -5, and -12 have been shown to participate in the formation of tight junctions between BMECs [9, 10, 39, 40]. Each claudin regulates the diffusion of a group of molecules of specific size.

Junction Adhesion Molecules (JAM). These proteins belong to the immunoglobulin superfamily. Three JAM-related proteins, JAM-A, JAM-B, and JAM-C, have been investigated in rodent brain sections. In human, it is observed that JAM-A and JAM-C are expressed in the tight junctions of BBB but not JAM-B [41]. JAM-B can be found in seminiferous epithelial cells [42]. All JAM proteins comprise a single transmembrane domain; the extracellular portion has two immunoglobulin like loops. They regulate the formation of tight junctions during the acquisition of cell polarity [43].

Cytoplasmic Accessory Proteins. Cytoplasmic proteins like zonula occludens proteins (ZO 1, ZO 2, and ZO 3), cingulin, 7H6, and several others are also involved in tight junction formation. Zonula occludens are proteins belonging to the family of membrane associated guanylate kinase (MAGUK) [44]. They provide the cytoskeletal anchorage for the transmembrane tight junction and control spatial distribution of claudins [24]. Cingulins are actomyosin-associated proteins with large globular N-terminal "head" domain, coiled-coil "rod" domain, and small globular C-terminal "tail." Cingulin helps in BBB formation by interacting with ZO proteins and junction adhesion molecules.

2.2. Transporters of BBB. Endogenous compounds and drugs may cross BBB by different mechanisms such as passive diffusion, carrier-mediated transport (like GLUT1 mediated transport), endocytosis, and active transport [45–52]. Participation of various transport proteins is there in most of these transport systems. These different transport proteins of brain mediate the uptake and extrusion of various metabolites and compounds. The efflux and influx transporter systems of BBB comprise transporters like ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters.

2.2.1. ABC Transporters. ABC (ATP-binding cassette) transporters are ATP-driven drug efflux pumps present in the BBB which include P-glycoprotein, breast cancer resistance protein, and members of the multidrug resistance related proteins [53]. These proteins form a key characteristic of the BBB by localizing at the luminal side of brain capillaries. They collectively impede brain uptake of a large variety of lipophilic molecules, xenobiotics, potentially toxic metabolites, and drugs. ABC transporters show broad substrate specificity and have been characterized by one or two cytoplasmically located nucleotide binding domains acting as a catalytic domain for nucleotide hydrolysis. There are 48 genes encoding ABC transporter superfamily of proteins, which are subdivided into 7 distinct subfamilies (ABCA to ABCG) [54]. All ABC transporters have three highly conserved motifs known as Walker A, Walker B motifs and the ABC signature C motif (i.e., ALSGGQ) [55]. It has been suggested that this domain may be involved in substrate recognition and ATP hydrolysis [56].

(1) P-glycoprotein (P-gp). It is a 170-kDa efflux transporter discovered in Chinese hamster ovary cells [57]. P-gp is encoded by multidrug resistant (MDR) genes [58]. Two MDR isoforms have been identified in human tissues, MDR-1 and MDR-2 [59, 60]. MDR1 encoded P-gp is a major efflux transporter of BBB, the expression of which is likely evolved to protect the brain from exposure to potentially neurotoxic xenobiotics. Thus, it is considered that P-gp has a key role in the maintenance of accurate homeostatic environment required for proper neuronal function [61]. The MDR1 gene product is 1280 amino acids in length and has two homologous halves; each consists of six transmembrane domains and ATP- binding site. On the first extracellular loop, two to four glycosylation sites are present [62]. In the brain, P-gp is localized to both the luminal and abluminal sides of BBB endothelium [63] and to the apical plasma membrane of choroid plexus epithelial cells [64]. Substrates of P-gp are usually nonpolar, weakly amphipathic compounds which significantly vary in molecular size. The different types of endogenous substrates of P-gp include cytokines, lipids, steroid hormones, and peptides [65]. P-gp has a vast endogenous and exogenous substrate profile that renders difficulty in drug delivery across the BBB.

(2) Breast Cancer Resistance Protein (BCRP). It was first identified in the MCF-7/AdrVp breast cancer cell line [66]. It is also known as a "half-transporter." Its molecular weight is approximately 72 kDa and it is composed of 655 amino acids. It has six transmembrane domains and both the C- and

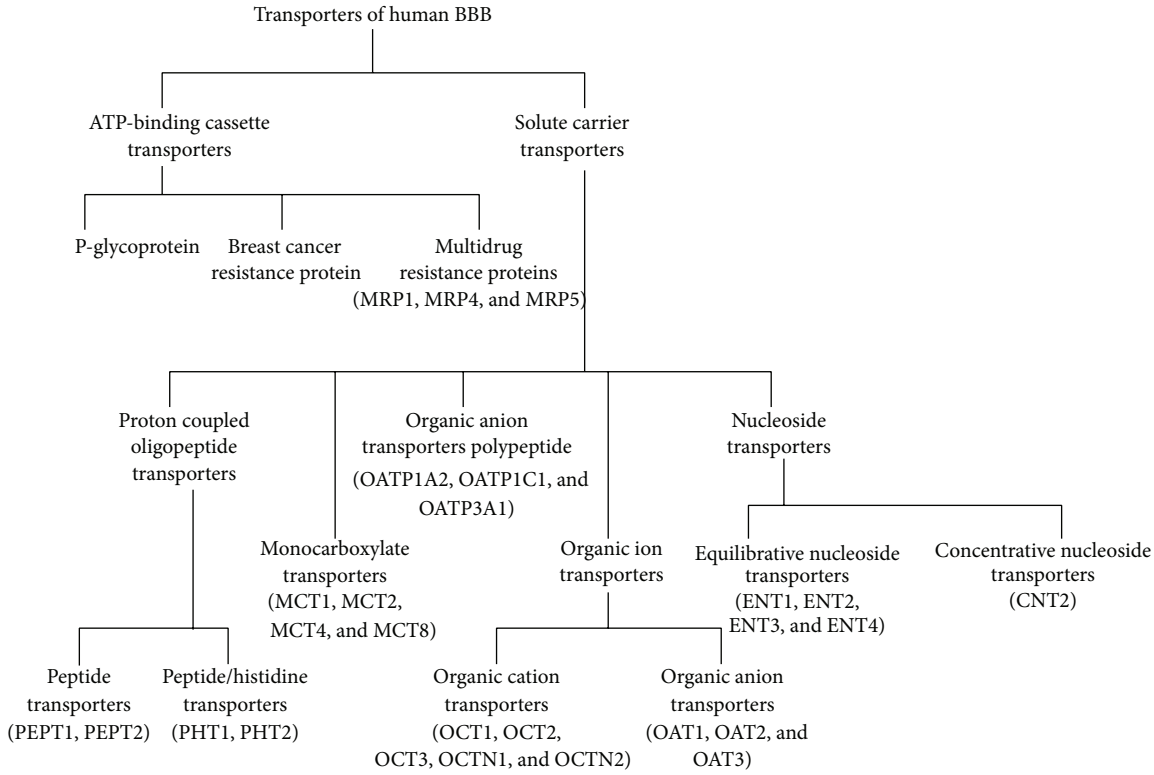

Figure 2: Schematic classification of transporters of human BBB. Two main classes of drug transporters are ATP-binding cassette (ABC) transporters and solute carrier transporters. Each of them is further classified into several other transporters mentioned in the flowchart. More information about each of the transporters is mentioned in the text.

N-terminus regions are located on the intracellular side of the plasma membrane [67].

Furthermore, the extracellular loops of the protein contain two to three sites for N-linked glycosylation. According to the earlier reports, the functional capabilities of the transporter and its cellular localization are not dependent on these glycosylation sites [67, 68]. It is also known that BCRP forms functional homo- or heterodimers to maintain the efflux activity [69]. BCRP is expressed at the luminal side of capillary endothelial cells, in astrocytes and microglia [70–72]. The substrate specificity of BCRP not only is limited to the physiological substrates, such as glutathione, steroid hormones, and folic acid [73], but also transports many structurally diverse therapeutic compounds. Significantly, the specificity of BCRP to the substrates overlaps with the substrate specificity of P-gp [74]. It is also known that high expression of these BCRP proteins causes significant resistance to different cancer chemotherapeutic drugs [75, 76].

(3) Multidrug Resistance Proteins (MRPs). It is well established that MRP family has 9 homologues, designated as MRP1–9, and these isoforms have overlapping substrate profiles. Out of these, expression of MRP1–6 has been observed in human brain [77], whereas multiple MRPs like MRP1, 4, and 5 have been detected in the human BBB [72, 78, 79]. Existence of other MRPs, namely, MRP2, 3, and 6 and along with these MRP1, 4, and 5, has also been noticed in other vertebrates like rat, cow, pig, and fish. But presence of them in human BBB is still questionable. Structural similarity can be observed in MRP1, 2, 3, and 6, as each of them possesses 3 transmembrane domains (TMD) designated as TMD0, TMD1, and TMD2, respectively. TMD1 and TMD2 contain 6 alpha helices, whereas TMD0 contains only 5 alpha helices [80, 81]. It is believed that TMDs are assembled in the plasma membrane pore through which the transport of substrates occurs [80]. On the contrary, MRP4 and MRP5 have structural similarity with P-gp that lack TMD0 [80, 82], but in all the MRP homologues, the conserved cytoplasmic linker (L0) portion is essential for transport function. MRP1, 4, and 5 are restricted to the luminal membrane of human brain capillary endothelial cells [81]. The localization of MRPs suggests that they play a crucial role in drug efflux transport through BBB.

2.2.2. Solute Carrier (SLC) Transporters. SLC transporters belong to SLC superfamily which comprises 43 known subfamilies of SLC transporters (SLC1–SLC43). At the BBB, SLC15A1, SLC16, SLC21, SLC22, SLC28, and SLC29 are expressed [83]. The major SLC transporters include proton coupled oligopeptide transporters, monocarboxylate transporters, organic anion polypeptide transporters, organic ion (anion and cation) transporters, and nucleoside transporters (see Figure 2) [84, 85]. Most of these transporters of BBB regulate the transport of brain tumor drugs by hindering their entry into the tumor regions. Generally, these SLC

transporters do not require ATP to translocate substrates across BBB; however, the electrochemical or concentration gradients of solute are essentially required for this type of transportation.

(1) Proton Coupled Oligopeptide Transporters (POT). POT belongs to SLC15A family solute carrier transporters. Names of the subfamilies of POT are peptide transporters (PEPT) and peptide/histidine transporter (PHT). Peptide transporter-1 (PEPT1; SLC15A1) and peptide transporter-2 (PEPT2; SLC15A2) are the members of PEPT subfamily, whereas PHT comprises peptide/histidine transporter-1 (PHT1; SLC15A4) and peptide/histidine transporter-2 (PHT2; SLC15A3) [86, 87]. These oligopeptide transporters are able to transport small peptides across the BBB by an electrochemical proton gradient [88]. Structural similarity can be observed in POT family members due to the presence of 12 α-helical transmembrane domains with intracellularly located C- and N-terminal regions. Two to seven glycosylation sites exist in the extracellular loops, while intracellular loops have protein kinase A and C phosphorylation sites [86, 89]. Other than the above-mentioned peptide transporters, peptide uptake and distribution in brain are also determined by peptide transport system (PTS) expressed endogenously at the BBB endothelium [90]. In the BBB, seven transport systems have been found for transport of peptides, which includes PTS1–PTS7. PTSs, PTS2, PTS4, and PTS6, are bidirectional, whereas the rest are unidirectional. The unidirectional PTSs, PTS1 and PTS5, facilitate brain-to-blood peptide transport, whereas PTS3 and PTS7 are known for reverse process [90].

(2) Monocarboxylate Transporters (MCTs). Generally, the MCTs facilitate the rapid transport of monocarboxylates across the biological membranes. In brain, MCTs not only assist the transport of the monocarboxylates for uptake into the neurons but also mediate the transport of some drugs across the BBB [91]. These MCTs are members of solute carrier family 16 (SLC16). SLC16 has 14 members, out of which only six have been functionally characterized and those MCTs are MCT1–4, MCT8, and the T-type amino acid transporter-1 (TAT-1/MCT10) (326, 327). MCT1, MCT2, and MCT4 are the most important BBB transporters, whereas active MCT8 expression has also been detected in BBB [92–94]. The MCT1 protein is present in the membrane of the capillary endothelium and astrocytes, while MCT2 and MCT4 are found on neurons and astrocytes, respectively [95, 96].

(3) Organic Anion Transporters Polypeptides (OATPs). These membrane influx transporters are present in BBB to regulate cellular uptake of a number of endogenous compounds and clinically important drugs [97]. The human OATP comprises 11 members: OATP1A2, 1B1, 1B3, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1, 5A1, and 6A1 [98–100], where OATP1A2 is the first discovered human member of the OATP family [101]. The OATP genes are classified within the SLCO (formerly SLC21A) family. Members of the same OATPs family share ~40% [99], whereas members of individual subfamilies possess ~60% amino acid sequence similarity. This group of transporters has broad substrate specificity. The OATP dependent transport of the substrates does not require ATP as energy source, yet it is conducted by electrochemical gradients that utilize an inorganic or organic solute as a driving force. The OATPs family members OATP1A2, 1C1, 2A1, 2B1, 3A1, and 4A1 are present in human brain [99]. OATP1A2 is the only human OATP isoform whose expression and function are widely established at BBB. The localization of OATP1A2 can be observed at both the luminal and abluminal membranes of human BBB endothelial cells [102]. The endogenous substrates of OATP1A2 are bilirubin, bromosulfophthalein, cholate, deltorphin-II, estradiol-17β-glucuronide, estrone-3-sulfate, glycocholate, hydroxyurea, PGE2, reverse-T3, taurocholate, taurochenodeoxycholate, tauroursodeoxycholate, T4, T3, and so forth [103], whereas a broad exogenous therapeutic substrate specificity can be noticed for this kind of OATPs. OATP1C1 and OATP3A1 are known to be present in both apical and basal sides of the brain endothelial cells and blood cerebrospinal fluid barrier, respectively, while the exact role of other OATPs is yet to be determined [104, 105].

(4) Organic Ion Transporters. These transporters can be classified into two specific types: (i) organic anion transporters (OATs) and (ii) organic cation transporters (OCTs). These transporters are the members of SLC transporter 22 superfamily (SLC22A) [83, 106].

(i) Organic Anion Transporters (OATs). The OAT family comprises OAT 1–6 and the renal specific transporter (RST) [107–111]. This classification is based on ATP-dependent energy requirements and involvement of Na^+ ion. [112]. Movement of the organic anions across biological membranes is determined by these OATs. Various endogenous molecules like anionic metabolites of neurotransmitters, hormones, prostaglandins, and exogenous molecules such as different drugs are known to cross the biological membrane by these OATs [113]. The general structure of OATs comprises 12 membrane-spanning α-helices and several glycosylation and PKC sites, which can be found on extracellular loops connecting helices 6 and 7 [113]. In brain, OAT3 is the most highly expressed isoform. It is reported earlier that OAT3 is present in the abluminal (brain side) and brush-border membrane (CSF side) of brain capillary endothelial cells and choroid plexus epithelial cells, respectively [114, 115]. Other than this, OAT1, OAT2, and OAT4–6 are also expressed in brain [78, 105, 109, 114–117]. But the proper localization and function of these OATs are yet to be known.

(ii) Organic Cation Transporters (OCTs). OCTs regulate the transport mechanisms to facilitate the passage of organic cations through biological membranes [118]. According to their transport capabilities, OCTs are categorized into two subgroups, namely, oligospecific organic cation transporters and polyspecific organic cation transporters. Apart from this, organic cation transporters can also be classified as chemical potential sensitive organic cation transporters (OCTs) and H^+ gradient-dependent novel organic transporters (OCTNs). OCTs comprise OCT1–3, whereas OCTN transport system

includes OCTN1 and OCTN2 [119]. Cellular influx and efflux of various cationic substrates are maintained by OCTs and OCTNs, respectively [120, 121]. All OCT family members generally contain 12 α-helical transmembrane domains with intracellular N- and C-termini. Furthermore, large extracellular loop between TMD1 and TMD2 and small intercellular loop connecting TMD6 and TMD7 are also present in OCT family members. In brain, OCT1–3 are localized to the basolateral membrane of BMECs and choroid plexus epithelial cells [122–124], and OCTN2 is reported to be localized to the luminal side of the BBB [125–127], whereas OCTN1 is reportedly absent in human CNS tissue [128]. Other than the transport of endogenous organic cations, OCT family members may also play crucial role in drug penetration through BBB [129].

(5) Nucleoside Transporters. The nucleosides play a major role as second messengers in many signal transduction pathways. Thus, their regulation of them is crucial for proper neuronal function [130]. The recycling pathways for nucleosides transportation into CNS tissue are needed, as brain cannot synthesize nucleosides *de novo*. Depending on the Na^+ dependence nucleoside, the membrane transporters are again classified into two subcategories: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs). ENTs are the members of the SLC29A transporter family and are Na^+-independent, whereas CNTs are the members of the SLC28A transporter family and are Na^+-dependent [131, 132]. In humans four isoforms of ENTs have been discovered, which are ENT1–4 [133–135]. All of them possess 11 α-helical transmembrane domains with intracellular N-terminus and extracellular C-terminus regions. Each and every isoform of ENTs also possesses a large cytoplasmic loop and an extracellular loop [136, 137]. ENT1, ENT2, and ENT4 are ubiquitously expressed in brain tissue and are localized to cellular membranes [138–140]. CNTs also have three isoforms: CNT1–3. They are integral membrane proteins with 13 transmembrane α-helices and a large extracellular C-terminal region, present in various regions of brain [85] and work as antiporters. CNT1 and CNT2 transport nucleosides into the cell in exchange for sodium ions, while CNT3 transports nucleosides in exchange for either sodium ions or protons [141]. But prominent expression of CNT2 protein has been observed at the luminal side of the BBB endothelium. Other than the endogenous nucleoside transporters, CNTs are also responsible for the cellular uptake of a number of nucleoside-derived drugs [85].

2.3. Aberrant Expression of BBB Components in Brain Tumors. BBB components claudins and occludins are either downregulated or not at all expressed in brain tumors. Loss of claudin-1 and downregulation of claudin-3 and claudin-5 expressions in high grade glioma are reported earlier [9, 142]. This variation of expression of claudins causes loosening of BBB tight junctions, but the involvement of claudins in the mechanism for the compromised tight junction function in BBB is not very clear. Claudin-1 proteins are known to regulate different signaling pathways, which in turn alter the expression and function of different cell-cell adhesion molecules [143]. It is also reported that claudin-5 regulates BBB permeability during the metastasis of brain tumors [144]. Loss of expression of another transmembrane protein occludin in microvessels is also observed in astrocytomas and metastatic adenocarcinomas. The probability of their contribution to endothelial tight junction opening is also very high [11]. High grade astrocytomas secrete vascular endothelial growth factor (VEGF), which downregulates the expression of occludins and increases endothelial cell permeability [145]. However, besides VEGF, cytokines and scatter factor or hepatocyte growth factor are also secreted by astrocytoma and other brain tumors. These factors are believed to be involved in the downregulation of tight junction molecules leading to its leakage [146, 147].

3. Drug Delivery Approaches and Current Advances in Brain Tumor Therapy

Most of the brain tumor drugs are ineffective due to their limited entry through BBB. Nowadays scientific communities are interested in providing solutions to this problem, and it is not surprising that most of the brain tumor patients could benefit from the improved drug delivery approaches. Few established approaches are intra-arterial drug delivery, intrathecal and intraventricular drug administration, intratumoral delivery, receptor-mediated transport, disruption of BBB, inhibition of drug efflux by BBB, and the use of intranasal drug delivery route.

The clinical trials of intra-arterial delivery in brain tumor drugs show minimal improvement in survival of brain tumor patients [148–154], but recently the neurosurgeons of New York Presbyterian Hospital/Weill Cornell Medical Center for the first time showed the successful intra-arterial delivery of monoclonal antibody like bevacizumab to the tumor region by means of transient blood brain barrier disruption [155]. In case of intrathecal drug administration, the drugs possess limited ability to enter the extracellular space of brain from the CSF [156–159]. The convection enhanced diffusion (CED) technique is used in transcranial brain drug delivery approaches to evade the BBB for forceful delivery of fluid into the brain and to increase the effective infiltration of drug into tumor region [160]. Application of microdialysis in neurooncology is also well established since it has been proposed as an efficient method of intratumoral drug delivery. This method employs the passive diffusion of a drug across the BBB [161, 162] and distributes drugs away from the dialysis catheter [163]. On the other hand, the receptor-mediated endocytosis and exocytosis facilitate the entry of the therapeutic compounds across the BBB of brain tumors. Receptor targeted monoclonal antibody-based drugs are delivered across the BBB by the help of receptor-mediated transport systems [164, 165]. Another traditional approach to solve the problem of drug delivery into the brain is BBB disruption. Osmotic disruption technique, bradykinin-analogue or alkylglycerol mediated disruption technique, MRI-guided focused ultrasound BBB disruption technique, and so forth are used to disrupt the BBB [166–169]. Though, bradykinin analogue mediated delivery of drug is abandoned

due to its ineffectiveness when administered in combination with carboplatin. Recently, MRI-guided focused ultrasound BBB disruption technique is used to disrupt BBB for effective drug delivery [170].

P-glycoproteins (P-gp) of the ABC drug efflux transporters are present not only in low grade brain tumors but also in different malignant glioma cells [171]. Modulation of P-gp may cause effective delivery of drugs to the tumor niche. The poor *in vivo* efficacy of the first generation P-gp modulators (verapamil, cyclosporine A, tamoxifen, and several calmodulin antagonists) is due to their low binding affinities, which necessitated the use of high doses, resulting in intolerable toxicity [172]. The coadministration of the second-generation P-gp modulators (dexverapamil, dexniguldipine, valspodar (PSC 833), and biricodar (VX-710)) [173, 174] and chemotherapy agents in clinical trials has provided limited success; hence, third-generation P-gp modulators come in the scenario. These modulators include anthranilamide derivative tariquidar (XR9576), cyclopropyldibenzosuberane zosuquidar (LY335979), laniquidar (R101933), and elacridar (GF120918) [175–178]. Kemper et al. showed 5-fold increase in brain uptake of paclitaxel by combinatorial treatment with elacridar (GF120918) [178]. Other than P-gp inhibitors, MRP inhibitors (like sulfinpyrazone, probenecid, etc.) and BCRP inhibitors (fumitremorgin C and its analogues) are also reported as transporter inhibitors [172, 179]. Ongoing clinical trials with these new P-gp inhibitors should prove whether this approach will result in increased survival of brain tumor patients.

A promising drug delivery technique that can bypass the BBB is the usage of intranasal drug delivery route. This technique eliminates the risk of surgery and the nonspecific spillover effect of drug to normal tissue. Intranasal delivery provides successful drug targeting mechanism which utilizes the unique anatomic connections of olfactory and trigeminal nerves of nasal mucosa and the central nervous system [180, 181]. The drugs administered through this path reach the cerebrospinal fluid (CSF), spinal cord, and brain parenchyma very rapidly. This delivery system has been proven to be successful in delivering anticancer agents to the brain, like raltitrexed, 5-fluorouracil, GRN163, and methotrexate [182–185]. Further studies about intranasal therapeutic agents are needed and it could be a major candidate for clinical trials in brain tumor patients.

Current techniques and new approaches in drug delivery across the BBB can be classified as follows.

3.1. Modification of Existing Drugs. The ability of drug to cross the BBB depends on few factors like molecular size (should be less than 500 Da), charge (should have low hydrogen bonding capabilities), and lipophilicity (should have high lipophilicity) [186]. Thus, chemical modification of brain tumor drugs refers to the process of making an existing drug smaller in size, more perfectly charged, and more lipid soluble [187] (Table 2). Existing brain tumor drugs may also be modified to make analogue of the ligand to the particular receptor present in the BBB or the ligand or a peptide can be linked to a drug against the cellular receptors of BBB. The drug melphalan has been modified by using this approach where melphalan nitrogen mustard (mechlorethamine) was linked to phenylalanine [188]. Another approach of drug modification is the use of lipid carriers for efficient transport through BBB. One example of such modification is incorporation of small drugs in fatty acids like N-docosahexaenoic acid (DHA) [189, 190]. Drugs are also modified in such a way that they acquire increased capillary permeability, but after crossing the BBB they undergo an enzymatic reaction and return to their active state. This approach is also known as prodrug therapy [191, 192].

3.2. Nanosystem Based Delivery. Nanosystems are colloidal carriers that mainly consist of liposomes and polymeric nanoparticles while other systems, including solid lipid nanoparticles, polymeric micelles, and dendrimers, have also been studied recently. Sizes of these nanosystems vary within 1–1000 nm. These kinds of functionalized drug colloidal carriers can act as a vehicle to deliver antitumor drugs to brain tumor tissues. These nanosystems generally use passive diffusion mechanism as they rely on increased vascular permeability of brain tumor location, but usage of active chemically modified drugs with nanoparticles and receptor-mediated or adsorptive endocytosis processes of nanoparticle delivery have also been reported [219–221]. Conjugation of ligands targeting BBB on the surface of the nanosystem increases their specificity for brain tumors. One of the important features of these nanosystems is that they can circulate in the bloodstream for a prolonged time period. But the interaction of the nanosystems with the reticuloendothelial system (RES) causes its rapid removal from systemic circulation [222]. Therefore, to minimize the interactions of nanosystems with the RES, polyethylene glycol (PEG) coating or direct chemical linking of PEG to the particle surface is a widely accepted approach. These colloidal nanosystems comprise liposomes and nanoparticles, which have shown potential to target brain tumors as drug carriers. Furthermore, studies are going on for the development of novel transport-enhancing nanocarriers for brain tumor treatment.

3.2.1. Liposomes. Liposome is a good carrier system for the delivery of therapeutic agents for brain tumors. They are easy to prepare, biocompatible, less toxic, and commercially available. Along with PEGylation, the liposomes can also be modified with monoclonal antibodies against transferrin receptors (OX-26), glial fibrillary acidic proteins (GFAP), or human insulin receptors [223]. Effective delivery of drugs like 5-fluorouracil (5-FU) and sodium borocaptate ($Na_2B_{10}B_{12}H_{11}SH$, BSH) to high grade brain tumors has been achieved by liposome mediated delivery [224, 225]. Modified liposomes like p-aminophenyl-α-D-mannopyranoside (MAN) and transferrin conjugated daunorubicin liposomes and *trans*-activating transcriptional peptide (TATp) modified liposomes have also been used *in vitro* and *in vivo* for targeting brain tumors [226, 227].

3.2.2. Nanoparticles. Polymeric nanoparticles (NP) are colloidal particles which can be found in the form of nanocapsules or nanospheres. The drugs are dissolved, entrapped,

Table 2: Recent modifications of few important brain tumor drugs.

Drug name	Mode of action	Modification type	Examples	Usual route of administration	Targeted brain tumor type	Reference
Temozolomide	Alkylating agent	Nanoparticle based	Polysorbate-80 coated PBCA nanoparticles as feasible carrier for TMZ delivery to the brain	Oral	Glioblastoma multiforme	[193]
			Transferrin-appended PEGylated nanoparticles for TMZ delivery to brain			[194]
			TMZ solid lipid nanoparticles (TMZ-SLNs)			[195]
			Polysorbate-80 coated TMZ loaded PLGA based supermagnetic nanoparticles			[196]
			TMZ loaded in PLGA nanoparticle			[197]
			TMZ loaded in chitosan nanoparticle			[198]
			TMZ loaded in albumin nanoparticle			[199]
Carmustine (BCNU)	Alkylating agent	Liposomes, polymer microchips, and microspheres	Gliadel	Wafer implant/IV/oral	Glioblastoma multiforme, medulloblastoma, and low grade astrocytoma	[200]
		Nanoparticles	Chitosan surface-modified poly(lactide-co-glycolide) nanoparticles loaded with BCNU			[201]
			Catanionic solid lipid nanoparticles (CASLNs) carrying BCNU			[202]
			BCNU-loaded poly(lactic acid) (PLA) nanoparticle			[203]
Doxorubicin (DOX)	Anthracyclines, inhibiting nucleic acid synthesis	Liposome	Long-circulating PEGylated liposomes to cross blood brain barrier	IV	Glioblastoma multiforme	[204]
		Nanoparticle	Cationic solid lipid nanoparticles (CASLNs), loaded with DOX			[205]
			Human serum albumin nanoparticles loaded with DOX			[206]
Lomustine (CCNU)	Alkylating nitrosourea compound	Liposomes or microcapsules	Administration of CCNU-Lips and inclusion complex solution of CCNU with hydroxypropyl-β-cyclodextrin (CCNU-Sol)	Oral	Oligodendrogliomas and mixed oligoastrocytomas	[207]
Vincristine (Oncovin)	Vinca alkaloid	Liposome	Vincristine sulfate liposome, PEGylated liposome	IV	Anaplastic oligoastrocytoma and oligodendroglioma, metastatic secondary brain tumors	[208, 209]

Table 2: Continued.

Drug name	Mode of action	Modification type	Examples	Usual route of administration	Targeted brain tumor type	Reference
Cisplatin	Platinum-containing anticancer drugs	Liposome	Transferrin-modified cisplatin liposome Cis-lipo(Tf)	IV	Glioma, medulloblastoma, and other types of brain tumors	[210]
Carboplatin	Platinum-based antineoplastic agents	Liposomes	Liposomal carboplatin	IV	Glioma, medulloblastoma, and other types of brain tumors	[211]
Methotrexate	Antimetabolite and antifolate	Nanoparticle	Magnetic nanoparticles	Oral/injection	Malignant brain tumors, brain lymphoma	[212]
Etoposide (ETP)	Topoisomerase inhibitor	Nanoparticle	ETP-encapsulated cationic solid lipid nanoparticles (ETP-CASLNs) grafted with 5-HT-moduline	IV/oral	Malignant brain tumors	[213]
			Liposomal etoposide			[211]
Actinomycin (dactinomycin)	Polypeptide antibiotics	Liposome	Liposome encapsulated actinomycin	IV	Secondary brain tumor, child brain tumor	[214]
Irinotecan	DNA topoisomerase I inhibitor	Liposome	Nanoliposomal irinotecan	IV	Glioblastoma multiforme	[215]
Paclitaxel (Taxol)	Taxanes	Chemical	Tx-67,10-O-deacetylpaclitaxel 10-monosuccinyl ester	IV	High grade glioma, oligodendroglioma	[216]
		Liposomes	Polysorbate 80 coated poly (ε-caprolactone)-poly (ethylene glycol)-poly (ε-caprolactone) (PCEC) micelles			[217]
			Paclitaxel plus artemether liposomes			[218]

encapsulated, adsorbed, or chemically linked to the surface of the NPs. The polymer structure and the drug trapping method determine the drug characteristics and its release kinetics from the nanoparticles [228]. One example of nanoparticle drug delivery approach is the usage of nanoparticles coated choline derivative that is reported to be transported across brain-derived endothelial cells by the cation transporter system [229]. Other remarkable systems are polysorbate-coated doxorubicin nanoparticles and doxorubicin-loaded folic acid-decorated nanoparticles, which cause effective penetration of drugs through BBB [230, 231]. Brain tumors can also be selectively targeted by bionanocapsules conjugated with anti-human EGFR antibody that recognizes EGFRvIII known to be overexpressed in high grade brain tumors like glioblastoma multiforme [232]. Those bionanocapsules may also contain virus, active proteins, vaccines, genes, or small interference RNA for targeted therapy of brain tumors. Solid lipid nanoparticles (SLNs), which are the dispersions of solid lipid stabilized with emulsifier or emulsifier/coemulsifier complex in water, are also known for delivering brain tumor drugs like camptothecin, doxorubicin, and paclitaxel to brain effectively [233]. Furthermore, gold nanoparticles and carbon nanoparticles (like carbon nanotubes, graphene, and carbon dots) are also able to deliver drugs (like doxorubicin) successfully [234–237]. Thus, nanoparticles may be considered as one of the most promising tools to deliver therapeutic drugs across the BBB to treat brain tumors [238].

Other nanosystems like polymeric micelles and dendrimers are also effective for targeted delivery of drugs to the tumors in the brain. Formation of polymeric micelles occurs spontaneously in aqueous solutions of amphiphilic block copolymers, whereas dendrimers are highly branched polymer molecules formed by a central core. These types of nanopreparations loaded with anticancer drugs should be considered as highly potential antitumor nanomedicines as they have the ability to cross the BBB by modulating BBB transporters like P-gp or glucose transporters [239–241].

3.3. Delivery Systems Used in Gene Therapy. Effective treatment of brain tumor can be obtained from intracerebral implantation of a therapeutic gene, inserted into a viral vector. It is a specifically targeted therapy where volume of the implantation is very low ($<1\,mm^3$). Thus, the expression of exogenous gene is highly localized. But gene reformulation may cause the generalised expression of exogenous gene in the total brain tumor niche. Few examples of carriers in this type of therapeutic systems are viral vectors like adenovirus, herpes simplex virus (HSV), and nonviral gene delivery system like cationic liposome-DNA complexes [242–244]. The O6-methylguanine-DNA methyltransferase (MGMT) upregulation in GBM makes it resistant to Temozolomide (TMZ), a well-known drug for glioma. Therefore, upregulation of wild-type (wt) p53 expression is needed which downmodulates MGMT. Since p53 therapy for GBM is not very efficient due to the presence of the blood brain barrier (BBB), a systemic nanodelivery platform (scL) for tumor-specific targeting (primary and metastatic) has been developed by Kim et al. It has been observed that the combination of scL-p53 and TMZ increased the antibrain tumor efficacy of TMZ [245]. Another report shows the efficacy of CMV-specific T cell therapy, as it is reported that the expression of human cytomegalovirus (CMV) antigens in GBM tissues is pretty high. Distinct gene expression correlated with the better clinical response is recorded for the high grade brain tumor patients, who availed themselves of CMV-specific T cell therapy [246].

3.4. Effective Delivery of Therapeutic Peptides. Towards fulfilling the goal of effective therapy, recently selective peptides have been developed against brain cancer. Discovery of novel peptide as novel specific chemical entity is encouraged by the identification of several protein/peptide receptors and tumor-related peptides/proteins, those expressed in brain cancer cells. Small sized, less toxic peptides are advantageous over the monoclonal antibodies (mAbs) and large proteins that have large size and high toxicity have poor rate of BBB crossing. Other major advantages of peptides are their BBB penetrating ability in brain tumors, ease of synthesis and modification, and good biocompatibility [247]. Chlorotoxin is such a peptide which selectively binds to glioma cells [248]. Somatostatin analogues, which can be defined as peptide receptor radionuclide therapeutic agents, are the only approved cancer therapeutic peptides in the market [249] and there are reports of their binding to the cellular receptors in brain tumors *in vivo* [250]. Another new approach of brain tumor therapy is developing vaccines consisting of peptides derived from the protein sequence of brain tumor-associated or specific antigens [251]. Autologous DC vaccine against CD133 (a marker of GBM), survivin peptide vaccine, rindopepimut (also known as CDX-110) against EGFRVIII, and so forth are the examples of peptide vaccines for high grade brain tumors and these are now under clinical trials [252–254].

3.5. Molecular Trojan Horses (MTH). Recently a new technique is used to ferry drug molecules across the BBB, which is called Molecular Trojan Horse (MTH) mediated drug delivery. Delivery of particular substances to the brain after attaching them to a protein, which can cross BBB, is the main focus of this type of delivery system. One of the recent progresses of MTH is "Trojan horse liposome" (THL) technology [255–257]. The application of this technology to transvascular nonviral gene therapy of brain represents a potential way out of the transvascular brain gene delivery problem. The THL is constructed with PEG-conjugated lipids which encapsulate plasmid DNA encoding proteins or shRNA/siRNA. Marked decrease in expression of EGFR protein in the tumor region was noticed after using THL mediated RNAi gene therapy. This resulted in a 90% increase in survival time of brain tumor patients [258].

3.6. Drug Delivery Targeting Brain Cancer Stem Cells. Cancer stem cells (CSCs) are the tumor initiating cells present in the tumor niche. These cells cause drug resistance, metastasis, and relapse of cancer. Most of the current chemotherapeutic molecules are able to destroy the cancer cells but not the CSCs. Thus, to kill these CSCs in brain tumors, effective treatment modalities are needed, which should also have the ability to cross the BBB; for example, curcumin encapsulated in nanoparticles caused a dose-dependent growth inhibition of brain tumor CSCs and neurospheres [259]. Other than this, targeting active genes like MGMT in brain CSCs by liposomes with anti-MGMT siRNA for oral Temozolomide therapy and destruction of brain CSCs niche by mAb-vectorized SWNT (single-walled carbon nanotubes) for hypothermic treatment also resulted in destruction of CSCs [260, 261]. The efficacy of the CSC targeting drugs can be improved by optimisation of chemo- and nanotherapies, novel gene-silencing techniques, and drug efflux inhibition techniques which may increase survivability of the brain tumor patients.

4. Concluding Remarks

Modern era of brain cancer therapy is characterized by novel target specific drugs with efficient delivery strategies. However, the prognosis and median survival of the brain tumor patients are not satisfactory till date. This is due to molecular heterogeneity of the brain tumors, presence of CSCs, and lack of effective drug delivery because of the presence of BBB. Rapid progress is needed in the sector of brain tumor characterization and BBB research. Till now, most effective drugs for brain tumor therapies are Temozolomide, Procarbazine, Carmustine (BCNU), Lomustine (CCNU), and Vincristine. Better modification of these drugs or identification of new chemical entities with enhanced efficacy and low side effect is always commendable. Alternatively, identification of drugs which can modulate BBB components or transporter systems could be an effective future strategy. Another potential future approach is combinatorial therapy, where through BBB destruction/modification, tumor cells/CSCs could be targeted easily. Modern techniques like nanotherapy may facilitate this kind of approach. Therefore, future research is needed to focus on the development of more specific targeting strategies to cure brain cancer, overcoming the above-mentioned difficulties arising due to the presence of the BBB.

Abbreviations

BBB: Blood brain barrier
GBM: Glioblastoma multiforme
CSF: Cerebrospinal fluid
VE: Vascular endothelium
BMEC: Brain macrovascular endothelial cells
CNS: Central nervous system
ZO: Zonula occludens
JAM: Junction adhesion molecules
GLUT1: Glucose transporter 1
ATP: Adenosine triphosphate
ABC: ATP-binding cassette
SLC: Solute carrier
P-gp: P-glycoprotein
MDR: Multidrug resistant
BCRP: Breast cancer resistance protein
MRP: Multidrug resistance proteins
TMD: Transmembrane domains
POT: Proton coupled oligopeptide transporters
PEPT: Peptide transporters
PHT: Peptide/histidine transporter
PTS: Peptide transport system
MCT: Monocarboxylate transporters
TAT: T-type amino acid transporter
OATP: Organic anion transporters polypeptides
OAT: Organic anion transporters
OCT: Organic cation transporters
OCTN: Organic cation transporter novel
ENT: Equilibrative nucleoside transporters
CNT: Concentrative nucleoside transporters
VEGF: Vascular endothelial growth factor
CED: Convection enhanced diffusion
LDL: Low density lipoprotein
RMP: Receptor-mediated permeabilizer
MRI: Magnetic resonance imaging
RES: Reticuloendothelial system
PEG: Polyethylene glycol
GFAP: Glial fibrillary acidic proteins
NP: Nanoparticle
EGFR: Epidermal growth factor receptor
EGFRvIII: Epidermal growth factor receptor variant III
SLN: Solid lipid nanoparticle
HSV: Herpes simplex virus
MGMT: O6-Methylguanine-DNA methyltransferase
CMV: Cytomegalovirus
mAbs: Monoclonal antibodies
DC: Dendritic cell
MTH: Molecular Trojan Horse
THL: Trojan horse liposome
shRNA: Short hairpin RNA
siRNA: Small interfering RNA
RNAi: RNA interference
CSC: Cancer stem cell
SWNT: Single-walled carbon nanotubes.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Mrinal Kanti Ghosh, Arijit Bhowmik, and Rajni Khan wrote and analyzed the paper.

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Review Article

Importance of pH Homeostasis in Metabolic Health and Diseases: Crucial Role of Membrane Proton Transport

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Protons dissociated from organic acids in cells are partly buffered. If not, they are transported to the extracellular fluid through the plasma membrane and buffered in circulation or excreted in urine and expiration gas. Several transporters including monocarboxylate transporters and Na^+/H^+ exchanger play an important role in uptake and output of protons across plasma membranes in cells of metabolic tissues including skeletal muscle and the liver. They also contribute to maintenance of the physiological pH of body fluid. Therefore, impairment of these transporters causes dysfunction of cells, diseases, and a decrease in physical performance associated with abnormal pH. Additionally, it is known that fluid pH in the interstitial space of metabolic tissues is easily changed due to little pH buffering capacitance in interstitial fluids and a reduction in the interstitial fluid pH may mediate the onset of insulin resistance unlike blood containing pH buffers such as Hb (hemoglobin) and albumin. In contrast, habitual exercise and dietary intervention regulate expression/activity of transporters and maintain body fluid pH, which could partly explain the positive effect of healthy lifestyle on disease prognosis.

1. Introduction

Body fluid pH is determined by the content of protons (H^+) generated from organic acids produced in living cells. Lactic acid (lactate$^-$/H^+) is a typical proton source and is involved in the regulation of physiological pH. In metabolic tissues such as skeletal muscle and adipose tissue, the glycolytic anaerobic metabolism mediates the conversion of glucose and glycogen into lactic acid. Because the pKa of lactic acid is 3.80, it is immediately dissociated into lactate (lactate$^-$) and protons under physiological conditions, resulting in reduced intracellular pH. Pyruvic acid (pyruvate$^-$/H^+), an intermediate metabolite in the glycolytic system, is also a source of protons, although it generates much less protons compared to lactic acid. In addition, metabolites such as ketone bodies also act as proton sources. Beta-hydroxybutyric acid (beta-hydroxybutyrate$^-$/H^+), a typical ketone body, is generated as a result of fatty acid metabolism in the liver and is also dissociated into beta-hydroxybutyrate anions and protons, leading to the reduction of intracellular pH.

The intracellular pH in most living cells is alkaline compared to the pH generated by protons that are transported passively through the plasma membrane by electrochemical forces. In addition to buffering systems such as the bicarbonate-carbonate system, protein-proton binding, and phosphoric acid, several membrane transporters are responsible for proton removal from the cytosol and play important roles in maintaining the alkaline pH in cells (Figure 1). In most mammalian cells, H^+-monocarboxylate cotransporters (MCTs) participate in the transport of monocarboxylic acids such as lactate, pyruvate, beta-hydroxybutyrate, and acetoacetate across the cellular membrane by cotransporting protons and monocarboxylate anions [1–3]. Other transporters such as the Na^+/H^+ exchanger (NHE) and bicarbonate-dependent

exchanger also contribute to proton extrusion from the cytosol to the extracellular space [4, 5]. This review focuses on the critical role of the membrane transport system of protons in regulation of intracellular and extracellular fluid pH and its importance in maintaining physiological homeostasis and preventing diseases development.

2. Proton Transport across the Plasma Membrane in pH Regulation

Regulation of body fluid pH is one of the most important physiological functions of homeostasis, because activity of most chemical reactions via enzyme proteins is dependent on fluid pH. To maintain homeostasis of body fluid pH, various buffering systems are utilized in addition to proton excretion from the cytosol to the extracellular space and ultimately outside of the body. However, if production of organic acid is elevated or the buffering and excretion systems are impaired, body fluid turns acidic, leading to abnormal conditions. A typical example is elevation of lactic acid production in skeletal muscle in response to strenuous exercise, which leads to body fluid acidosis, preventing muscle contraction [6, 7]. Proton transport across the plasma membrane of muscle cells is important for maintaining the appropriate intracellular pH. Skeletal muscle is a major metabolic organ that generates acids, in particular during contraction. Strenuous muscle contractions can cause a drastic reduction in intramuscular pH to −6.5 with accumulation of more than 40 mM lactate [6–8], regardless of cellular buffering capacity. Several studies have shown that intracellular pH is reduced during muscle contraction and has a delayed recovery to basal conditions during the recovery phase in the absence of proton transporters [9]. This delay suggests that proton transporters play a key role in maintaining pH homeostasis. Indeed, the function of proton transporters is involved in the capacity for pH maintenance [9, 10]. In particular, over 80% of intracellular proton is transported through lactate cotransport in contracting muscle, although remaining parts are transported through NHE and bicarbonate-depending transport [8, 11]. The liver, another organ that is closely associated with the metabolism of organic acids, generates ketone bodies (i.e., acetoacetic and β-hydroxybutyric acids), metabolizes lipids, and converts lactate to glucose via gluconeogenesis. Therefore, this organ generates acidic conditions [12–14] and intracellular pH should be maintained by proton extrusion along with buffering function.

MCTs, a part of the solute carrier (SLC) 16 that contains 14 members in total, play a crucial role in proton transport across the plasma membrane by cotransporting monocarboxylates. Each isoform has different transport kinetics and is specifically located on a particular subcellular site. It has been shown that MCT1–MCT4 transport aliphatic monocarboxylates such as lactate, pyruvate, and ketone bodies [2] and that the direction of transport across the plasma membrane in a 1 : 1 manner is determined by the concentration gradients of protons and monocarboxylate both inside and outside of the cell [15–17]. Thus, these isoforms play important roles in proton transport maintaining intracellular pH. In particular, the expression of two MCT isoforms (MCT1 and MCT4) is associated with lactate disposal in muscles. MCT1 is highly expressed and located in both the sarcolemmal and the mitochondrial membranes of oxidative muscles [18–20]; on the other hand, MCT4 is predominantly located on the plasma membrane of glycolytic muscle and is assumed to contribute to lactate efflux [19, 21]. In contrast, MCT2 is mainly located on the membranes of liver cells and contributes to the extrusion of ketone bodies [22]. Other members of the family have different substrate specificities. For example, MCT6 has been shown to transport bumetanide, a diuretic drug [23], MCT 8 acts as a thyroid hormone transporter [24], MCT9 is a potential carnitine extrusion transporter [25], and MCT10 is identified as a low-affinity transporter of aromatic amino acids along with iodothyronines [26]. In addition, NHE is known as another major proton transporter that plays an important role in intracellular pH homeostasis by exchanging intracellular proton with extracellular Na^+ using the chemical gradient between intra- and extracellular Na^+ concentrations [4, 27]. Currently, 10 isoforms are known to exist in mammals. NHE1–NHE5 are located on the plasma membrane of their specific tissues, while NHE6–NHE9 are located on the membrane of subcellular organelles [27–29]. In particular, NHE1 has been recognized as a ubiquitous isoform and plays an important role in maintaining homeostasis in metabolic organs.

Proton transport across the plasma membrane is important for maintenance of intracellular and extracellular fluid pH. In particular, proton excretion and bicarbonate reabsorption are recognized as important function of renal tubules. Proton excretion into urine is mainly mediated by both proton-ATPase and NHE3 located on the apical plasma membrane of the proximal convoluted tubule participating in approximately 80% of bicarbonate reabsorption occurring in the whole kidney, acting as the major buffering system in blood [30, 31], which has also pH buffers such as Hb (hemoglobin) and albumin. Bicarbonate reacts with protons via catalytic carbonic anhydrase on the apical membrane and generates CO_2. Then, it is transported into the blood by sodium-bicarbonate cotransporters on the basolateral side [32].

3. pH Disturbance and Disease Development

The normal physiological pH of mammalian arterial blood is strictly maintained at 7.40; blood has pH buffers such as Hb (hemoglobin) and albumin. A decrease of more than 0.05 units from the normal pH results in acidosis. The body fluids of diabetic patients are chronically acidic and exhibit characteristic ketoacidosis caused by an increased level of ketone bodies in the blood [33, 34]. Insulin resistance in metabolic tissues such as skeletal muscle, adipose tissue, and the liver accelerates the utilization of lipids as an energy substrate instead of glucose. Excess lipolysis caused by impaired glucose metabolism leads to free fatty acids in circulation, which facilitate hepatic gluconeogenesis by the oxidation of fatty acids resulting in large quantities of ketone bodies. This further accelerates proton overloads, leading to the metabolic ketoacidosis found in diabetic patients.

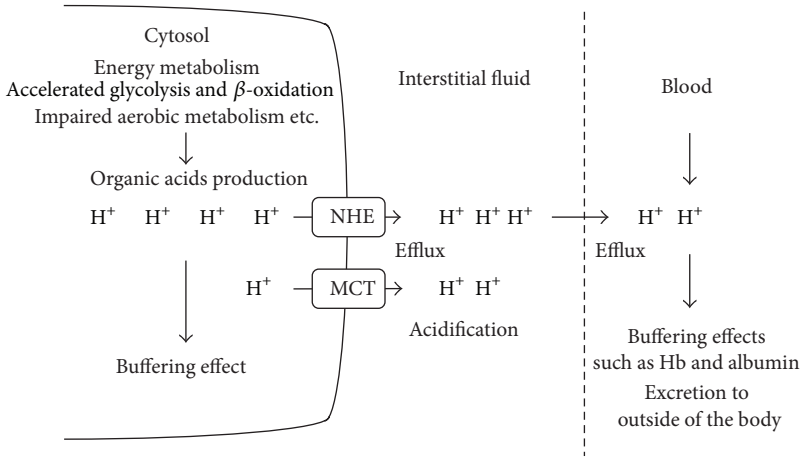

Figure 1: Proton production and its transporting kinetics in intracellular and extracellular fluid in metabolic tissues. The production of organic acids such as lactic acid and ketone bodies is accelerated by elevating glycolytic anaerobic metabolism and β-oxidation in metabolic cells. Body fluid pH is strictly maintained by buffering systems, efflux across plasma membrane, and acid excretion. Monocarboxylate transporter (MCT) and Na^+/H^+ exchanger (NHE) contribute to proton extrusion from the cytosol to the extracellular space. In contrast to intracellular fluid and blood containing pH buffers such as Hb (hemoglobin) and albumin, the interstitial fluid pH could be easily reduced by acid stress owing to the limited availability of the buffering factors such as proteins.

Such acidic conditions prevent the activity of metabolic enzymes such as phosphofructokinase and further accelerate the progression of pathological conditions [33–35]. Acidic conditions can also result in physical fatigue of diabetic patients. Therefore, maintaining normal pH is important for physiological homeostasis.

It has been suggested that loss of function of MCTs causes a change of body fluid pH. Several point mutations of the MCT gene have been shown to affect both specificity and transport activity. The spontaneously occurring mutation of arginine 306 to threonine in domain 8 of MCT1 resulted in reduced transport activity [36]. In addition, it has been shown that subjects who have mutations in MCT1 cDNA have drastically lower transport rates and a delayed decline of blood lactate after exercise [37, 38]. Healthy subjects feel severe chest pain and muscle cramping after strenuous exercise, along with a defect in lactate efflux from muscle. Furthermore, many amino acid differences that are not attributable to polymorphisms are found in MCT1 obtained from muscle tissues in these subjects [37, 39]; thus, mutations in MCT1 are related to physical fatigue and exercise performance. MCT dysfunction may lead to metabolic disorder. Indeed, lower level expression of MCT1 and MCT4 is found in the skeletal muscle of obese rats compared to normal rats [40]. In addition, the activity of lactate transport in muscle is also decreased by both denervation and aging [41, 42]. A significant negative correlation between the level of circulating lactate and degree of insulin sensitivity is found in humans [43], suggesting that lower lactic acid disposal caused by reduction of MCT function is associated with insulin resistance.

4. Interstitial Fluid pH and Disease Development

Body fluid acidosis could also contribute to the development of metabolic diseases. Our recent study indicates that before the development of diabetic symptoms the interstitial fluid pH in ascites and metabolic tissues of Otsuka Long-Evans Tokushima Fatty (OLETF) rats is lower than the normal pH (7.40) [44]. The buffering capacity is relatively high in the cytosol and blood but low in the interstitial fluid due to limited buffering factors such as proteins [45, 46]. Therefore, interstitial fluid pH in metabolic tissues easily changes (Figure 1) and may contribute to the onset of insulin resistance. We have shown the inhibitory effect of extracellular pH on the insulin signaling pathway in the L6 rat myotube. The phosphorylation level and binding affinity to insulin of insulin receptors were significantly diminished in media with low pH [47]. In addition, the levels of Akt phosphorylation, a downstream of the insulin receptor, are also decreased in low pH media, along with a reduction in glucose uptake. These *in vitro* observations support the hypothesis that lower extracellular pH may cause insulin resistance in skeletal muscle cells. Other studies [48–50] have suggested a close correlation between organic acid production and insulin sensitivity in both type 2 diabetes patients and healthy subjects. In a cross-sectional study of over 1,000 subjects [48], it has been demonstrated that body weight and waist circumference have a negative correlation with both insulin sensitivity and urine pH. Patients with metabolic syndrome have also reported a significantly lower pH of 24 h urine compared to the normal subjects and a negative correlation between the mean 24 h urine pH and the number of metabolic syndrome abnormalities [49, 50]. It has been suggested that lower levels of serum bicarbonate and higher levels of anion gap resulting from metabolic acidosis are associated with lower insulin sensitivity [51]. Hyperlactacidemia is found in patients with obesity and type 2 diabetes [43], which supports the strong relationship between acidic condition and insulin sensitivity. Even in healthy subjects, acids level could be an independent risk factor for the development of type 2 diabetes [52].

Insulin resistance is one of the major symptoms of metabolic disorders and is frequently associated with hypertension, high blood glucose levels, visceral obesity, and dyslipidemia. Insulin resistance also causes type 2 diabetes and plays a key role in developing cancer and cardiovascular disease. Thus, pH abnormalities can cause abnormal metabolic regulation in a predisease state. We recently found an observation that the interstitial pH around the hippocampus, an important region for memory [53], is lower in diabetic OLETF rats (26 weeks of age) than in normal Wistar rats [54]. It has been reported that diabetic patients have a high risk of developing dementia and Alzheimer's disease [55] and may experience defective memory functions. The insulin action is required for neuronal survival within the central nervous system [56]. Fluctuating glucose levels resulting from defective insulin have been suggested to lead to apoptosis, energy starvation, formation of neuritic plaques and neurofibrillary tangles, hallmark lesions of Alzheimer's disease, and altered acetylcholine levels in the hippocampus [57, 58]. Therefore, we indicate that maintenance of the interstitial fluid pH within the normal range or the recovery of the interstitial pH to the normal range could be one of the most important factors in developing molecular and cellular therapies for metabolic brain disorders.

5. pH Regulation by Diet and Exercise Intervention

The maintenance of pH in metabolic organs is achieved through various regulatory systems. Physical exercise and appropriate diet contribute to pH homeostasis. Habitual exercise adaptively accelerates the entry of fatty acids both from the plasma into the muscle cell and from the cytosol into the mitochondria, while also enhancing Krebs cycle function in the resting state. Their actions are caused by elevation of activity and expression of related enzymes in skeletal muscles [59–61]. Since the energy consumed in muscle during exercise is mainly supplied by carbohydrates and lipids, the exercise-induced lipid utilization may decrease the energy obtained from carbohydrates, further decreasing the lactate/proton production, or lactic acidosis. In addition, circulating and intramuscular buffering capacities are improved via habitual exercise increasing proteins, amino acids, and phosphate [62–64]. Peripheral circulation is also improved through vasodilation caused as a physiological adaptation to exercise [65], which further facilitates the proton washout. In particular there is evidence suggesting that excretion of protons from the cytosol to the extracellular space or into circulation via transporters located on the plasma membrane contributes to the prevention of intracellular acidosis. It has been reported that exercise training increases the MCT1 and MCT4 levels in the skeletal and cardiac muscle of humans and animals [66–68]. Although the regulation of MCT expression levels is not clearly understood, it has been suggested that protein kinases A and B are involved in the regulation of MCT expression [69] as an adaptation mechanism, which may be mediated by an increase in lactate movement across the membrane. In addition, our recent study has reported that MCT1 content in erythrocyte membranes is elevated by exercise training in rats [70, 71]. A proportion of the lactate released from skeletal muscles into the plasma is taken up by erythrocytes. The mature erythrocytes generate ATP only through the glycolytic pathway, since they have no mitochondrial machinery. Thus, erythrocytes cannot utilize lactate produced as a respiratory fuel and this necessitates the release of lactate into the plasma via MCT1 [72]. However, one of the most important roles of erythrocytes is to distribute released monocarboxylates by taking up monocarboxylates, since erythrocytes produce much less lactate than other tissues. Based on the results of our *in vitro* study, the skeletal muscle may be entirely dependent on MCT1-mediated lactate uptake by erythrocytes to maintain pH homeostasis [71]. In addition, there is a high correlation between the athletic performance of horses and their erythrocyte lactate concentrations after racing [73]. Therefore, efficient proton transport via MCTs induced by habitual exercise may contribute to the improvement of insulin sensitivity and muscle fatigue caused by lowered pH.

It is well known that adequate diet is important for controlling pathological conditions in patients with metabolic disorders. In addition, intervention studies in humans have reported that several bioactive factors included in foods such as antioxidants [74–77] and n-3 unsaturated fatty acids [78, 79] improve energy metabolism. Additional factors such as carotenoids, alpha lipoic acids, amino acids/peptides, and minerals may also offer preventive or therapeutic effects to combat hyperglycemia and several animal and culture studies have demonstrated their efficacy in improving insulin sensitivity [80–84]. The effects of these nutrients are only beneficial when administered in combination. In contrast to the successful application of dietary approaches or combined nutrients [85–87], various types of intervention studies using single nutrients have failed to clarify their beneficial action on cardiovascular risk and insulin resistance [88, 89]. Therefore, administration of multiple nutrients is considered more effective when compared to administration of a single bioactive factor. Propolis, a natural product derived from the plant resins collected by honeybees, contains various types of compounds including polyphenols, phenolic aldehydes, sesquiterpene quinones, coumarins, amino acids, steroids, and inorganic compounds [90] and has been reported to reduce the metabolic defects caused by abnormal blood glucose and insulin in young (18 weeks of age) OLETF rats [42] characterized by hyperphagia, obesity, decreased glucose infusion rate in a euglycemic clamp at 16–18 weeks of age, hyperinsulinemia around 25 weeks of age responding to an intravenous glucose infusion, and developing type 2 diabetes [91, 92]. Thus, our study indicates that propolis has a beneficial and preventive action on type 2 diabetes mellitus at early stages developing insulin resistance. Further, we have obtained evidence that intake of propolis elevates the pH of ascites and metabolic tissues compared with normal diet, indicating that dietary propolis diminishes production of organic acids or increases buffering capacity in those tissues. Therefore, propolis may be a useful compound to improve insulin sensitivity via prevention of metabolic acidosis. The molecular mechanism of how propolis improves interstitial

pH is unclear, and we should strive to better understand the mechanism of this bioactive supplement.

6. Conclusion

Membrane transport of protons is required for preventing acidic states of body fluid, maintaining physical performance, and improving metabolic impairments. In contrast to the intracellular and blood pH, interstitial fluid pH can easily be reduced by acid stress. This can disturb homeostasis of the intracellular metabolism, leading to the development of metabolic diseases. However, detailed mechanisms including the involvement of membrane transport of protons responsible for the reduction of interstitial fluid pH are unknown. In addition, activity and expression of proton transporters such as MCT and NHE are easily altered by various changes in the cell environment. More studies are required to examine the detailed regulatory mechanisms of proton transporters, including gene expression, protein modification, and membrane trafficking, in addition to their contributions to metabolic homeostasis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Vascular Endothelial Growth Factor Increases during Blood-Brain Barrier-Enhanced Permeability Caused by *Phoneutria nigriventer* Spider Venom

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Phoneutria nigriventer spider accidental envenomation provokes neurotoxic manifestations, which when critical, results in epileptic-like episodes. In rats, *P. nigriventer* venom (PNV) causes blood-brain barrier breakdown (BBBb). The PNV-induced excitotoxicity results from disturbances on Na^+, K^+ and Ca^{2+} channels and glutamate handling. The vascular endothelial growth factor (VEGF), beyond its angiogenic effect, also, interferes on synaptic physiology by affecting the same ion channels and protects neurons from excitotoxicity. However, it is unknown whether VEGF expression is altered following PNV envenomation. We found that adult and neonates rats injected with PNV showed immediate neurotoxic manifestations which paralleled with endothelial occludin, β-catenin, and laminin downregulation indicative of BBBb. In neonate rats, VEGF, VEGF mRNA, and Flt-1 receptors, glutamate decarboxylase, and calbindin-D28k increased in Purkinje neurons, while, in adult rats, the BBBb paralleled with VEGF mRNA, Flk-1, and calbindin-D28k increases and Flt-1 decreases. Statistically, the variable age had a role in such differences, which might be due to age-related unequal maturation of blood-brain barrier (BBB) and thus differential cross-signaling among components of the glial neurovascular unit. The concurrent increases in the VEGF/Flt-1/Flk-1 system in the cerebellar neuron cells and the BBBb following PNV exposure might imply a cytokine modulation of neuronal excitability consequent to homeostatic perturbations induced by ion channels-acting PNV neuropeptides. Whether such modulation represents neuroprotection needs further investigation.

1. Introduction

Accidents with venomous animals have been considered a neglected health public issue. Accidents caused by *Phoneutria nigriventer*, popularly known as the armed spider, are common in Southeast Brazil. The majority of them only cause pain, intense sudoresis, and local inflammation. However, accidents graded as severe (less than 0.5%) cause in addition cramps, tremors, blurred yellow vision, tachypnea, cardiovascular alterations, priapism, and convulsion and rarely death, those symptoms appearing to be more severe in children [1, 2].

Experimental studies in rats showed that the clinical symptoms of envenomation paralleled those of humans. Our studies have shown that the *Phoneutria nigriventer* venom induces blood-brain barrier breakdown through increased vesicular transcellular transport [3] followed by upregulation of the glycoprotein P (P-gp) efflux protein and by impairment

of the paracellular pathway due to the displacement and phosphorylation of junctional proteins [4]. Apart from that, the venom induces vasogenic edema in hippocampus [5] and cytotoxic edema in rat cerebellum [6], reactive gliosis in the two regions [7], and neuronal activation (FOS+ induction) in regions associated with stress and motor activity [8]. In the cerebellum gray matter, mainly in the granular layer, PNV upregulates the water channel aquaporin-4 [9], a protein which regulates edema formation and resolution. All the changes have a pace and tend to be restored with time. The mechanism(s) underlying these effects is unknown, but hypoxia secondary to the respiratory and cardiovascular distress exhibited by animals could be a primary cause.

VEGF, an angiogenic cytokine which mediates vascular permeability [10], has been reported to protect neurons in adverse conditions by modulation of glutamatergic synaptic excitability and interference on Ca^{2+}, K^{+}, and voltage-gated Na^{+} channels physiology [11–13]. In hippocampus, the cytokine is considered an endogenous anti-convulsing factor to preserve pyramidal neurons from hyperexcitability [14]. VEGF exerts its action through the binding and activation of two transmembrane tyrosine kinase receptors: VEGFR-1 or Flt-1 [15] and VEGFR-2 or Flk-1 [16]. The receptors, in turn, modulate the VEGF signals that affect different cellular processes, so contributing for counteracting or aggravating the noxious stimuli.

In the light of the above, the current study aimed at determining whether the expression of VEGF, Flt-1, and Flk-1 and respective mRNAs of cerebellar neurons is altered in the course of BBB impairment caused by PNV in rats. Age-related alterations were investigated in neonate and young adult rats. We further analyzed the expression of glutamate decarboxylase (GAD), responsible for the synthesis of gamma-amino butyric acid (GABA) and of the calcium-binding protein calbindin-D28k (CaB), a marker of Purkinje cells. Such events were investigated because postnatal development continues for several months in the cerebellum and blood-brain barrier interface is immature in neonates. Besides, BBB of cerebellum has been already shown to be disrupted by PNV, and our hypothesis is that changes in the expression of VEGF/Flt-1/Flk-1 system may occur in parallel with BBB opening induced by ion channels-acting *P. nigriventer* venom and those changes could be age dependent.

2. Material and Methods

2.1. Ethics Statement. This study was approved by the Institution's Committee for Ethics in Animal Use (CEUA/IB/Unicamp, protocol 2403-1) and the experiments were carried out according to the Brazilian Society of Laboratory Animal Science (SBCAL) guidelines for animal use.

2.2. Venom. PNV was obtained by the electrical stimulation of spiders living in Arachnid Laboratory at the Department of Biology, IBS, UFMG, Belo Horizonte, MG, Brazil, and stored at −20°C until use.

2.3. Animals and Envenoming Procedure. Male Wistar rats (*Rattus norvegicus*) were obtained from an established colony maintained by the Central Animal House Service at Unicamp (CEMIB/Unicamp) and housed under standard laboratory conditions. Rats aged 14 days (P14 animals—neonate group) and 8–10 weeks (adult group) were used; they received a single intraperitoneal (i.p.) injection of PNV (1.7 mg/kg in 0.5 mL of 0.9% sterile saline solution), while to control groups the same volume of vehicle was given. PNV concentration was selected based on previous laboratory studies [9, 17]. Neonate rats were used for comparison with adult rats, since severe accidents by *Phoneutria* generally occur in children [1]. Time limits of 2, 5, and 24 hours corresponded to periods of peak of intoxication, beginning of clinical recovery, and no sign of intoxication at all, respectively [17].

2.4. Evaluation of Blood-Brain Barrier (BBB) Permeability. The integrity of the BBB was determined using Evans blue extravasation method. Briefly, 10 mL/kg of Evans blue (Vetec Química, Duque de Caxias, RJ, Brazil) dye solution (2% in 0.9% saline) was injected into the tail vein of adult rats (i.v.) at 2, 5, and 24 hours after i.p injection of 0.9% saline or PNV (1.7 mg/kg). Fifteen minutes after Evans blue injection, the rats were killed with an overdose of anesthetics and brain and cerebellum were rapidly removed and photographed.

2.5. Immunohistochemistry. At designated time intervals and after anesthesia, the animals (n = 4/time interval, n = 12 control, and n = 12 PNV-treated per age) were perfused transcardially with saline solution followed by 4% paraformaldehyde in 0.1 M PBS (phosphate buffered saline), pH 7.4. The cerebella were dissected out and were embedded in paraffin. Antibodies utilized were anti-VEGF (1 : 50, sc-7269), anti-Flt-1 (1 : 500, sc-316), and anti-Flk-1 (1 : 50, sc-315), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA), CaB (1 : 1000, C2724, Sigma-Aldrich, St. Louis, MO, USA), and GAD (1 : 500, AB1511, Millipore, Billerica, MA, USA). Immunohistochemistry was performed in sequential coronal 5 μm thick paraffin sections of the cerebellum as previously described [17]. To avoid procedure differences between control and envenomed groups, the immunohistochemistry for each protein (VEGF/Flt-1/Flk-1 and CaB/GAD) was performed concomitantly. Three images per region (molecular, granular, and Purkinje) of n = 4 rats, totaling 12 images per time/age/treatment, were captured using a BX51 microscope (Olympus Optical C. Ltd., Tokyo, Japan). Objective (20x), lens aperture, and light intensity were set for all images captured.

2.6. Western Blot (WB). Standard Western blot analysis [4] of cerebellum lysates (n = 6/time interval, n = 18 control, and n = 18 PNV treated per age) was performed using rabbit polyclonal antibody against CaB (1 : 2000, C2724, Sigma-Aldrich, St. Louis, MO, USA), Flt-1 (1 : 500, sc-316), Flk-1 (1 : 250, sc-315), both from Santa Cruz Biotechnology (Santa Cruz, CA, USA), GAD (1 : 1000, AB1511, Millipore, Billerica, MA, USA) and laminin (1 : 500, L9393, Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal antibody

against VEGF (1 : 500, sc-7269) and β-Catenin (1 : 600, sc-7963), both from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and β-actin (1 : 1000, A2228, Sigma-Aldrich, St. Louis, MO, USA) and goat monoclonal antibody against occludin (1 : 500, sc-8144, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were visualized using chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA). For quantification, the density of pixels of each band was determined by the NIH Image J 1.45s software (available at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, NIH, Bethesda, MD). For each protein investigated the results were confirmed in three sets of experiments and data were normalized using the respective loading controls. Values were normalized to the corresponding value for β-actin internal control and expressed as a ratio.

2.7. RNA Isolation and Real-Time Quantitative Reverse Transcription-PCR (qPCR). Total RNA was isolated from the cerebellum of each group (n = 4/time interval, n = 12 control, and n = 12 PNV treated per age) using Trizol reagent (Life Technologies, Gaithersburg, MD, USA). Primers used in this study and their respective assay identification numbers in the Applied Biosystem catalogue were VEGF: Rn01511601_m1, Flt-1: Rn00570815_m1, Flk-1: Rn00564986_m1. The levels of VEGF, Flt-1, and Flk-1 mRNAs were calculated relative to amplicon-specific standard curves by qPCR using 50 ng total RNA in triplicate and analyzed on an ABI Prism 7500 sequence detector, using a TaqMan Universal Master Mix. The optimal concentrations of cDNA and primers, as well as the maximum efficiency of amplification, were obtained by five-point, twofold dilution curve analysis for each gene. Each PCR contained 3.0 ng of reverse-transcribed RNA, 200 nM of each specific primer, SYBR SAFE PCR master mix, and RNAse-free water to a final volume of 20 μL. All samples were run in triplicate with water as a no-template control and GAPDH as an endogenous control. Real-time data were analyzed using the Sequence Detection System 1.7 (Applied Biosystems).

2.8. Statistics. Data were assessed by a three-way ANOVA to compare the variables: treatment (saline treated and PNV treated), age (P14 and 8–10 weeks), and time (2, 5, and 24 hours). Groups' comparison was done using unpaired Student's t-test. The results were expressed as the mean ± SEM. Values of $P \leq 0.05$ indicate significance.

3. Results

Animals of both ages injected with saline solution were alive and exhibited no sign of discomfort. Animals injected with PNV showed neurotoxic manifestation as described elsewhere [5]. P14 rat excitotoxic effects were more precocious than in adult rats, but the recovery was delayed relative to adults. Nevertheless, signs of recovery of intoxication started 5 hours after envenoming; one adult and one neonate rat died.

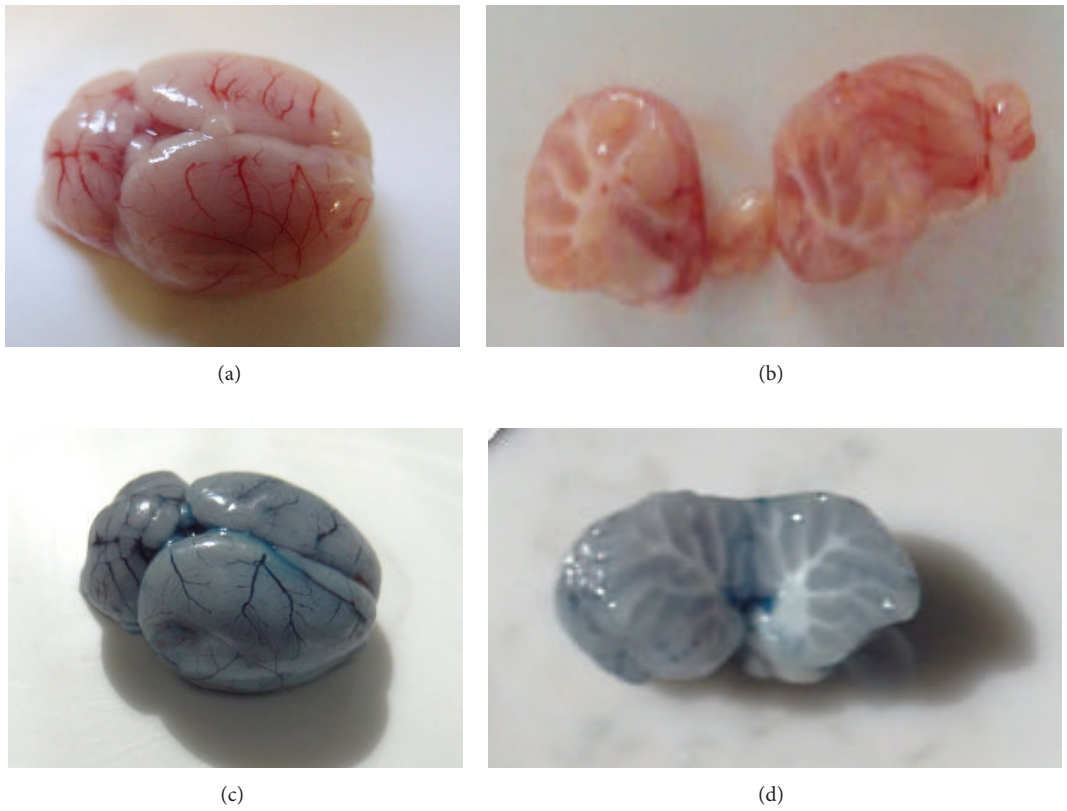
3.1. Blood-Brain Barrier Permeability. Control rats (saline injected) that received i.v. infusion of Evans blue showed absence of any blue color on the brain surface or in the interior of cerebellum hemispheres which indicaes that there is no extravasation of Evans blue dye from microvasculature circulation (Figures 1(a) and 1(b)). On the contrary, PNV-treated rats showed brain and cerebellum surface and cerebellum interior shaded blue which indicates disruption of microvascular blood bed and leakage of Evans blue (Figures 1(c) and 1(d)). The leakage of Evans blue was detected at 2 hours, but not at 5 and 24 hours suggesting BBB return to integrity (not shown).

3.2. BBB Assessment: PNV Decreased Transiently Occludin, β-Catenin, and Laminin. All the three BBB-associated proteins, occludin from tight junction, β-catenin from adhesion junction, and laminin from the endothelial basal membrane, were decreased significantly by 34%, 35%, and 36%, respectively, 2 hours after administration of PNV to neonates. In adult rats, PNV induced a 33% decrease of occludin at 2 hours followed by 23% increase at 24 hours and 60% decrease of β-catenin at 2 hours and did not alter laminin expression (Figures 2(a) to 2(c)). The changes were transitory in animals of both ages.

Age-related changes showed occludin and β-catenin basal level lower in saline-injected neonate rats at 2 hours relative to adult rats. Moreover, baseline expression of laminin was lower in neonates at 5 hours and higher at 24 hours compared to adults. In envenomed rats, occludin was higher in adult rats at 5 hours than in P14 rats.

Immunohistochemistry: PNV Increased the VEGF, Flt-1, and Flk-1 Reactivity. In P14 and adult controls, VEGF immunolabeling was weakly detected in the perikaryum, outlining the cell profile of Purkinje cells (PCs); the nucleus was negative. Some cells in the molecular (ML) and granular layer (GL) were stained (Figure 3(a)). PNV-administered animals induced anti-VEGF staining in the nucleus and increased it in the somata and marginal cytoplasm of PCs (Figure 3(b)). In addition, delicate VEGF+ tangled cell processes were labeled within the ML. Cells in the GL and ML remained VEGF negative. The labeling pattern was fairly similar throughout time; however, the intensity of the reaction was marginally strongest at 24 hours.

In the cerebellum of controls, the anti-Flt-1 and anti-Flk-1 reactivity was seen in the PC nucleus and perikaryum or only in the peripheral perikaryum; a number of stained nuclei were labeled within the ML and GL (Figures 3(c) and 3(e)). PNV upregulated Flt-1 and Flk-1 level at all times in cells throughout the cerebellar cortex such as in the PCs' nuclei, somata, and peripheral cytoplasm, in nuclei within the GL and ML, and in straight-lined processes present in the ML; in envenomed rats, regions of glomeruli were wider and density of glomerular neurons seemed lower than in controls in some parts of the GL (Figure 3(d)). Flk-1 upregulation was not as intense as for Flt-1 (compare Figures 3(e) and 3(f)). Flt-1 and Flk-1 staining increased with time, but visually no obvious difference in the intensity of the labeling could be perceived between P14 and adults (not shown).



(a) (b)

(c) (d)

Figure 1: Representative photographs of brain and cerebellum from adult rats injected with Evans blue 2 hours after administration of saline ((a) and (b)) or *Phoneutria nigriventer* venom ((c) and (d)). (a) and (c) show dorsal brain surface. (b) and (d) show interior of cerebellum hemispheres.

3.3. Western Blot and Real-Time PCR. VEGF, Flt-1, and Flk-1 expressions were higher in neonates than in adult animals, both basally and after envenomation. PNV altered level of the proteins and respective mRNAs but the dynamics of changes differed between neonates and adults.

Throughout the time interval examined, VEGF, Flt-1, and Flk-1 expressions were significantly higher in neonates than in adults, both controls and envenomed (Figures 4(a) to 4(c)). However, Flk-1 mRNA was higher in adults, both controls and envenomed, than in neonates at all three time points (Figure 5(c)).

PNV induced an 18% increase of VEGF expression in P14 animals at 5 hours (Figure 4(a)). VEGF mRNA content was quite similar in control and envenomed neonates and adults (Figure 5(a)), except at 5 and 24 hours when there was a twofold increase for neonates and an 18% increase for adults.

In relation to VEGF receptors, PNV caused an immediate 50% increase in the Flt-1 level in P14 rats (2 hours) that changed to 14% increase at 5 hours and 11% increase at 24 hours above baseline (Figure 4(b)). In contrast, in adult rats PNV promoted decreases in the level of Flt-1, 25% at 2 hours, and 93% at 5 hours followed by recovery to baseline values at 24 hours. In neonate and adult rats, Flt-1 mRNA expression was unaffected by PNV exposure (Figure 5(b)). PNV did not alter the expression of the Flk-1 and its mRNA in neonates (Figures 4(c) and 5(c)) but increased Flk-1 expression in adults at 5 hours.

The three-way analysis of variance showed that variables treatment versus age versus time influenced VEGF mRNA expression ($^{*}P \leq 0.05$) and Flt-1 receptor expression ($^{*}P \leq 0.05$). In addition, there was interaction between treatment versus age for the Flt-1 receptor ($^{***}P \leq 0.001$) and treatment versus time for VEGF ($^{*}P \leq 0.05$).

PNV increased CaB in neonate and adult rats while GAD was increased only in neonate rats.

In controls, adult and neonate, the Purkinje cell-specific calcium-binding protein, calbindin-D28k, was expressed in the nucleus, cytosol, and dendritic tree of PCs (Figure 6(a)). Following PNV injection anti-CaB labeling was stronger particularly in PC dendrites extending across the ML (Figure 6(b)). Western blot analyses confirmed that PNV induced a 16% significant upregulation of the protein at 24 hours in neonates and a 9% increase at 5 hours in adult rats (Figure 6(c)). The baseline expression of CaB was significantly higher in neonates than in adults at 2 hours (23%); also the PNV-induced CaB expression was higher in neonates than in adults at 2 hours (25%) and 24 hours (28%). The three-way analysis of variance showed that the variables treatment versus age versus time influenced CaB expression ($^{**}P \leq 0.01$).

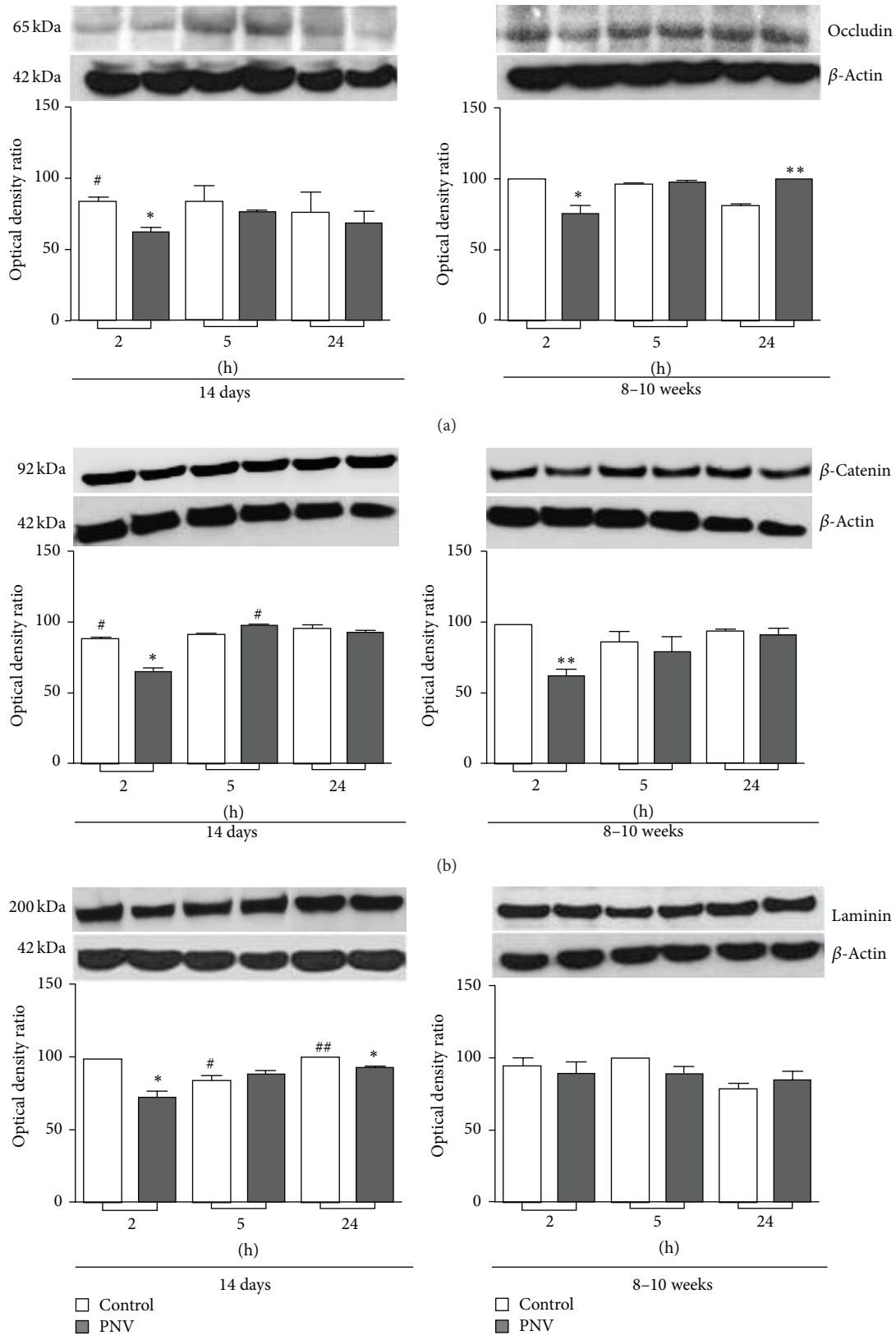


FIGURE 2: Immunoblots of occludin (a), β-Catenin (b), and laminin (c) show lower expression of the proteins in the cerebella lysates of envenomed animals relative to controls. Data are means ± SEM; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ $^{***}P \leq 0.001$ relative to controls; $^{\#}P \leq 0.05$; $^{\#\#}P \leq 0.01$ relative to corresponding adults at the same time interval. Unpaired Student t-test.

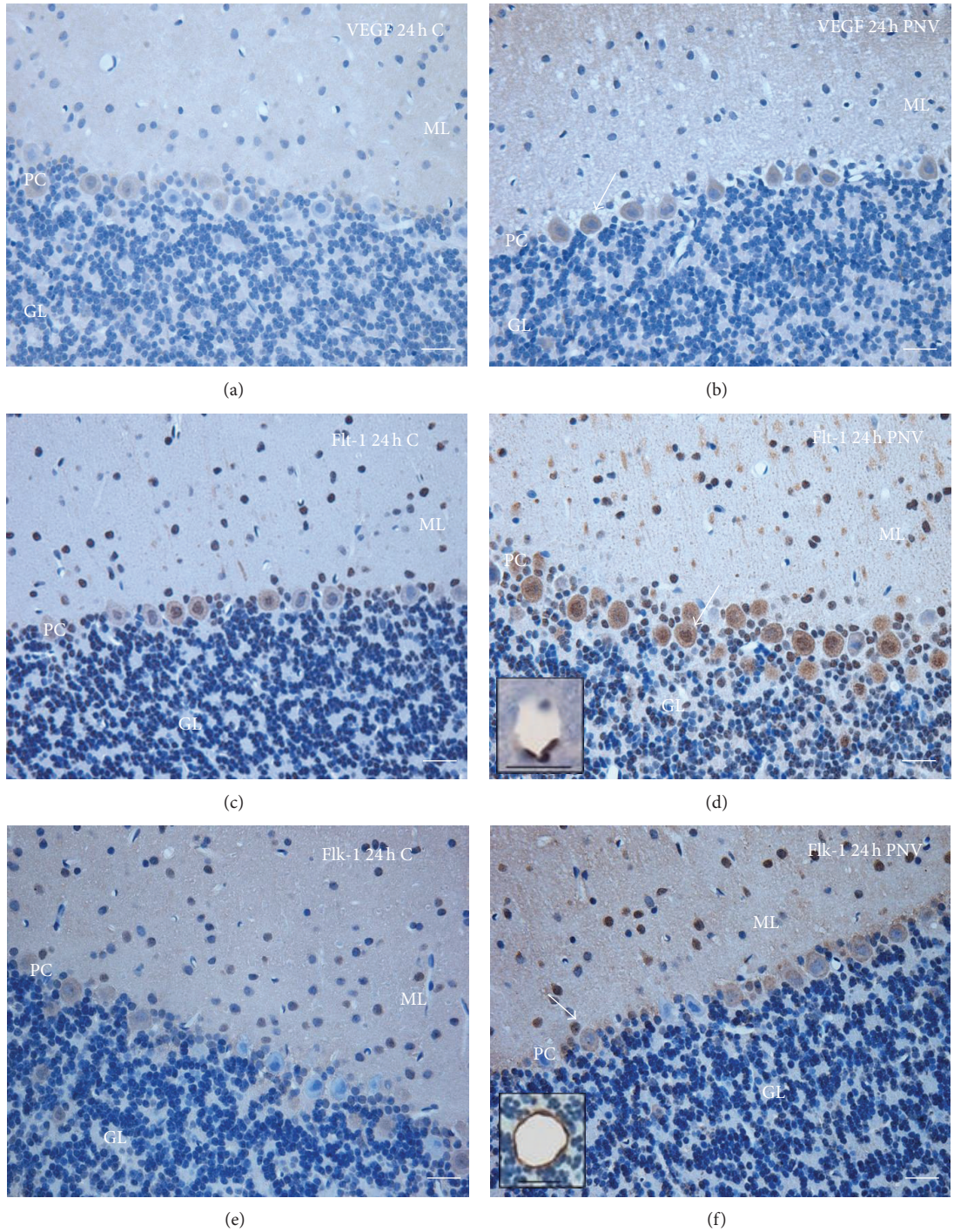


Figure 3: Photomicrographs of cerebellar cortex of rats aged 14 days 5 hours after administration of saline solution ((a), (c), and (e)) or *Phoneutria nigriventer* venom (PNV) ((b), (d), and (f)). PNV increased VEGF (b), Flt-1 (d), and Flk-1 (f) labeling. ML = molecular layer; PC = Purkinje cells, and GL = granular layer. Arrows point Purkinje cells positive for VEGF, Flt-1, and Flk-1; triangles show nonuniformly scattered labeled nuclei inside the molecular layer. Bars = 25 μm for all panels. Inserts illustrate the expression of Flt-1 receptor in the endothelium nuclei, whereas Flk-1 receptor is expressed in nuclei and cytoplasm of endothelial cells of envenomed rats (Bars = 10 μm (d) and 25 μm (f)).

GABA signaling was altered in PNV-administered rats, given the expression of GAD, responsible for GABA synthesis, and was noticeably increased in PCs somata and dendritic tree (Figures 6(e) and 6(f)). Immunoblots showed that the GAD protein expression of treated P14 rats was 10% upregulated at 2 hours and remained practically unchanged thereafter (Figure 6(g)). In adult rats exposed to PNV, GAD remained unchanged compared to baselines values (Figure 6(h)). There was an age-related difference at 24 hours with GAD expression of PNV-treated neonate rats surpassing

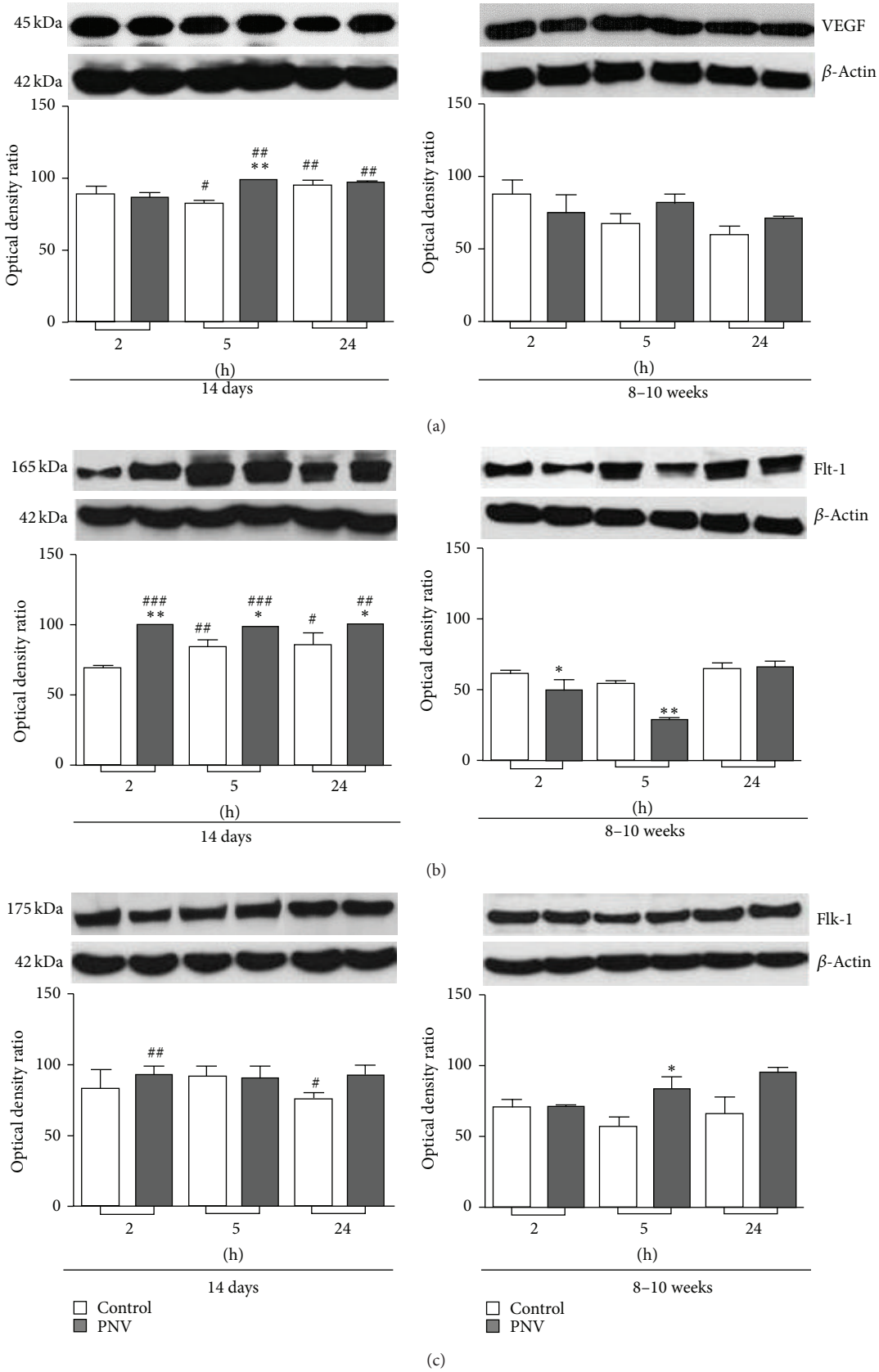


FIGURE 4: VEGF (a), Flt-1 (b), and Flk-1 (c) graph representation of western blot signals after densitometric measurement and normalization to internal β-actin at time points after PNV (1.7 mg/kg) or 0.9% saline i.p. injection. Data are means ± SEM. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ in relation to controls; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, and $^{\#\#\#}P \leq 0.001$ in relation to corresponding adults at the same time interval. Unpaired Student t-test was used.

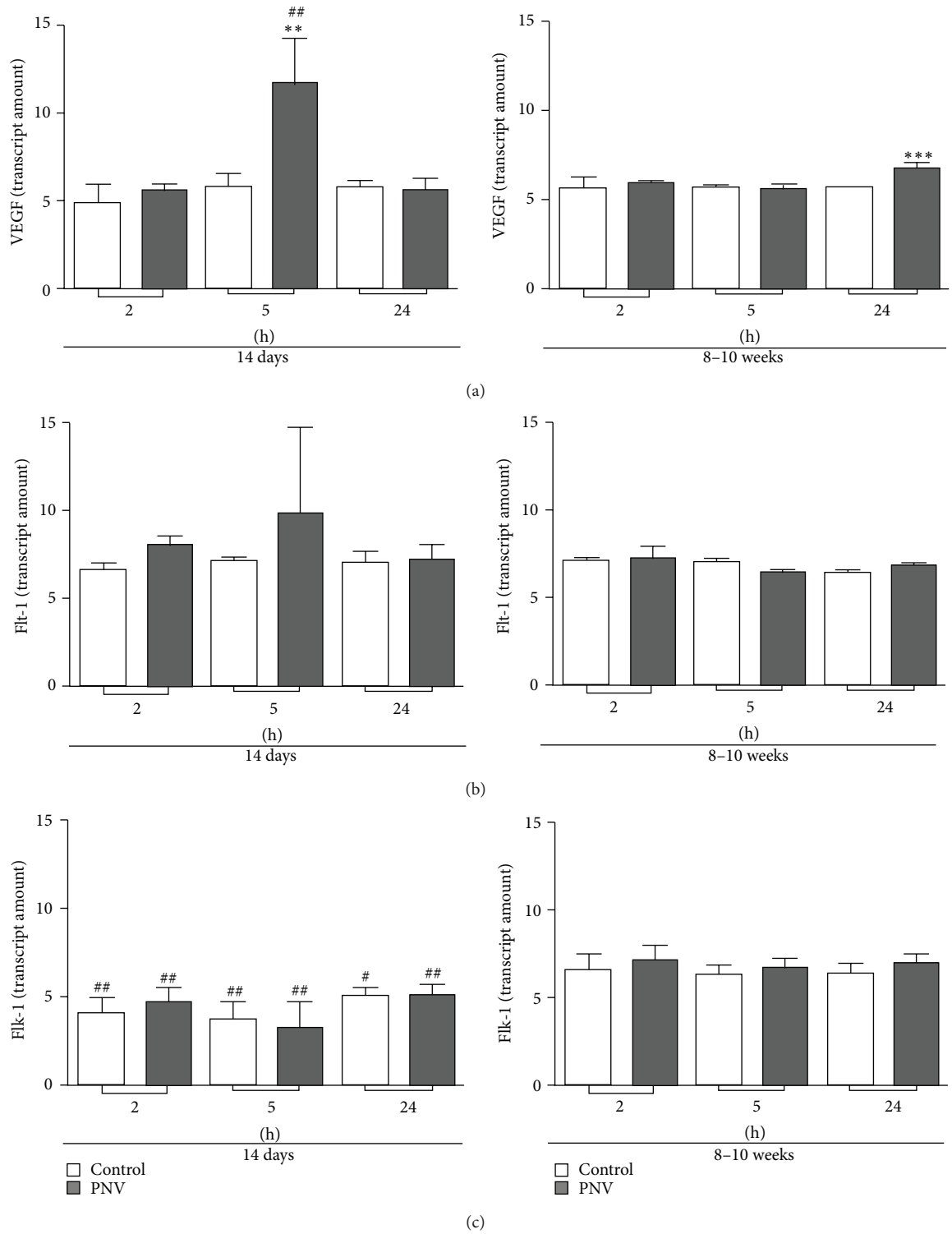


Figure 5: Quantitative real-time PCR analysis quantified and normalized to endogen control (GAPDH). VEGF (a), Flt-1 (b), and Flk-1 (c) mRNAs expression at time points after PNV (1.7 mg/kg) or 0.9% saline i.p. injection. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ indicate significant difference relative to controls; $^{\#}P \leq 0.05$ and $^{\#\#}P \leq 0.01$ denote significant age-related differences between PNV-treated or control group at corresponding time point. Student t-test; data were shown as means ± SEM.

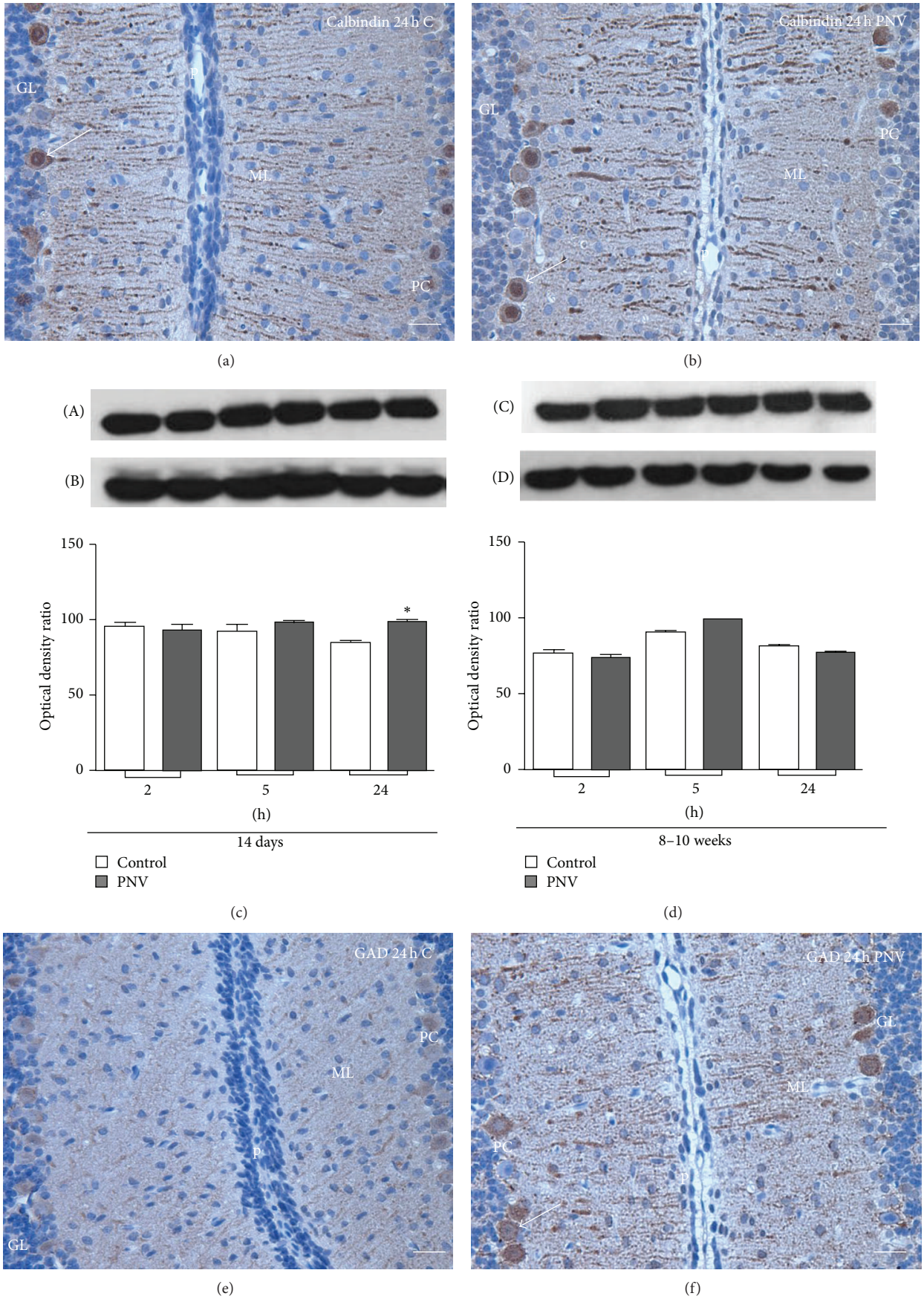


Figure 6: Continued.

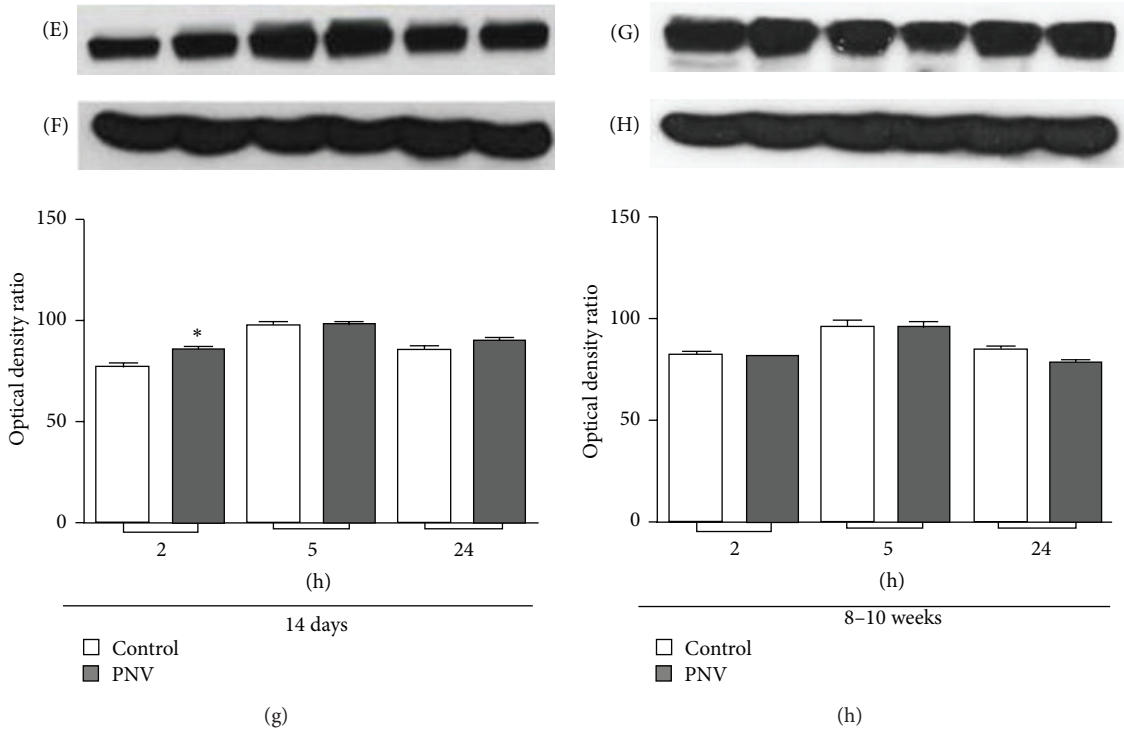


Figure 6: CaB and GAD immunohistochemistry in the cerebellar cortex of rats aged 14 days after i.p. injection of saline solution ((a) and (e)) or *P. nigriventer* venom PNV ((b) and (f)). (a) and (b) show CaB labeling (24 hours after saline or PNV exposure, resp.) in Purkinje cell bodies, including nuclei (arrows) and processes crossing the molecular layer (ML). (c) and (d) show immunoblots and representative histograms of the densitometric CaB values of rats injected with saline (A) or PNV (C) at different time points. (e) and (f) show GAD labeling in Purkinje cell bodies and cell processes within the molecular layer (arrow). The physiologic GAD labeling was weak in controls (e) whereas it was strong in envenomed animals (f). (g) and (h) show immunoblots and representative histograms of the densitometric GAD values of rats injected with saline (E) or PNV (G) at different time points (n = 6/time). The membranes were stripped and reprobed to β-actin, confirming equal protein loading in the gel ((B), (D), (F), and (H)). Values are mean ± SEM; unpaired Student t-test. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ compared to control at each time point; $^{\#}P \leq 0.05$; $^{\#\#}P \leq 0.01$; $^{\#\#\#}P \leq 0.001$ compared to corresponding adults at the same time interval. P = pia mater; PC = Purkinje cells; ML = molecular layer; GL = granular layer. Bars = 25 μm for all panels.

by 15% that of PNV-treated adults. The three-way analysis of variance showed interaction only between treatment versus age ($^{***}P \leq 0.001$).

4. Discussion

The present findings show that prominent increases of VEGF and receptor Flt-1 in cerebellar neurons course with neurotoxic effects caused by PNV in P14 rats. This is accompanied by upregulations of VEGF mRNA, CaB, and GAD. The data suggest that, upon systemic presence of PNV, endogenous signaling mechanisms may have activated transcription factors and promote proteins translational regulation in newborn rats.

The alterations involving the VEGF-Flt-1 binding in neonate rats were concurrent with prominent decreases of the proteins associated with BBB endothelium: occludin, β-catenin, and laminin. The data corroborate previous studies showing PNV-induced BBB permeation in adult rats [3–6], now reaffirmed by the extravasation of Evans blue injected peripherally. Herein, the time course data over a period of 2 hours to 24 hours revealed that expressional decreases of occludin, β-catenin, and laminin (at 2 hours) were simultaneous with the peak of Flt-1 upregulation (50%) but preceded the PNV-induced significant increases of VEGF and VEGF mRNA, which occurred later at 5 hours after PNV. Also, at 5 hours, occludin, β-catenin, and laminin levels were recovered markedly relative to corresponding control levels, which paralleled with content of VEGF and Flt-1 significantly above baseline. Such data disclose what seems to be a coordinated sequence of molecular events, which began with the BBB breakdown, and proceeded with increases of Flt-1, VEGF, and VEGF mRNA. The transient disturbance of BBB-associated proteins in P14 rats might be due to the fact that VEGF receptors once activated trigger signals directed to endothelial cells [18] and neuron cell [19], either to accentuate permeability [20–22] and/or to restore homeostasis.

Curiously, in contrast to neonates, PNV induced decreases over time of Flt-1 in adult rats reaching ~93% downregulation at 5 hours, which though was transient and shows great capacity for recovery since at 24 hours the

baseline level had been already reached. Consistent with this, at the same 24 hours, VEGF mRNA was significantly increased. However, PNV increased cerebellar Flk-1 and CaB at 5 hours, a time point in which BBB functionality had been restored as indicated by absence of extravasation of Evans blue dye. Interestingly, 5-hour time coincides with the beginning of the adult animals' recovery from neurotoxic manifestations caused by PNV [4, 6].

VEGF, Flt-1, and Flk-1 protein expressions were also detected by immunohistochemistry. The three proteins were constitutively present in Purkinje neurons and their dendritic processes extended across the ML in control animals. The exposure to PNV induced visible increases in the Purkinje neurons' immunoreactivity over a period of 2 to 24 hours; interestingly, nonuniformly dispersed cells within the GL and ML also showed increases in the Flt-1 and Flk-1 reactivity in envenomed P14 and adult rats. We do not know whether such cells are astrocytes and/or neurons. Studies have shown that VEGF and Flk-1 can be expressed by granule neurons during postnatal development of cerebellum [23–25] and by astrocytes under normal or pathological conditions [26–29]. Astrocytes also express Flt-1, but as far as we know there is no mention in the literature on Flt-1 expression by cerebellar granule cells. Since the ML is characterized by extensive synaptic contacts between granule neurons, interneurons, Begmann glia, and Purkinje neurons [30], it is conceivable that activation of a given cell may elicit a chain of stimuli in the others. The induction of VEGF and receptors in neurons and probably in astrocytes supports previous evidence showing these cells as targets of PNV concurrently with BBB dysfunction [3–9, 17].

VEGF family members, traditionally known as potent inducers of angiogenesis, have been recently recognized to exert various nonangiogenic effects on different cell types, among which there are neuron cells [13]. VEGF and receptors have been recognized as an important element for neuron survival and maintenance of endothelium in adults. It acts in the nervous system both through vascular and neuronal mechanisms. Molecules that dually affect both neural and vascular (neurovascular) functions are referred to as angioneurins; their action includes regulation of angiogenesis, BBB integrity, vascular perfusion, neuroprotection, neurodegeneration, and synaptic plasticity [31]. The protection of neurons by VEGF is exerted by interference on Ca^{2+}, K^{+}, and voltage-gated Na^{+} channels physiology and modulation of glutamatergic synaptic excitability [11–13]. VEGF inhibits outward delayed K^{+} currents and reduces Ca^{2+} influx through the high-voltage-activated Ca^{2+} channels; the cytokine also inhibits Na^{+} currents in cultured rat hippocampal neurons, thus modulating neuron excitability. In hippocampus, the cytokine is considered an endogenous anticonvulsing factor to preserve pyramidal neurons from hyperexcitability [14]. The interference of PNV peptides in calcium homeostasis and glutamate handling have been well-documented [32–36]. Herein, we found signs that PNV disturbed calcium buffering in Purkinje neurons. P14 and adult rats treated with PNV increased CaB immunoreactivity in the PC's nucleus, perikaryum, and dendritic ramification, which was confirmed by the WB data showing upregulation of the protein. Because PCs are the only output cell of the cerebellar cortex and because CaB functions as a calcium buffer, the changes in the PC's immunoreactivity and level of the protein in the cerebellum lysates suggests implication on PC functional changes related with PNV effect.

PNV induced an immediate, but transient, increase of GAD expression in neonate rats, while in adults it was unaffected. The key synthesizing isoforms for GABA, GAD types 65 and 67, were decreased in the Purkinje cells of neonate rats but not in adult rats treated with PNV. This suggests that the decarboxylation of glutamate to the major inhibitory neurotransmitter GABA supports the view that synaptic inhibition is vital for the control of neuron excitability in the central nervous system and a dynamic mechanism to restore brain homeostasis, when disturbed by neurotoxic peptides of PNV. The increase of GAD in neonate rats in response to PNV is in accordance with the major level of VEGF both basically and after PNV treatment in neonate than in adult rats (see Figure 4). Whether the increase of VEGF promoted by PNV, mainly seen in neonate rats, was a way to preserve neurons [11, 12] against the glutamate toxicity generated by PNV is uncertain. Nevertheless, it is well-known that the postnatal development of cerebellum continues during months after birth [37], which requires active angiogenesis, neurogenesis, and cell migration. These cell processes are regulated by angioneurins, that is, growth factors that act both in neural an vascular cells, like VEGF and receptors [10, 13, 19]. In contrast, angiogenesis, neurogenesis, and cell migration are very discrete or switched off in adult animals. This might explain the relative lower expression of VEGF and receptors of cerebellum in adult animals, what could imply a mature BBB able to better stand the toxic effects. In addition, the BBB in neonate rats is undergoing postnatal development [38], what could explain why junctional proteins remains below baseline at 24 h in P14 rats whereas it is above baseline in adult rats. It could be the reason why systemic PNV increases even more the expression of VEGF and its mRNA (at 5 hours) and the receptors Flt-1 (from 2 to 24 hours) for neonates and transiently decreases Flt-1 expression but does not alter VEGF and only increases VEGF mRNA at 24 h in adults. Interestingly, PNV causes release of kallikrein [39], and kallikrein causes BBB breakdown [40]. VEGF and kallikrein share a series of effects such as enhancing the survival and migration of neuronal and glial cells, promoting angiogenesis, protecting against ischemia, apoptosis, and glutamate-induced neurotoxicity [41]. However, we do not know if kallikrein released by PNV is able to increase VEGF.

5. Conclusions

VEGF and PNV have in common to act on BBB-enhanced permeability, interfere in ion channels physiology, affect synaptic plasticity, and disturb glutamatergic transmission. Here, we found that the evolution of the toxic manifestations exhibited by rats injected with PNV seems to be time-related

to the dynamics of immunochemical content of VEGF, Flt-1, and Flk-1 and respective mRNAs and CaB and GAD in the cerebellum. Age-related differences with neonate rats apparently were also found more susceptible to PNV than adult rats. Future studies are needed to determine whether VEGF and tyrosine kinase intracellular domains changes in Purkinje cells underlie BBB disturbances as a consequence of venom effect or a causal factor mediating the venom's homeostatic perturbations. Ionic and glutamate disturbances, induced by PNV, affect synaptic extracellular compartments and neuronal signaling and could underlie the neurotoxic manifestations of animals. Whether the increase of VEGF in neurons represents protective modulation of synaptic excitability and of Ca^{2+}, K^{+}, and Na^{+} channels functioning is a matter to be elucidated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

High Guanidinium Permeability Reveals Dehydration-Dependent Ion Selectivity in the Plasmodial Surface Anion Channel

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Malaria parasites grow within vertebrate erythrocytes and increase host cell permeability to access nutrients from plasma. This increase is mediated by the plasmodial surface anion channel (PSAC), an unusual ion channel linked to the conserved *clag* gene family. Although PSAC recognizes and transports a broad range of uncharged and charged solutes, it must efficiently exclude the small Na^+ ion to maintain infected cell osmotic stability. Here, we examine possible mechanisms for this remarkable solute selectivity. We identify guanidinium as an organic cation with high permeability into human erythrocytes infected with *Plasmodium falciparum*, but negligible uptake by uninfected cells. Transport characteristics and pharmacology indicate that this uptake is specifically mediated by PSAC. The rank order of organic and inorganic cation permeabilities suggests cation dehydration as the rate-limiting step in transport through the channel. The high guanidinium permeability of infected cells also allows rapid and stringent synchronization of parasite cultures, as required for molecular and cellular studies of this pathogen. These studies provide important insights into how nutrients and ions are transported via PSAC, an established target for antimalarial drug development.

1. Introduction

Malaria parasites are intracellular pathogens that invade, grow, and replicate asexually within erythrocytes; the clinical sequelae of malaria are, in large measure, determined by modification and eventual destruction of host erythrocytes. During its ~48 h intracellular cycle, the human pathogen *P. falciparum* remodels its host cell by generating a membranous network in erythrocyte cytosol and altering erythrocyte membrane properties such as adhesiveness and permeability to various organic and inorganic solutes [1–4].

This increased permeability is mediated by the plasmodial surface anion channel (PSAC), identified by patch-clamp studies of the host cell membrane [5]. PSAC activity and the associated *clag* multigene family are conserved in all *Plasmodium* spp. [6–9], suggesting a function required for intracellular parasite survival. *In vitro* growth inhibition studies using PSAC inhibitors and modified media have implicated an essential role in parasite nutrient acquisition [10], with sugars, amino acids, purines, and some vitamins all having established uptake [11–13].

As a shared ion channel for these structurally divergent nutrients, PSAC has broad selectivity for solutes of varying charge and size [11, 14, 15]. At the same time, this channel must efficiently exclude Na^+, an impermeant cation responsible for the osmotic stability of erythrocytes in plasma [16]. PSAC excludes Na^+ by $10^{3.5}$- to 10^5-fold when compared to Cl^-, a remarkable feat for a channel that allows large organic cations to pass [14]. Consistent with fine-tuning of PSAC solute selectivity by evolutionary pressures, this level of Na^+ exclusion is sufficient to prevent osmotic lysis of infected cells before completion of the parasite's intracellular cycle [17]. Although being low, Na^+ flux through this channel is sufficient to remodel the host erythrocyte's cation concentrations and affect parasite activities [17–19]. Structure-function

studies with mammalian ion channels have revealed mechanisms for selecting a specific ion and excluding nearly all other solutes [20], but the reverse problem—broad permeability with exclusion of the small Na^+ ion by this parasite channel—requires distinct solutions that will have fundamental implications for understanding permeation.

Here, we examine possible mechanisms for PSAC's unusual selectivity and identify guanidinium (Gdm^+) as a cation with high permeability. We show that monovalent cations have permeabilities that increase with cation ionic radius, contradicting pore sieving predictions and paralleling a similar relationship for anions in this channel. High Gdm^+ permeability also enables stringent synchronization of parasite cultures, as commonly needed for molecular and cellular studies of malaria parasites. These findings suggest a testable model for how the channel achieves its unusual solute selectivity.

2. Materials and Methods

2.1. Parasite Cultivation and Synchronization. Human erythrocytes were obtained from anonymous donors (Interstate Blood Bank, Memphis, TN) and used for *in vitro P. falciparum* cultivation of indicated parasite lines in RPMI-1640 medium supplemented with 0.5% lipid-rich bovine albumin (MP Biomedicals, Solon, OH); cultures were maintained at 37°C under 5% O_2, 5% CO_2, and 90% N_2.

To assess the efficiency of synchronization conditions, asynchronous parasite cultures were treated with either 300 mM D-sorbitol or 150 mM guanidinium chloride (Gdm-Cl) in a buffered solution (20 mM HEPES, 0.1 mg/mL BSA, pH 7.4 with NaOH); each experiment included treatment with standard culture medium as a matched control. Synchronization involved 5 or 30 min incubations at room temperature and was terminated by addition of 10 volumes of culture medium. After centrifugation to remove the lysis solution, the cells were resuspended in culture medium and returned to 37°C for cultivation without additional washing. Parasite stage and growth were evaluated after 24 h using microscopic examination of Giemsa-stained smears.

2.2. Osmotic Lysis Transport Assays. Solute transport assays were performed as described previously [21]. Trophozoite-infected erythrocytes were harvested and enriched using the percoll/sorbitol method, washed, and resuspended at 0.1% hematocrit in osmotic lysis solutions containing either 280 mM sorbitol or 150 mM Gdm-Cl buffered with 20 mM HEPES, 0.1 mg/mL BSA, pH 7.4. The permeability of other cations was identically measured; each salt produced negligible hemolysis of uninfected cells (not shown). Where present, inhibitors were added from DMSO stock solutions. Solute transport was quantified by tracking transmittance of 700 nm light through a 1 mL cell suspension; kinetics were measured at indicated temperatures using a spectrophotometer (DU640 with Peltier temperature control, Beckman Coulter, Fullerton, CA). Inhibitor dose response experiments were normalized to matched controls without inhibitor; a normalized permeability at each inhibitor concentration (P_i) was calculated according to $P_i = t_o/t_i$, where t_o and t_i correspond to the time required to reach a threshold level of lysis without and with inhibitor, respectively. This equation is based on a quantitative and inverse relationship between solute transport and time to cell lysis [21]. Permeability estimates and inhibitor affinities determined using this method match those obtained with tracer flux and patch-clamp [5, 7, 10, 21].

2.3. Sybr Green Measurements. Toxicity of Gdm-Cl was evaluated using parasite cultures after synchronization with two consecutive 30 min D-sorbitol treatments. These synchronized cultures were treated with either 150 mM Gdm-Cl, 20 mM HEPES, 0.1 mg/mL BSA, pH 7.4, or culture medium for 5 min at room temperature. After adding 10 volumes of culture medium, the cells were centrifuged to remove the medium and resuspended to 2% hematocrit in culture medium prior to plating in 96-well microplates. After cultivation for 72 h, parasite DNA production was quantified with SYBR Green I nucleic acid stain as described previously [17].

3. Results

3.1. High PSAC Permeability to Guanidinium$^+$. We sought to examine PSAC's unusual solute selectivity profile and recognized that blasticidin S and leupeptin, toxins that reach their intracellular parasite targets via PSAC [22–24], are both bulky guanidine derivatives with molecular weights >420 Da. Unsubstituted guanidine is positively charged at physiological pH and its conjugate acid, the guanidinium ion (Gdm^+, Figure 1(a) inset), has been used to study transport through other ion channels [25]. We therefore examined Gdm^+ permeability in infected erythrocytes and used a quantitative transmittance assay [26]. These studies revealed rapid osmotic lysis of infected cells in isotonic Gdm-Cl; the half-time, 0.89 ± 0.08 min, was significantly less than in isotonic sorbitol (6.7 ± 0.5 min, $P < 10^{-8}$), a highly permeant sugar alcohol. Although net uptake of the Gdm-Cl salt depends on both Gdm^+ and Cl^- permeabilities to maintain electroneutrality, conductive Cl^- transport at this membrane is greater and not rate-limiting [4, 21]. Because there is a quantitative and inverse relationship between osmotic lysis half-time and solute permeability [21], these measurements implicate 8-fold greater permeability for Gdm^+ than for sorbitol. In contrast, uninfected human erythrocytes exhibited low Gdm^+ permeability and were osmotically stable in Gdm-Cl (bottom trace, Figure 1(a)).

The nonspecific PSAC inhibitor, furosemide, inhibited Gdm^+ uptake, suggesting channel-mediated transport (Figure 1(b)) [27]. Notably, the levels of inhibition achieved with 200 μM and 2 mM concentrations of furosemide resembled those for a subset of PSAC substrates that access two different mechanisms of transport through this channel [15, 28]. These two mechanisms exhibit differences in inhibitor efficacy: while the transport of some solutes is abolished by 200 μM furosemide, other solutes, collectively referred to as "R+" solutes, exhibit significant residual uptake via PSAC that can be blocked by a higher furosemide concentration (2 mM). Remarkably, PSAC inhibitors from multiple chemical scaffolds exhibit a similar 10-fold reduction in potency when

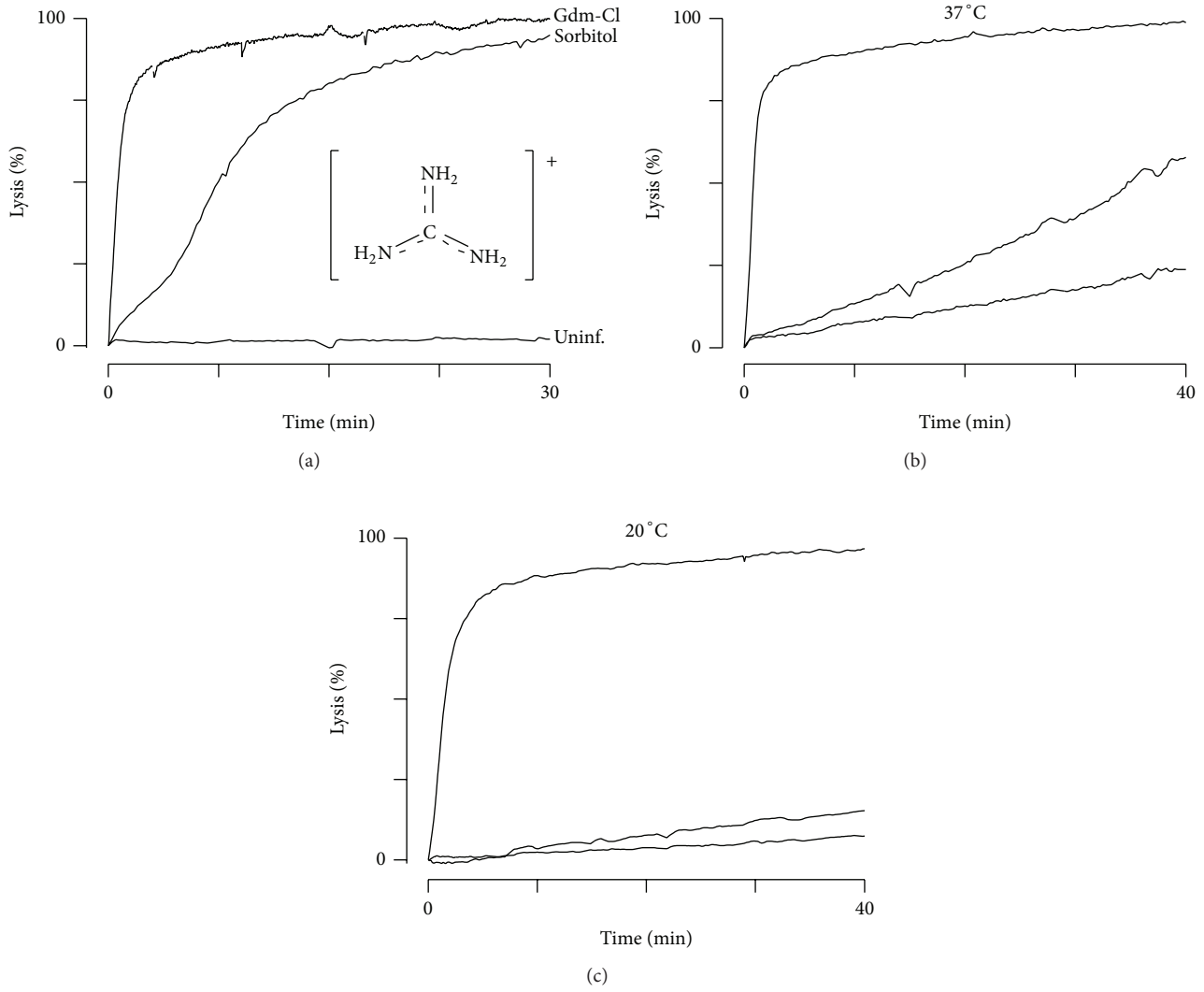

Figure 1: High Gdm^+ permeability in infected but not uninfected RBCs. (a) Osmotic lysis kinetics for infected erythrocytes in Gdm-Cl or sorbitol at 37°C (top two traces). Notice the faster kinetics in Gdm-Cl. Uninfected cells do not lyse in Gdm-Cl (bottom trace). Inset shows the structure of Gdm^+, which has a net +1 charge distributed amongst three primary amines. (b and c) Osmotic lysis kinetics for infected cells in Gdm-Cl with 0, 200, or 2000 μM furosemide (top to bottom traces, resp.). 200 μM furosemide produces incomplete inhibition at 37°C but is more effective at 20°C (panels b and c, resp.), suggesting that Gdm^+ is an R+ solute.

transport is examined with each R+ solute. This observation suggests two distinct mechanisms used by this channel to recognize and transport solutes. Because the residual transport mechanism has steep temperature dependence [28], we examined Gdm^+ transport at 20°C; under this condition, 200 μM furosemide largely abolished uptake (Figure 1(c)), as reported for all other known R+ solutes [15]. These experiments suggest Gdm^+ is transported via PSAC as an R+ solute.

Because furosemide is nonspecific, we examined the mechanism of Gdm^+ uptake further with ISPA-28, a potent and specific small molecule inhibitor identified by high-throughput screening [7]. ISPA-28 blocks PSAC activity associated with the Dd2 parasite line ($K_{0.5}$ = 56 nM) but is largely inactive against channel activity induced by other parasite lines such as HB3 ($K_{0.5}$ = 43 μM); this compound's unique specificity enabled identification of *clag3* genes and the channel's role in nutrient uptake through genetic mapping and DNA transfection experiments [7, 10]. A short variable motif on the CLAG3 protein is exposed at the host cell surface and has been implicated in ISPA-28 binding [29].

ISPA-28 inhibited Gdm^+ uptake into cells infected with Dd2 but not those infected with HB3 parasites (Figures 2(a) and 2(b)). To explore whether other transporters contribute to Gdm^+ uptake after infection, we quantified ISPA-28 inhibition and compared block to that for sorbitol, a solute whose uptake via PSAC is well-established [7, 26]; these transport inhibition studies were performed at 15°C to reduce errors due to the residual transport mechanism described above. In both Dd2 and HB3 parasite lines, these dose response studies revealed quantitatively concordant inhibition of Gdm^+ and

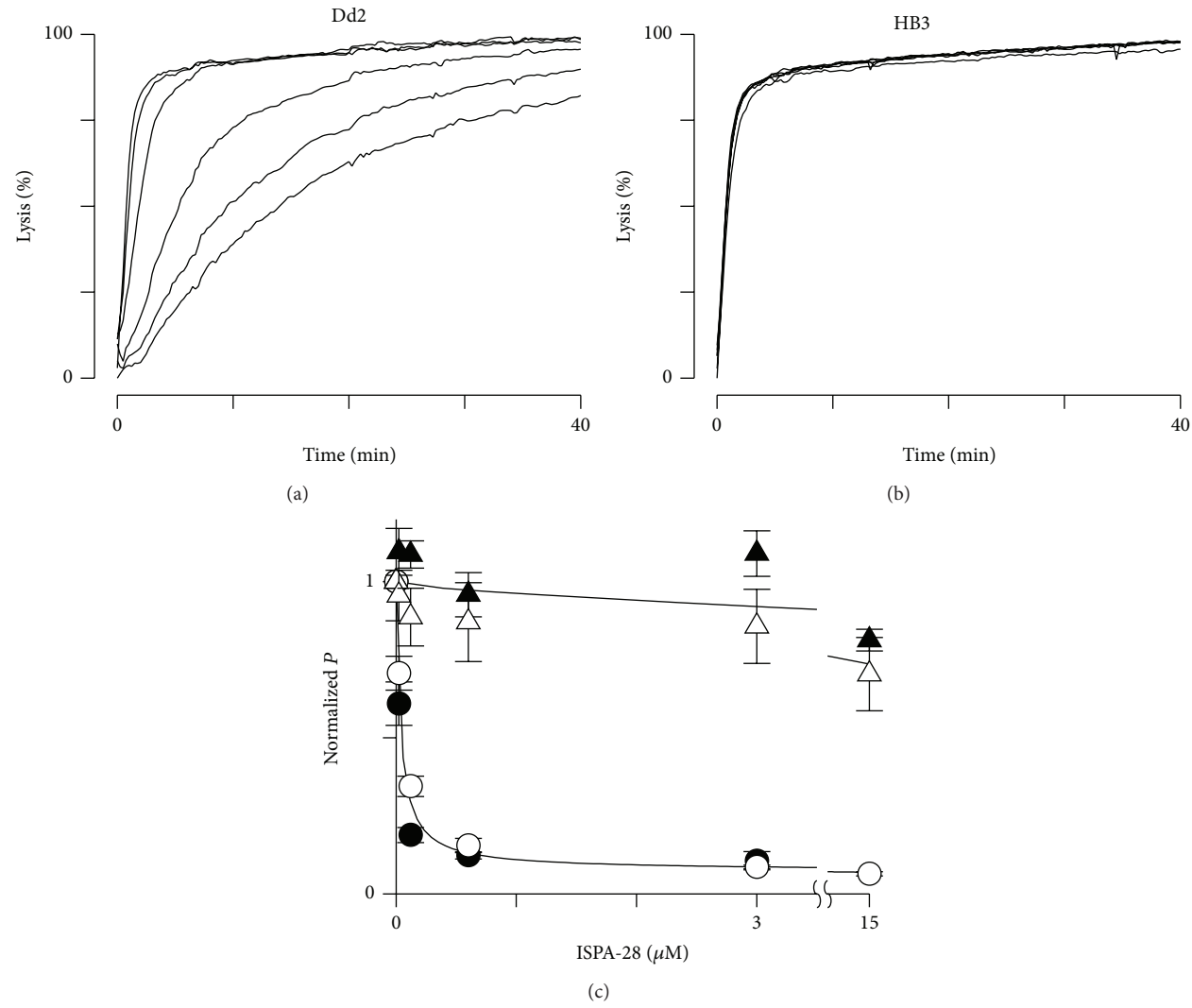

FIGURE 2: Gdm^+ uptake is primarily via PSAC. (a and b) Osmotic lysis kinetics for Gdm^+ uptake into Dd2 and HB3 at 15°C. Traces reflect inhibition dose responses using 0, 0.024, 0.12, 0.6, 3.0, and 15 μM ISPA-28 (top to bottom, resp., in each panel). While inhibition is clear with Dd2-infected cells, there is negligible effect with HB3-infected cells. (c) Symbols represent mean ± S.E.M. of tallied dose responses from experiments as in panels (a) and (b) for Gdm-Cl and sorbitol (white and black symbols, resp.) using Dd2- and HB3-infected cells (circles and triangles, resp.). The Gdm^+ and sorbitol dose responses do not differ ($n = 3$ trials at each concentration, $P > 0.1$ for comparisons between solutes in each parasite.).

sorbitol uptake by ISPA-28 (Figure 2(b)), indicating that Gdm^+ uptake is mediated primarily by PSAC.

3.2. Low Toxicity of Gdm^+ Permits Stringent Synchronization of Parasite Cultures. Sorbitol treatment, the current method of choice for synchronizing parasite cultures [30], is based on osmotic lysis of trophozoite-infected cells due to PSAC-mediated uptake [21]; it spares immature ring-infected cells, which lack this channel activity [31]. Two limitations include a requirement for relatively long incubations in sorbitol and poor stringency of synchronization. To achieve tighter synchrony for molecular studies such as stage-specific gene transcription, it is often necessary to use two or more rounds of sorbitol synchronization, making the procedure time- and effort-intensive. Alternative methods, such as gelatin floatation or enrichment of mature infected cells on magnetic columns [32, 33], have low yield or are also time-consuming.

We tested whether the greater permeability of Gdm^+ allows improved synchronization by treating asynchronous cultures with either Gdm-Cl or sorbitol. A 5 min Gdm-Cl treatment was more effective than either a 5 or 30 min sorbitol exposure, as quantified with examination of Giemsa-stained smears after subsequent cultivation for 24 h (Figure 3(a), $P < 0.05$). Although improved synchronization is presumably determined by the greater Gdm^+ permeability, the near-physiological ionic strength of the Gdm-Cl solution may also help; the lower ionic strength of sorbitol solutions may cause erythrocyte aggregation [34], leading to delayed solute uptake and osmotic lysis of some cells.

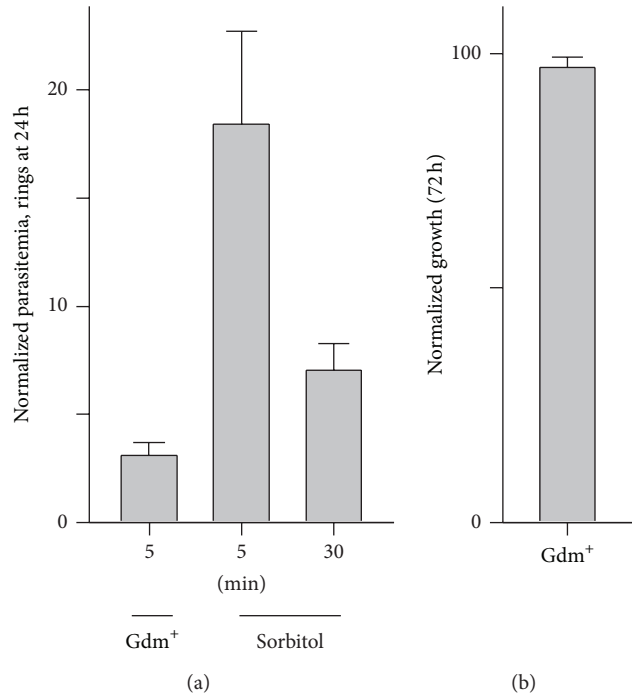

Figure 3: Stringent synchronization of cultures using Gdm-Cl. (a) Mature infected cells surviving synchronization with Gdm-Cl or sorbitol for indicated durations, as quantified using ring-stage parasitemia 24 h after treatment. A 5 min Gdm-Cl treatment is the most effective. (b) 72 h parasite growth after a 5 min treatment of synchronous cultures with Gdm-Cl, normalized to matched cultures sham-treated with culture medium. Bars represent mean ± S.E.M. of 9 replicates from 3 experiments.

Because Gdm^+ is a strong protein denaturant at high concentrations, we wondered whether this synchronization strategy would be toxic to parasite cultures. We therefore treated ring-stage cultures with isotonic Gdm-Cl solution and quantified subsequent parasite growth. Comparison to a sham treatment using standard culture medium revealed no change in parasite growth (Figure 3(b), $P = 0.36$), indicating that this treatment is not toxic to cultures. Experiments using a significantly longer Gdm^+ treatment of 30 min yielded measurable toxicity, but we did not detect accumulated toxicity with prolonged, regular use of 5 min Gdm-Cl treatments over consecutive asexual cycles (not shown).

Protein denaturation by Gdm-Cl also cannot account for the apparent high Gdm^+ permeability via PSAC. Such models would predict hemolysis of uninfected erythrocytes in Gdm-Cl solutions, which was not detected (Figure 1(a)). Denaturation would also not be consistent with block by ISPA-28, a highly specific PSAC inhibitor. Quantitatively concordant dose responses for inhibition of Gdm^+ and sorbitol uptake (Figure 2), when combined with insights from single channel patch-clamp using this inhibitor [7], implicate permeation through a channel pore not compromised by Gdm-Cl exposure.

3.3. PSAC Permeabilities to Other Monovalent Cations. In contrast to Gdm^+ and various organic cations [14, 35], PSAC maintains a very low Na^+ permeability [16]. To explore possible mechanisms, we quantified the relative permeabilities of organic and inorganic cations with osmotic lysis kinetics

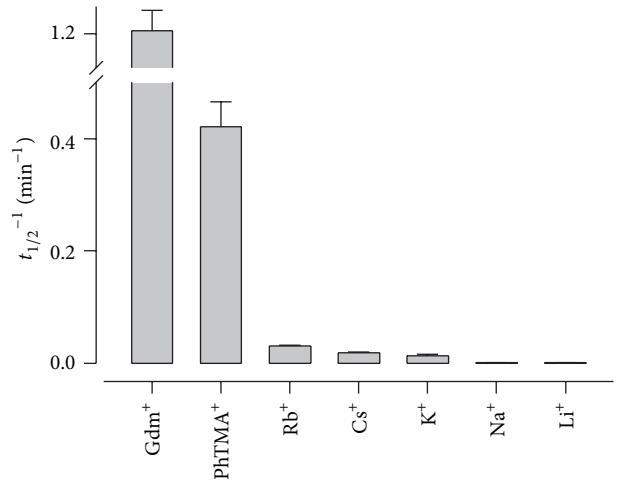

Figure 4: Dehydration-dependent permeation of cations. Mean ± S.E.M. apparent permeability coefficients for indicated cations, determined as the reciprocal of the osmotic lysis halftime in buffered solutions of each chloride salt at 37°C.

for infected cells in buffered solutions of each chloride salt (Figure 4). As expected, Gdm^+ was the most permeant of these cations. $PhTMA^+$ had substantial permeability that was 3-fold lower. Cs^+, Rb^+, and K^+, large group 1A alkali metals with ionic radii of 1.67, 1.48, and 1.33 Å, respectively, had more modest but still clearly resolved uptake. Na^+ and Li^+, smaller group 1A metals with radii of 0.98 and 0.68 Å,

respectively, had negligible permeabilities. While anion fluxes through PSAC have been studied using both single-channel and whole-cell patch-clamp configurations, the significantly lower permeabilities of the cations in Figure 4 prohibit measurement of cation-specific currents with patch-clamp methods. These currents would be overwhelmed by the larger fluxes of anions such as Cl^-, necessarily present at stoichiometric levels due to electroneutrality.

4. Discussion

The increased permeability of infected erythrocytes to small solutes is one of the earliest identified cellular phenotypes in malaria research [36]; studies from many groups have defined the range of permeant solutes and identified inhibitors [3, 14, 37–39]. Although several distinct channels have been proposed for the infected cell membrane [40], recent chemical screens and molecular studies have implicated PSAC as a shared route for most solutes with increased permeability [7, 26]. Parasite CLAG proteins, which lack homology to known channel proteins from other organisms, play a critical but incompletely understood role in formation of this channel [8, 10]. Permeating solutes may be uncharged or zwitterionic or may carry a net positive or negative charge. Solutes up to 670 Da in size have significant uptake [16], but the small Na^+ and Li^+ ions are effectively excluded (Figure 4). The combination of broad permeability to large solutes and effective exclusion of specific small ions is without precedent amongst other ion channels. This unusual selectivity profile appears to have been selected by evolutionary pressures that require uptake of diverse nutritive solutes, evasion of host immune responses, and a very low Na^+ permeability to prevent osmotic lysis of infected cells in the bloodstream, where Na^+ is the main osmotically active solute [41].

Here, we examined this unprecedented solute selectivity by quantifying cation transport through PSAC. We found that permeability increased with ionic size for group 1A cations (Figure 4), paralleling a similar relationship described for halide and pseudohalide anions identified through patch-clamp, $SCN^- \gg I^- > Br^- > Cl^-$ [4]. These findings contradict the predictions of simple pore sieving models, which expect the smallest solutes to have the greatest permeabilities. Instead, there appears to be a controlling effect of ion dehydration, the process of removing the shell of water molecules around dissolved ions [42, 43]. For both cation and anion series, greater PSAC permeability correlates precisely with lower energy requirement for dehydration.

In particular, Gdm^+ and SCN^- are notable as the cation and anion with the fastest transport rates. Gdm^+ is one of the most weakly hydrated ions known; it interacts poorly with water because a single positive charge is diffusely shared by three nitrogen atoms and because it has a rigid structure unable to interact well with water molecules [44]. SCN^- also has a low charge density and is the least hydrated of all the anions in the Hofmeister series [45]. The poor hydration of these ions also accounts for their strong denaturant properties when present at higher concentrations [44].

Studies on K^+, Na^+, and Ca^{++} channels suggest that permeating ions must be dehydrated to fit within the pore [20, 46]; dehydration is thought to allow specific interactions with the channel protein and enable selective transport. In this context, it is surprising that our studies implicate dehydration as a critical step in transport through PSAC: broad permeability to bulky organic solutes typically suggests a large pore capable of accommodating hydrated ions. We propose that ion dehydration may serve a distinct role in this channel's case by facilitating the selective exclusion of Na^+. The energy required to dehydrate Na^+, 91.2 kcal/mol [46], is very large indeed. Na^+ channels compensate for this energy barrier by providing a strong binding site for Na^+ in the pore; in contrast, the PSAC pore offers negligible compensation, with an Eisenman selectivity sequence that corresponds to the weakest theoretical binding site for permeating ions. Under such conditions, large, easily dehydrated ions and nutritive solutes will be preferred; Na^+ and Li^+ will be effectively excluded. Two observations implicate additional unknown factors in defining PSAC solute selectivity. First, it is not clear how small ions with intact water shells are excluded by a pore large enough to accommodate bulky organic solutes. Second, studies have found important differences in the transport of closely related organic solutes [15].

Our study also provides an improved, shorter protocol for synchronization of parasite cultures, as often required in basic research studies of transcription or translation stage-specificity. It may also be useful for parasite lines that do not tolerate extended exposure to ambient temperature and O_2 levels, as are invariably associated with longer protocols.

Although identification of parasite *clag* genes as determinants of PSAC activity addressed long-standing debates about whether the channel is host- or pathogen-derived [7, 9, 40], the structural basis of solute recognition, binding, and transport through this channel remains largely unknown. The CLAG proteins lack conventional transmembrane domains for pore formation; they have also been proposed to serve unrelated roles in erythrocyte invasion or cytoadherence [47, 48]. Functional studies, such as those presented here, should guide inquiries into the molecular and structural basis of permeation through this unusual channel and important antimalarial drug target.

Abbreviations

PSAC: Plasmodial surface anion channel
Gdm^+: Guanidinium
Gdm-Cl: Guanidinium chloride.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Sphingosine-1-Phosphate Transporters as Targets for Cancer Therapy

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Sphingosine-1-phosphate (S1P) is a pleiotropic lipid mediator that regulates cell survival, migration, the recruitment of immune cells, angiogenesis, and lymphangiogenesis, all of which are involved in cancer progression. S1P is generated inside cancer cells by sphingosine kinases then exported outside of the cell into the tumor microenvironment where it binds to any of five G protein coupled receptors and proceeds to regulate a variety of functions. We have recently reported on the mechanisms underlying the "inside-out" signaling of S1P, its export through the plasma membrane, and its interaction with cell surface receptors. Membrane lipids, including S1P, do not spontaneously exchange through lipid bilayers since the polar head groups do not readily go through the hydrophobic interior of the plasma membrane. Instead, specific transporter proteins exist on the membrane to exchange these lipids. This review summarizes what is known regarding S1P transport through the cell membrane via ATP-binding cassette transporters and the spinster 2 transporter and discusses the roles for these transporters in cancer and in the tumor microenvironment. Based on our research and the emerging understanding of the role of S1P signaling in cancer and in the tumor microenvironment, S1P transporters and S1P signaling hold promise as new therapeutic targets for cancer drug development.

1. Introduction

It is well recognized that the tumor microenvironment (TME) plays a key role in cancer progression and metastasis [1–3]. Tumors influence the surrounding microenvironment through the release of extracellular signals, such as cytokines, chemokines, and lipid mediators [4–7]. These bioactive molecules secreted from cancer cells and noncancer components in the TME, such as blood vessels, lymphatic vessels, and inflammatory cells, are considered to be potential therapeutic targets.

Sphingosine-1-phosphate (S1P) has emerged as a new player in the TME and cancer progression during the last decade [4]. S1P generated inside cells is exported outside of cells into the TME where it stimulates specific S1P receptors on the cell surface (Figure 1). This "inside-out" signaling of S1P is considered to play a fundamental role in cancer progression [8]. To date, several S1P transporters have been identified [9–13]. Here, we review the "inside-out" signaling of S1P with a focus on S1P transporters. We will discuss the importance of S1P signaling in cancer and the possibility of targeting S1P transporters for cancer treatment.

2. "Inside-Out" Signaling of S1P

S1P is a pleiotropic lipid mediator that regulates cell survival, proliferation, migration, angiogenesis, lymphangiogenesis,

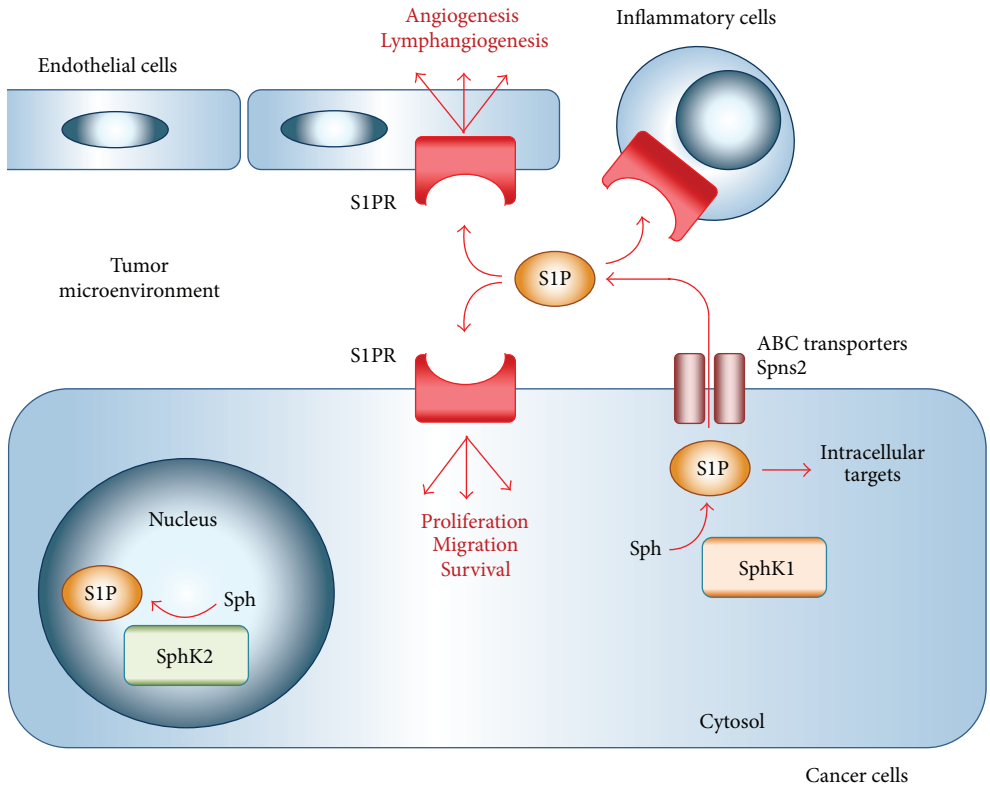

Figure 1: "Inside-out" signaling of sphingosine-1-phosphate (S1P). S1P is generated from Sph (sphingosine) by SphK1 (sphingosine kinase 1) in the cytosol of cancer cells and exported via ABC (ATP-binding cassette) transporters or Spns2 (spinster 2) outside of cells (tumor microenvironment). S1P stimulates specific S1P receptors (S1PR1-5) to promote numerous cellular functions, such as cell proliferation, migration, angiogenesis, and lymphangiogenesis.

and the recruitment of immune cells (Figure 1), making it involved in many physiological and pathological conditions including immune function, inflammation, and cancer [14–19]. S1P is generated from sphingosine inside the cells by two sphingosine kinases (SphK1 and SphK2) [20–23]. The balance between the levels of S1P and its metabolic precursors of ceramide and sphingosine has been regarded as a rheostat that could determine whether a cell proliferates or dies [24]. S1P generated within cells is exported out from cells via S1P transporters on the cell membrane, which will be described in detail later in this review. S1P outside of the cells can stimulate any of five specific G protein coupled receptors (S1PR1-5) [10, 12], with each S1P receptor coupled to different G proteins, which regulate activation or inhibition of the downstream intracellular signaling involved in various cellular functions (Figure 2).

Cancer cells and each type of cell in the TME, such as inflammatory cells and endothelial cells, express different combinations of S1P receptors, which contribute to each cellular function regulated by S1P. For example, S1PR1 is important for B and T lymphocyte egression from secondary lymphatic organs, such as lymph nodes [25]. In endothelial cells, S1PR1 and S1PR2 are known to play an important role in vascular development [26–29]. Stimulation of S1PR1 and/or S1PR3 often promotes cell proliferation and migration in normal and cancer cells, while S1PR2 may inhibit the signaling that promotes cell proliferation and migration [30–32]. Altogether, this "inside-out" signaling of S1P plays a pivotal role in cancer cells and in the TME by stimulating the S1P receptors on each type of cell [33, 34].

In addition to its "inside-out" signaling, S1P is also known to have a variety of intracellular functions. Intracellular S1P produced by SphK1 can bind to TRAF2 (tumor necrosis factor receptor-associated factor 2) and function as a cofactor required for its E3 ubiquitin ligase activity and consequently, Lys-63-linked polyubiquitination of RIP1 (receptor-interacting protein 1) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation [35]. Similarly, it has been shown that S1P enhances cIAP2 (cellular inhibitor of apoptosis 2) mediated K63-linked polyubiquitination of IRF-1 (interferon regulatory factor-1), which is essential for IL-1-induced production of chemokines CXCL10 and CCL5 [36]. Furthermore, S1P produced by SphK2 in the nucleus acts as an endogenous inhibitor of specific histone deacetylases (HDAC1 and HDAC2), thereby regulating gene transcription, including that of the cyclin dependent kinase inhibitor p21 [37].

The relative levels of S1P within body fluids and tissues are important to a variety of physiologic processes [25, 38]. Levels of S1P are maintained by S1P synthesis and degradation, which create an S1P gradient within the tissue [39]. S1P is dephosphorylated to regenerate sphingosine by S1P

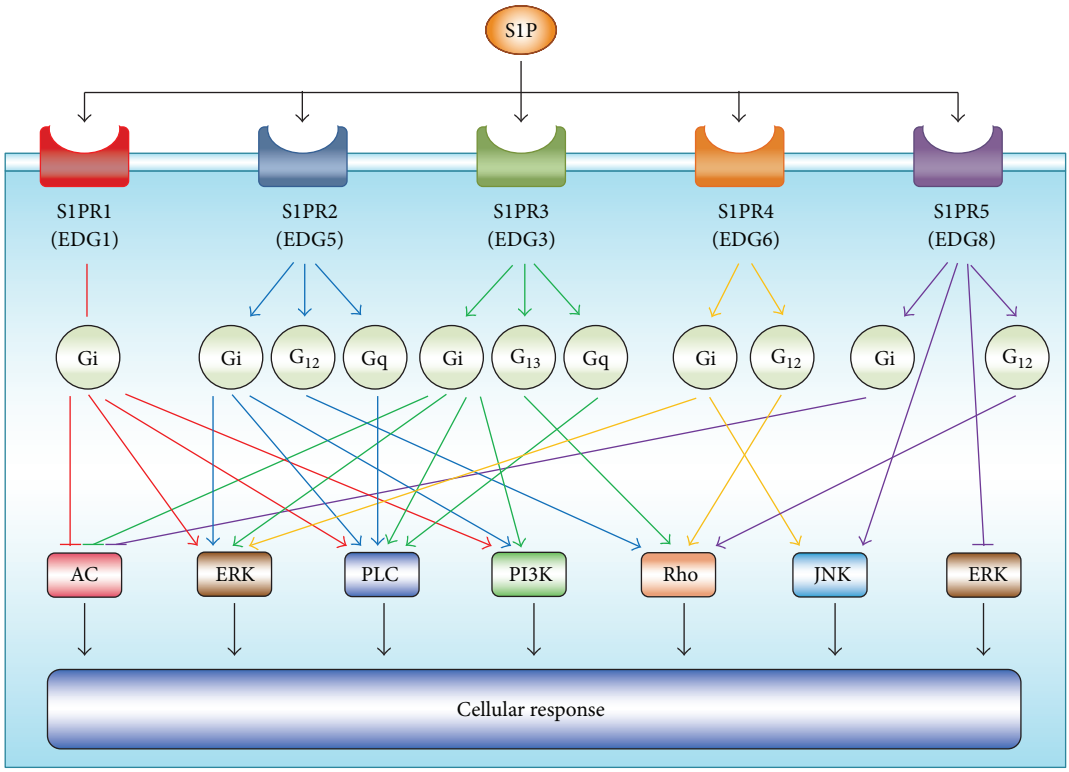

Figure 2: Downstream signaling pathways of sphingosine-1-phosphate (S1P) receptors. S1P is a ligand for the five different specific G protein coupled receptors: S1PR1/EDG1; S1PR2/EDG5; S1PR3/EDG3; S1PR4/EDG6; and S1PR5/EDG8. Each S1P receptor is coupled to different G proteins, which regulate activation or inhibition of the indicated downstream signaling pathways, such as adenylyl cyclase-cyclic AMP, AC; extracellular signal-regulated kinase, ERK; phospholipase C, PLC; phosphatidylinositol 3-kinase, PI3K; the small GTPases of the Rho family; and Jun amino terminal kinase, JNK. Only a few examples of these pathways are illustrated.

phosphatases (SPPs) and/or lipid phosphate phosphatases (LPPs). S1P is also irreversibly degraded to hexadecenal and ethanolamine phosphate by S1P lyase (SPL). The current consensus is that trafficking of immune cells is controlled by this S1P gradient. For example, in the blood and lymph, S1P levels are relatively high, but in secondary lymphatic tissue, such as lymph nodes and the thymus, S1P is maintained at very low levels [40]. Importantly, any inhibition of S1P signaling, and therefore altering of this S1P gradient, results in alteration of immune cell trafficking. S1P also regulates vascular integrity. In the plasma, a decrease in the amount of S1P causes increased vascular permeability, likely due to loss of signaling through S1PR1 on endothelial cells [41–43]. S1P through its tissue gradients, intracellular functions, and "inside-out" signaling is important in both physiologic and pathologic processes.

3. Export of S1P via ATP-Binding Cassette (ABC) Transporters and Spinster 2 (Spns2) Transporter

Until recently, the process through which S1P produced inside cells by the two SphKs reaches its receptors on the cell surface remained obscure. Membrane lipids, including S1P, do not spontaneously exchange through the lipid bilayers of the plasma membrane since the polar head groups do not readily go through the hydrophobic interior. Though there are many transporter proteins on the membrane for lipid exchange [9], sphingosine is known to spontaneously translocate without the aid of a transporter across intracellular membranes when added to cells or produced intracellularly [9].

Studies from several laboratories, including ours, have suggested the involvement of ABC transporters in the export of S1P from various types of cells *in vitro* [10]. S1P has been shown to be exported from mast cells via ABCC1 (also known as multidrug resistant protein 1; MRP1) [9], from astrocytes via ABCA1 [44], from endothelial cells via ABCA1 and ABCC1 [45], and from thyroid carcinoma cells via ABCC1 [46]. Using pharmacological and molecular approaches, we demonstrated that ABCC1 and ABCG2 (also known as breast cancer resistance protein; BCRP) are involved in estradiol-mediated transport of S1P and dihydro-S1P out of MCF-7 human breast cancer cells [12]. S1P is exported from erythrocytes and platelets by other transporters in the ABC transporter family [47, 48]. In erythrocytes, S1P is exported by an ATP-dependent and vanadate- and glyburide-sensitive transporter [47], while in platelets, S1P export requires an extracellular stimulus such as thrombin and is exported through two independent transporters, a Ca^{2+}-dependent transporter and an ATP-dependent glyburide-sensitive transporter [48, 49].

Collectively, these studies suggest that members of the large family of ABC transporters are responsible for export of S1P in various types of cells; however, in studies using mice with ABC transporter deficiencies, including animals with knockout of ABCA1, ABCA7, and ABCC1, S1P levels and related functions have been found to be unaltered [45], indicating the existence of compensatory mechanisms with other transporters.

Spns2, a member of the MFS (major facilitator superfamily) that does not have a typical ATP binding motif, has been recently discovered to export S1P from cells [11, 50–52]. Spns2 was identified independently by two groups, both of whom revealed that it transports S1P through observations in zebrafish. They showed that a mutation in Spns2 caused abnormal development resulting in cardia bifida (two hearts) [53] and that the phenotype of the Spns2 mutation is rescued by providing exogenous S1P [11]. The cardia bifida phenotype in the Spns2 knockout zebrafish was the clue that linked Spns2 to S1P, since the same phenotype was seen in S1PR2 knockout zebrafish [11, 53]. We have also shown that Spns2 can export endogenous S1P and dihydro-S1P from cells [13]. In addition, human Spns2 can transport several S1P analogues, including phosphorylated FTY720 [54]. Importantly, Spns2 was the first S1P transporter discovered to be physiologically functional *in vivo*, in contrast to the ABC transporters [54].

It has been suggested that Spns2 is important for vascular development [29]. We have observed that lymph nodes from Spns2 knockout mice have aberrant lymphatic sinuses that appear collapsed, with reduced numbers of lymphocytes [13]. Our data suggest that Spns2 is an S1P transporter *in vivo* that plays a role in regulation of levels of S1P not only in the blood, but also in the lymph nodes and lymphatic fluid, influencing lymphocyte trafficking and lymphatic vessel network organization [13]. The recent finding that blood endothelial cells purified from the aorta of Spns2-deficient mice are unable to release S1P [52] seems to support a strong role for Spns2 in S1P regulation. The levels of S1P in the plasma of Spns2 knockout mice have been observed to be decreased to 60% of that of wild type mice with endothelial cells contributing 40% of the total plasma S1P [54]. There was no difference between Spns2 knockout mice and wild type in S1P release activity in both erythrocytes and platelets, showing that a disruption of Spns2 does not affect the S1P release from erythrocytes or platelets [52]. Furthermore, bone marrow reconstitution studies revealed that Spns2 was not involved in S1P release from blood cells and suggested a role for Spns2 in other cells [51]. Consistent with these data, specific deletion of Spns2 on endothelial cells has been shown to result in a lack of lymphocyte egress, mimicking observations in global Spns2-knockout mice. These data suggest that Spns2 functions in endothelial cells, not blood cells, to establish the S1P gradient required for T and B cells to egress from their respective lymphoid organs [51].

4. Targeting S1P Transporters for Treatment of Cancer Patients

There have been an increasing number of studies, implicating roles for S1P in different stages of cancer progression in subtypes of both adult and pediatric malignancies. SphKs and S1P signaling have been suggested to also have a role in acquisition of drug resistance [55, 56]. There is a growing body of literature, with several clinical and pathological reports revealing the importance of SphK1 on cancer metastasis and prognosis [57–61]. Previous clinical studies have shown that SphK1 is overexpressed in human breast cancer and its expression correlates with poor patient outcomes [58, 62].

Studies linking the molecular interactions of S1P signaling with other oncogenic pathways, such as Ras, STAT3 (signal transducer and activator of transcription 3), NF-κB, and estrogen signaling, have been published. K-Ras mutations are known to increase the production of S1P in a SphK1-dependent manner and expression of the K-Ras oncogene leads to plasma membrane localization of SphK1. The RAS/RAF (rapidly accelerated fibrosarcoma)/MEK (mitogen-activated protein kinases)/ERK (extracellular signal-regulated kinases) pathway likely mediates this process, as constitutively active B-Raf or MEK are capable of activating SphK1 [63]. S1P produced by upregulated SphK1 in tumor cells activates S1PR1, which has been shown to lead to activation of STAT3 [34]. S1P is also involved in the activation of NF-κB, thereby regulating the transcription of the proinflammatory cytokines TNF-α (tumor necrosis factor-alpha) and IL-6 (interleukin-6) [34]. Estradiol is known to also stimulate SphK1 activation and the release of S1P, through which estradiol is capable of activating the S1PR3, resulting in EGFR transactivation in a matrix metalloprotease-dependent manner [31]. Altogether, these molecular interactions of S1P signaling with other oncogenic pathways suggest the importance of S1P signaling in cancer.

Communication among tumor cells, the host microenvironment, and inflammatory cells via systemic S1P regulates metastasis. S1P generated in cancer cells is secreted into tissue interstitial fluid in the body and affects the TME by altering immune cells, evoking inflammation, and inducing angiogenesis and lymphangiogenesis thereby promoting cancer metastasis [34, 64]. We have recently measured S1P levels within tumor interstitial fluid and found that significantly higher levels of S1P compared with the interstitial fluid of normal tissue and that inhibiting the "inside-out" signaling of S1P by FTY720 (known as fingolimod) significantly decreased S1P levels in the tumor interstitial fluid (unpublished data). Altogether, the "inside-out" signaling of S1P with overexpressed SphK1 and S1P transporters plays an important role in cancer progression through its effects on the TME.

It has recently been reported that a specific pharmacological inhibitor of SphK1 had no effect on cell proliferation *in vitro* [65]. This result generated some arguments that S1P, the product of SphK1, may not be an ideal anticancer target. On the other hand, targeting S1P signaling leads to a significant suppression of cancer progression *in vivo* [64], especially under conditions in which cancer is associated with inflammation [34]. Considering the importance of "inside-out" signaling of S1P within the TME through promotion of angiogenesis and lymphangiogenesis [4, 66, 67], the effects of targeting S1P signaling may only be adequately assessed *in vivo*.

ABC transporters were originally described as multidrug resistance genes and have been shown to be overexpressed in various solid and hematological cancers [68]. Expression of ABC transporters has been correlated with resistance to chemotherapies and poor prognosis for patients with certain types of cancer [68]. ABCB1, also known as multidrug resistant gene 1 (MDR1), has been targeted in a number of clinical trials that failed to demonstrate significant benefit [69]. Since it has been suggested that several ABC transporters are involved in secretion of S1P from stromal, endothelial, and cancer cells, it is possible that these transporters may play an important role in the pathological processes regulated by S1P and may worsen the biology of cancer cells. Considering that ABCB1 does not have a role for S1P transport, targeting ABCC1 and/or G2 may be a more promising treatment option for cancer patients. In contrast to the ABC transporters, with some of them originally described as multidrug resistant genes, the roles of Spns2 in cancer remain unknown.

Spns2, a 549 amino acids protein, belongs to the MFS transporter family as determined based on its predicted amino acid sequence [49]. The crystal structure of Spns2 and the precise mechanism of S1P transport via this transporter are still under investigation. S1P import by this transporter also remains to be described. To elucidate the role of Spns2 in cancer, we are currently in the midst of a process of developing gene targeting techniques utilizing nanoparticles to downregulate spns2 expression in cancer cells. How effective this may be in light of other transporters that contribute to S1P export, particularly *in vivo* setting, is yet to be determined. Further investigation is needed to clarify the function and role of Spns2 in normal physiological conditions and in the pathological condition of cancer.

The ABC transporter family and Spns2 are found in various types of cells and have different roles in each type of cell, so that it is possible that targeting a specific transporter may present some difficulties for cancer therapy for patients. In addition to targeting S1P transporters, a possible strategy for cancer treatment involves targeting S1P itself using a monoclonal antibody. In an animal model, neutralization of systemic S1P using Sphingomab, an anti-S1P monoclonal antibody, was shown to suppress lung metastasis [70]. The humanized version of Sphingomab, Sonepcizumab/ASONEP [71], has finished phase I trials and has recently entered phase II efficacy and safety studies for the treatment of renal cell carcinoma and age-related macular degeneration [72]. Targeting S1P itself may be one of the promising strategies for controlling cancer progression and metastasis.

5. Conclusion

S1P promotes a variety of intracellular and extracellular biological functions and is transported from inside cells through several members of the ABC transporter family and the enigmatic Spns2 transporter. Once through the plasma membrane, S1P exerts its "inside-out" signaling in the context of the TME and promotes cancer progression. Targeting S1P transporters and S1P signaling in cancer progression is a promising direction for development of the next generation of cancer therapeutics.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Molecular Mechanisms for Biliary Phospholipid and Drug Efflux Mediated by ABCB4 and Bile Salts

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On the canalicular membranes of hepatocytes, several ABC transporters are responsible for the secretion of bile lipids. Among them, ABCB4, also called MDR3, is essential for the secretion of phospholipids from hepatocytes into bile. The biliary phospholipids are associated with bile salts and cholesterol in mixed micelles, thereby reducing the detergent activity and cytotoxicity of bile salts and preventing cholesterol crystallization. Mutations in the *ABCB4* gene result in progressive familial intrahepatic cholestasis type 3, intrahepatic cholestasis of pregnancy, low-phospholipid-associated cholelithiasis, primary biliary cirrhosis, and cholangiocarcinoma. *In vivo* and cell culture studies have demonstrated that the secretion of biliary phospholipids depends on both ABCB4 expression and bile salts. In the presence of bile salts, ABCB4 located in nonraft membranes mediates the efflux of phospholipids, preferentially phosphatidylcholine. Despite high homology with ABCB1, ABCB4 expression cannot confer multidrug resistance. This review summarizes our current understanding of ABCB4 functions and physiological relevance, and discusses the molecular mechanism for the ABCB4-mediated efflux of phospholipids.

1. Introduction

ABCB4, also called multidrug resistance 3 (MDR3), is a 1279-amino acid transmembrane protein. ABCB4, belonging to the ATP-binding cassette (ABC) transporter family, consists of two homologous halves, each of which contains six transmembrane helices (TMHs) and a cytoplasmic nucleotide-binding fold (NBF) (Figure 1) [1]. ABCB4 has two *N*-glycosylation consensus sites in the first extracellular loop. The human *ABCB4* gene on chromosome 7q21.1 has 28 exons and 27 introns and is located adjacent to the *ABCB1* gene [2]. ABCB1, also called MDR1 or P-glycoprotein, has a 1280-amino acid sequence with 76% identity and 86% similarity to ABCB4 (Figure 2). ABCB1 is normally present in various tissues, including the liver, kidney, intestinal mucosa, and capillary endothelial cells at the blood-brain barrier [3]. On the other hand, ABCB4 protein is mainly expressed in the liver, although low levels of ABCB4 mRNA expression are found in the adrenal gland, muscle, tonsil, spleen, placenta, testis, and ileum [4]. ABCB1 exports a large number of structurally unrelated hydrophobic compounds and is responsible for multidrug resistance of cancer cells. However, ABCB4 is unable to export most ABCB1 substrates efficiently and to confer equivalent multidrug resistance properties [5]. In the liver, ABCB4 is localized to the canalicular membranes of hepatocytes and is necessary for the secretion of phospholipids into bile.

2. Discovery of ABCB4

In 1976, Juliano and Ling demonstrated the overproduction of a large membrane protein called P-glycoprotein in multidrug resistant cells [6]. In 1986, Roninson et al. found two kinds of MDR gene, called *MDR1* and *MDR2*, amplified in multidrug resistant cell lines from KB cells [7]. Ueda et al. have shown that a full-length cDNA for the *MDR1* gene encodes P-glycoprotein and confers multidrug resistance phenotype [8, 9]. In 1987, van der Bliek et al. isolated the human *MDR3* gene from liver cDNA libraries and determined its sequence [1, 10]. *MDR2* is actually identical in sequence to *MDR3* [1].

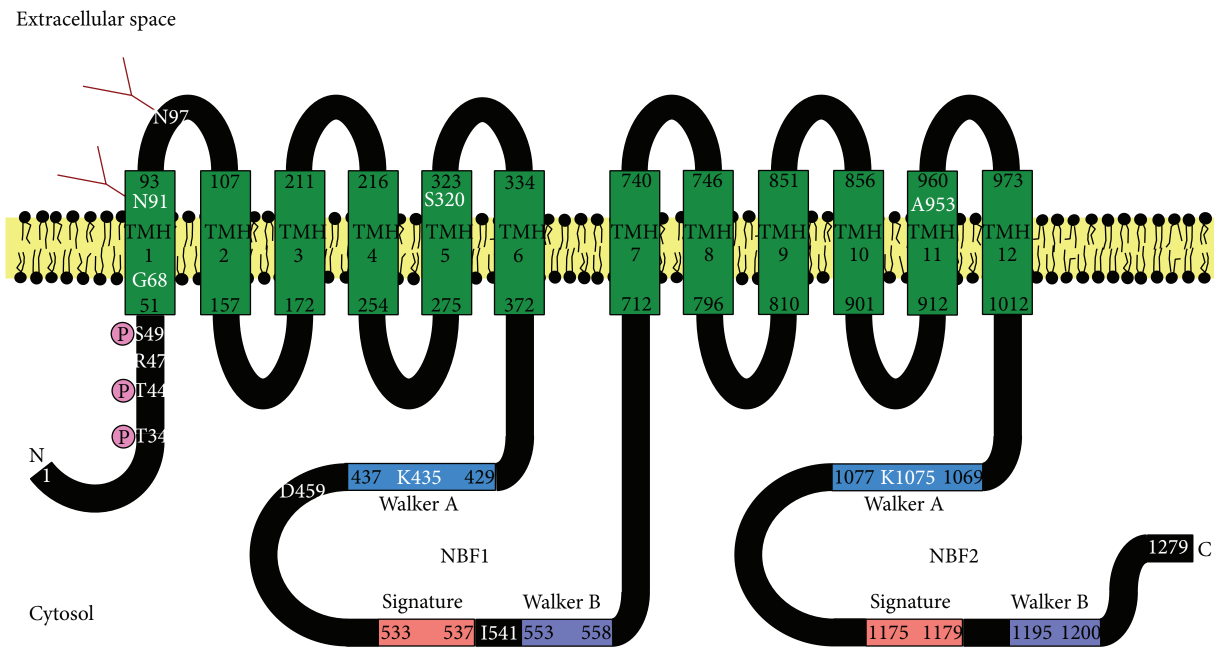

Figure 1: Schematic model of ABCB4. ABCB4 consists of twelve TMHs spanning the plasma membrane and two cytosolic NBFs containing the Walker A, Walker B, and signature motifs. N and C indicate the N- and C-termini of ABCB4, respectively. TMHs are predicted from the crystal structure of mouse Abcb1a (PDB code 3G61) reported by Aller et al. [60]. The residues N91 and N97 are *N*-glycosylated with complex oligosaccharides. The residues T34, T44, and S49 are phosphorylation sites. The T34M, R47G, K435M, and K1075M mutations impair the phospholipid efflux activity of ABCB4. The G68H, S320F, D459H, I541F, and A953D mutations result in the intracellular retention of ABCB4.

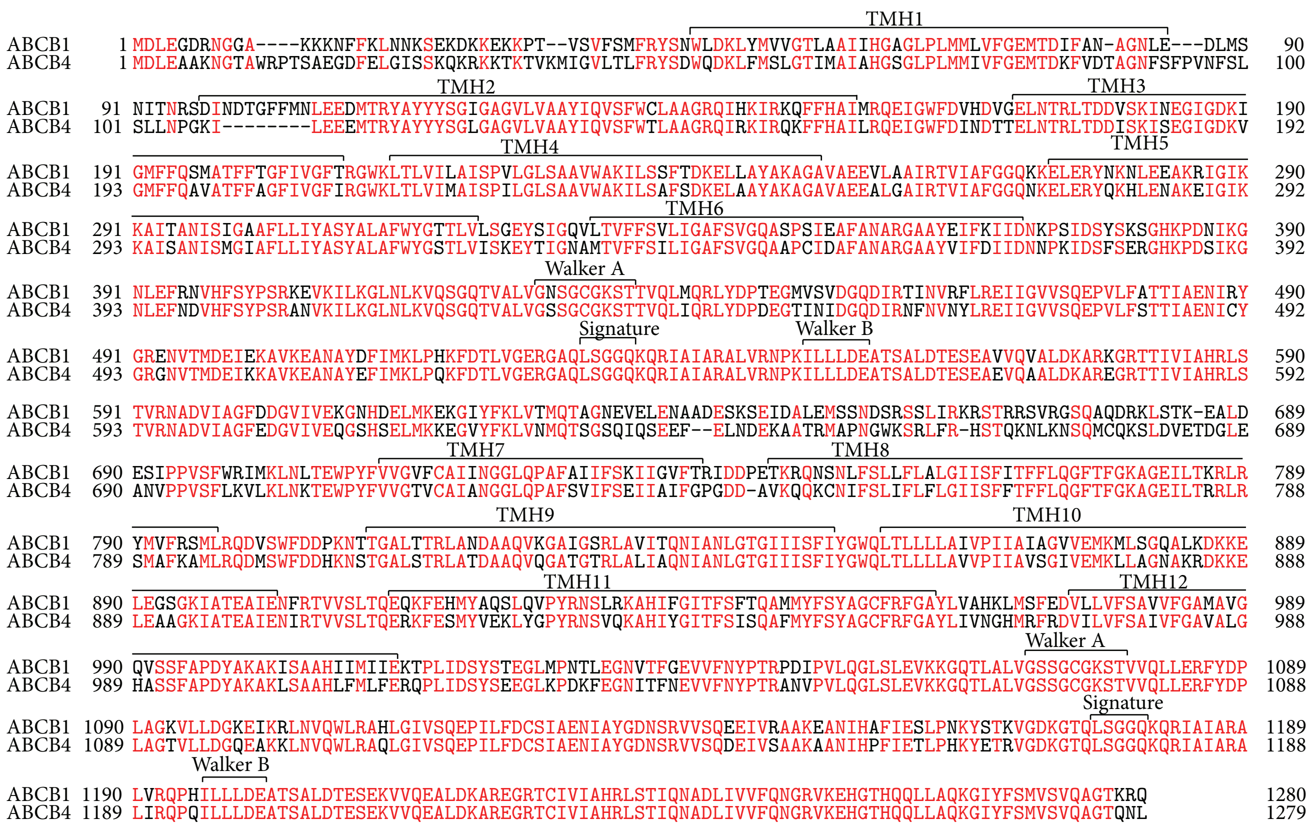

Figure 2: Amino acid sequence alignment of human ABCB1 and ABCB4. Identical amino acids are *red*. Functional domains are shown above the sequence alignment. TMHs are predicted from the crystal structure of mouse Abcb1a (PDB code 3G61) reported by Aller et al. [60].

3. Physiological and Pathophysiological Roles of ABCB4

The function of biliary phospholipid secretion is to protect the membranes of cells facing the biliary tree against bile salts. Biliary phospholipids also play a key role in solubilizing cholesterol. The complexation of bile salts with phospholipids and cholesterol into mixed micelles strongly reduces the cytotoxic detergent effect of bile salts. The concentration of bile salts as monomers and simple micelles is responsible for the potentially damaging effects on membrane bilayers [11]. The hepatocyte plasma membrane is functionally divided into an apical region adjacent to the bile canalicular lumen and a basolateral region in contact with sinusoidal blood. The major structural phospholipids in the outer leaflet of canalicular membranes are phosphatidylcholine (PC) and sphingomyelin (SM) [12]. In bile, however, the predominant (~95%) phospholipid is PC, while SM is present only in trace amounts [13].

Mouse Abcb4, formerly known as Mdr2, is the homolog of human ABCB4. In 1993, Smit et al. generated the mice with homozygous disruption of the *Abcb4* gene, which suffer from liver disease characterized by severe necrotic damage of hepatocytes, strong portal inflammation, and proliferation and destruction of the canalicular and small bile ductular tracts [14]. Spontaneous gallstone formation is also a feature of the phenotype of *Abcb4* knockout mice [15]. These *Abcb4* knockout mice show almost complete absence of PC from their bile, although their bile salt secretion is normal in these mice, suggesting that Abcb4 is required for the secretion of phospholipids into bile [14]. In addition, the cholesterol secretion is strongly suppressed in these mice [14].

Elferink et al. have investigated the relationships among the biliary secretion of bile salt, phospholipids, and cholesterol using *Abcb4* knockout mice [16, 17]. In wild-type mice (+/+), the biliary phospholipid secretion increases with increasing bile salt secretion, and a curvilinear relationship between phospholipid and bile salt secretion is observed [16]. In *Abcb4* homozygous (−/−) mice, the phospholipid secretion is negligible at all bile salt output rates [16]. The bile in wild-type (+/+) mice contains almost exclusively PC with a small amount of phosphatidylethanolamine (PE), but no SM can be detected [17]. The cholesterol secretion does not differ between wild-type (+/+) and *Abcb4* heterozygous (+/−) mice, although the phospholipid secretion rate in *Abcb4* (+/−) mice is 30–50% lower than that in wild-type (+/+) mice [16]. In *Abcb4* (−/−) mice, the biliary secretion of cholesterol was very low [16]. However, the cholesterol secretion in *Abcb4* (−/−) mice is completely restored by infusion of a sufficiently hydrophobic bile salt, taurodeoxycholate, to allow solubilization of cholesterol in the absence of phospholipids [17]. Mixed micelles of bile salts and phospholipids have a much higher capacity to take up cholesterol than simple bile salt micelles [18], and the cholesterol secretion in the absence of phospholipids depends on the cholesterol-solubilizing capacity of the secreted bile salts [17]. These results provide the first evidence that the biliary cholesterol secretion is at least partially independent of ABCB4.

Human ABCB4 mutations result in a wide spectrum of phenotypes, ranging from progressive familial intrahepatic cholestasis type 3 (PFIC3) to adult cholestatic liver disorders [19]. PFIC3 is characterized by high γ-glutamyl transpeptidase and early onset of persistent cholestasis that progresses to cirrhosis and liver failure before adulthood [20, 21]. In many cases of PFIC3, liver transplantation is the only therapy. The biliary phospholipid level in PFIC3 patient is dramatically decreased despite the presence of bile acids [20]. This cholestasis may be caused by the toxicity of detergent bile salts that are not associated with phospholipids, leading to bile canaliculus and biliary epithelium injuries. ABCB4 defect is also involved in intrahepatic cholestasis of pregnancy (ICP), low-phospholipid-associated cholelithiasis (LPAC), and primary biliary cirrhosis [4, 22]. ICP is a reversible form of cholestasis in the third trimester of pregnancy and rapidly ameliorated after childbearing. LPAC is characterized by intrahepatic hyperechoic foci, intrahepatic sludge, or microlithiasis [23]. The absence of biliary phospholipids may lead to the destabilization of micelles and promote the lithogenicity of bile with the crystallization of cholesterol. The association between cholangiocarcinoma, a rare malignant tumor of the biliary tract, and ABCB4 mutations has been recently reported [24]. Chronic biliary inflammation may increase cholangiocyte turnover, leading to the growth of altered cholangiocytes and increased susceptibility to cholangiocarcinoma.

Besides ABCB4, several ABC transporters expressed on the canalicular membranes of hepatocytes are involved in the secretion of lipids and thus in canalicular bile formation (Figure 3). ABCB11, also called bile salt export pump, is implicated in most of the bile salt transport from hepatocytes into the bile canalicular lumen [25]. PFIC2 is caused by mutations in ABCB11 [25]. Patients with PFIC2 usually suffer from severe cholestasis and severe pruritus, with markedly elevated serum bile acids and normal serum γ-glutamyltransferase activity.

ABCG5 and ABCG8 are responsible for the secretion of biliary cholesterol [26, 27]. ABCG5 and ABCG8 form a heterodimer in the endoplasmic reticulum, which is required for their movement into the Golgi and onto the apical membranes [28]. Disruption of the *Abcg5* and *Abcg8* genes in mice strongly decreases the biliary cholesterol secretion but results in modest nonsignificant reductions in the biliary phospholipid levels [27]. On the other hand, Abcg5 and Abcg8 independent routes at least partially contribute to the biliary secretion of cholesterol [29]. The expression of the human *ABCG5* and *ABCG8* transgenes does not increase biliary cholesterol in *Abcb4* knockout mice, suggesting that ABCG5 and ABCG8 require ABCB4 for the secretion of cholesterol into bile [30]. The mixed bile salt/phospholipid micelles generated by ABCB11 and ABCB4 are probably essential for the cholesterol secretion mediated by ABCG5/ABCG8.

Abcb4 protein is also expressed in mouse macrophages. Bone marrow transplantation replaces all bone marrow-derived cells. Pennings et al. have created mice specifically lacking Abcb4 in bone marrow-derived cells, including macrophages, by bone marrow transplantation in *LDLr*

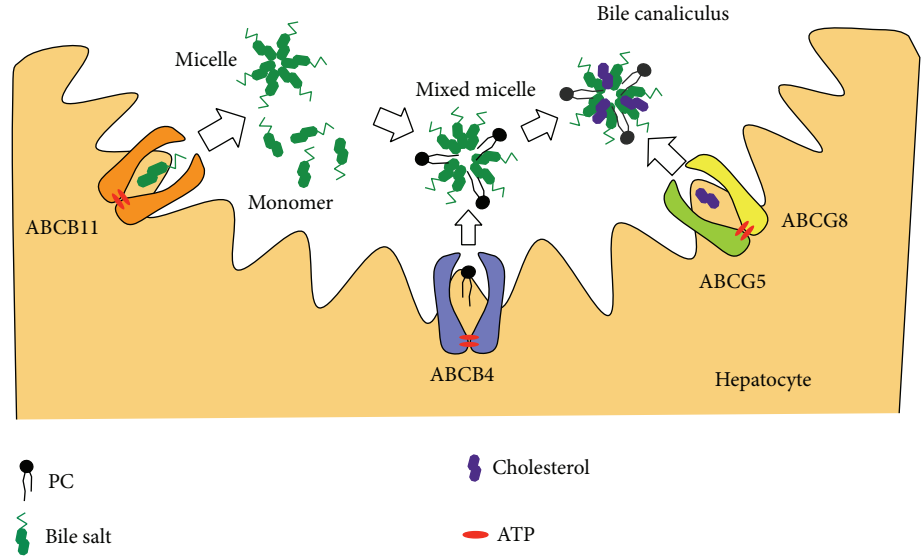

FIGURE 3: Formation of mixed micelles of bile salts, phospholipids, and cholesterol in bile canaliculus. ABCB11 mediates the efflux of bile salts into bile. The bile salt monomers are essential for the phospholipid efflux mediated by ABCB4. In the presence of mixed bile salt/phospholipid micelles, ABCG5/ABCG8 heterodimer mediates the efflux of cholesterol.

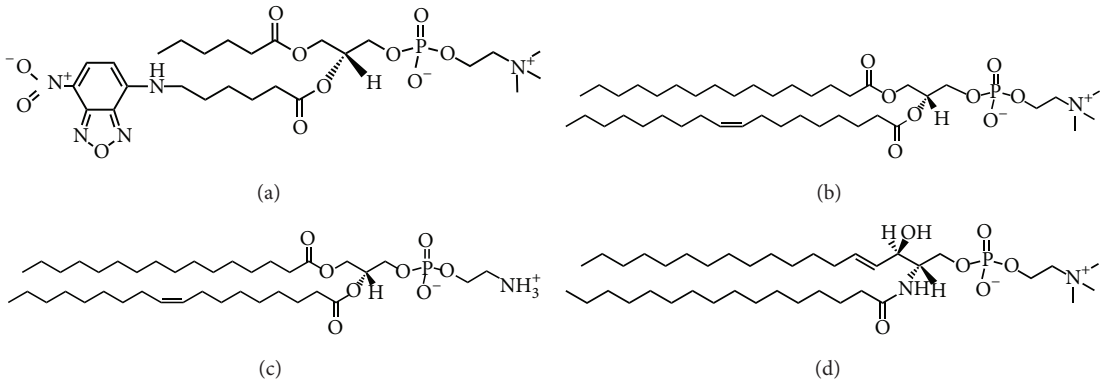

FIGURE 4: Phospholipid substrates for ABCB4. (a) C_6-NBD-PC, (b) PC, (c) PE, and (d) SM.

knockout mice [31]. Abcb4 deficiency in bone marrow-derived cells leads not only to lower serum cholesterol levels but also to an increase in the atherosclerotic lesion size, suggesting an important atheroprotective function of bone marrow-derived Abcb4 [31]. However, there is no difference in the cholesterol or phospholipid efflux to HDL or apolipoprotein A-I between *Abcb4* (+/+) and *Abcb4* (−/−) macrophages [31].

4. ABCB4-Mediated Efflux of Fluorescence-Labeled Phospholipids

Ruetz and Gros have shown that the expression of mouse Abcb4 in secretory vesicles from the yeast mutant *sec6-4* enhances the translocation of a fluorescence-labeled short-chain PC analog, C_6-NBD-PC (Figure 4), from the outer to the inner leaflet of the vesicle bilayer, suggesting the function of Abcb4 as a phospholipid translocase [32]. Dithionite, a membrane-impermeant anion, has been used to reduce only C_6-NBD-PC molecules on the outer leaflet, but not inner leaflet, of the bilayer to their nonfluorescent derivatives. Increased Abcb4-mediated translocation of C_6-NBD-PC is strictly dependent on ATP and Mg^{2+} and abrogated by the ATPase inhibitor, vanadate, and the ABCB1 modulator, verapamil [32]. Addition of the bile salt taurocholate results in an enhancement of Abcb4-mediated PC translocation activity in the secretory vesicles, suggesting that the stimulation of Abcb4 activity is provoked by the formation of intravesicular aggregates or mixed micelles of taurocholate and C_6-NBD-PC [33].

Van Helvoort et al. have made the stable transfectants of pig kidney epithelial LLC-PK1 cells expressing human ABCB4, which is localized in the apical membranes [34]. C_6-NBD-diacylglycerol is efficiently converted by cells to the homologous C_6-NBD-PC and C_6-NBD-PE, while C_6-NBD-ceramide yields the corresponding SM (C_6-NBD-SM). The newly synthesized C_6-NBD-PC, but not C_6-NBD-PE or C_6-NBD-SM, is exclusively secreted to the apical

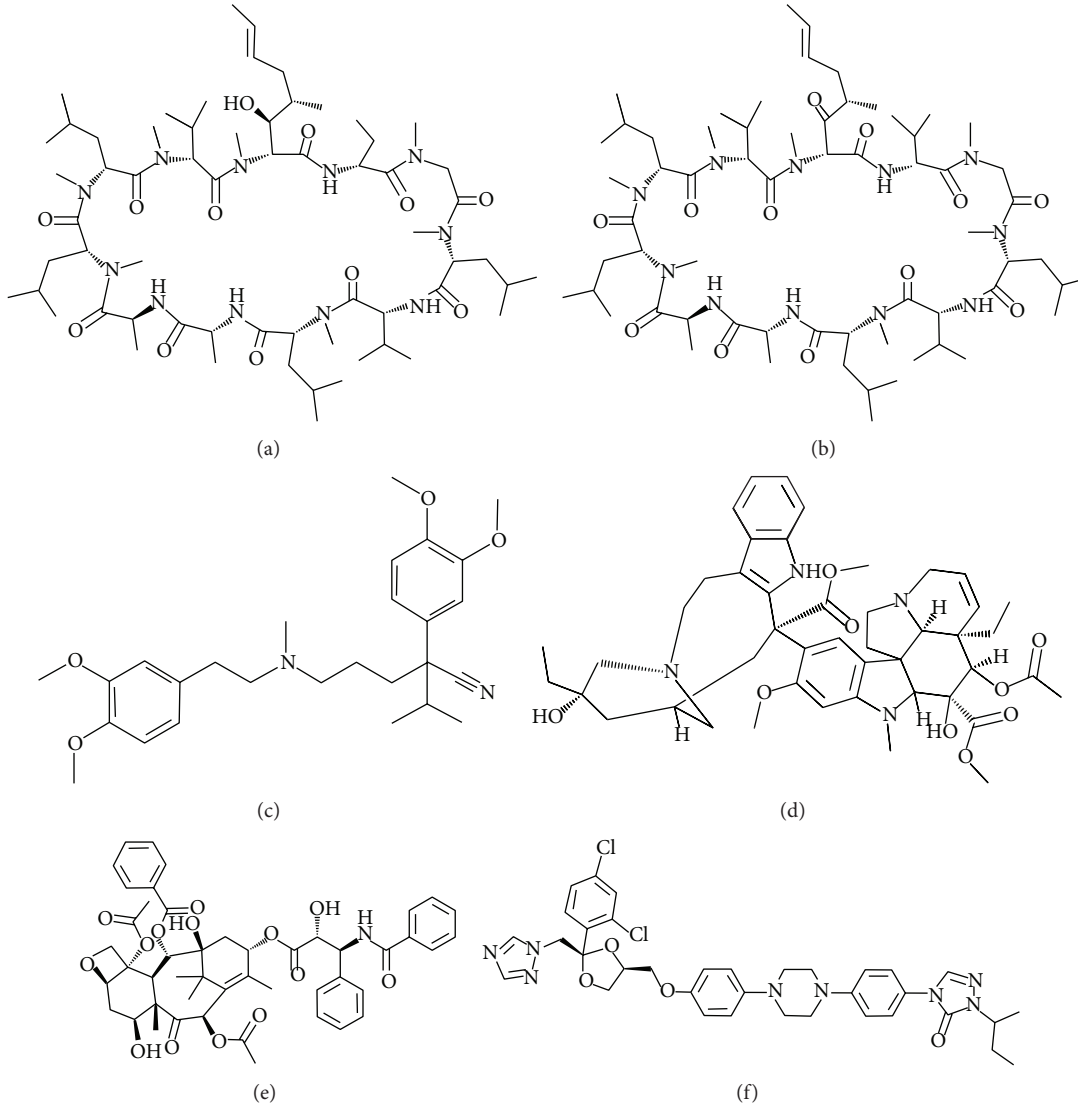

Figure 5: Inhibitors of ABCB4: (a) cyclosporine A, (b) valspodar, (c) verapamil, (d) vinblastine, (e) paclitaxel, and (f) itraconazole.

albumin-containing medium of ABCB4-expressing LLC-PK1 cells [34]. The energy depletion markedly reduces the apical release of C_6-NBD-PC mediated by ABCB4 [34]. Cyclosporine A, valspodar, verapamil, vinblastine, and paclitaxel (Figure 5), but not digoxin, decrease the rate of C_6-NBD-PC secretion by ABCB4-expressing LLC-PK1 cells [35]. On the other hand, ABCB1-expressing cells secrete C_6-NBD-PC and C_6-NBD-PE, but not C_6-NBD-SM, to the apical albumin-containing medium [34]. Nevertheless, ABCB1 has quite low, if any, ability to mediate the secretion of endogenous long-chain phospholipids into bile. Indeed, *Abcb4* knockout mice do not secrete any phospholipids into bile, despite the substantial expression of *Abcb1a* and *Abcb1b* on the canalicular membranes of hepatocytes.

ABCB4 is expressed in well-differentiated human hepatoblastoma HepG2 cells and distributed to the pseudocanaliculi formed between adjacent cells. The functional activity of ABCB4 has been estimated by the transport of C_6-NBD-PC into the pseudocanaliculi of HepG2 cells [36]. Additionally, in collagen sandwich cultured rat hepatocytes, the fluorescence-labeled PC is secreted into the bile canaliculi [37].

5. ABCB4-Mediated Efflux of Endogenous Phospholipids

The above-mentioned studies have assessed the function of ABCB4 by using C_6-NBD-PC as a substrate. However, C_6-NBD-PC is structurally different from the endogenous phospholipids in cells, and its bulky fluorescent probe probably affects the recognition by the transporters. Actually, ABCB1 is not involved in the biliary phospholipid secretion but can transport C_6-NBD-PC molecules. Hence, to further clarify the function of ABCB4, we have established HEK293 cells stably expressing ABCB1 or ABCB4 and have quantified the efflux of endogenous phospholipids from cells by using enzymatic assays [38]. There is no significant difference in

the phospholipid efflux into the medium among the host HEK293 cells, ABCB1-expressing cells, and ABCB4-expressing cells [38]. However, the phospholipid efflux from ABCB4-expressing cells is remarkably enhanced by the addition of taurocholate, while the phospholipid efflux from ABCB1-expressing cells is not affected by taurocholate [38]. The phospholipid efflux mediated by ABCB4 is increased with increasing concentrations of taurocholate and shows concentration dependence from 0.2 mM to 1 mM taurocholate [38]. The critical micelle concentration of taurocholate is estimated to be 2.5 mM in the medium by light scattering measurements, and thus, below 2.5 mM, taurocholate molecules are present as monomers [38]. Based on these results, the monomer forms of bile salts most likely function, at least initially, in supporting the ABCB4-mediated phospholipid efflux. The majority of bile salts are conjugated in the liver to glycine or taurine, which increases their polar surface. The phospholipid efflux from ABCB4-expressing cells increases in the order of taurocholate > glycocholate > cholate [38], which is inversely correlated with the bile salt hydrophobicity index, from the least to the most hydrophobic: taurocholate (0) < glycocholate (+0.07) < cholate (+0.13) [39]. ABCB4-K435M and ABCB4-K1075M, Walker A lysine mutants in NBFs, do not mediate the phospholipid efflux in the presence of taurocholate, suggesting that ATP hydrolysis is essential for the ABCB4-mediated efflux [38]. Verapamil also completely blocks the taurocholate-dependent efflux of phospholipids from ABCB4-expressing cells, suggesting that ABCB1 and ABCB4 have quite similar substrate binding domains [38]. Mass spectrometry has revealed that ABCB4-expressing cells preferentially secrete PC (16:0-16:1 PC, 16:0–18:2 PC, 16:0–18:1 PC, and 18:0–18:2 PC) rather than SM (16:0–18:1 SM) in the presence of taurocholate [38]. Moreover, we have developed enzyme-based fluorometric methods for quantifying PC, PE, and SM, which are simple, rapid, and sensitive and have high throughput [40, 41], and have demonstrated that the addition of taurocholate significantly increases the efflux of PC, PE, and SM from ABCB4-expressing cells, although the enhancement of PE or SM efflux by taurocholate was less marked than that of PC efflux [42]. Notably, apolipoprotein A-I or HDL cannot stimulate the ABCB4-mediated phospholipid efflux, despite their ability to accept phospholipids from ABCA1-expressing cells [31, 43], whereas taurocholate also promotes the phospholipid efflux mediated by ABCA1 [43].

Gautherot et al. have recently identified two point mutations of the ABCB4 N-terminal domain, T34M and R47G, in patients with LPAC or ICP [44]. The PC secretion activities of both mutants in HEK293 cells are low compared with that of wild-type ABCB4 [44]. In the N-terminal domain of ABCB4, Thr34, Thr44, and Ser49 are phosphorylatable [44]. The T34M mutation directly abolishes the phosphorylation, while the R47G mutation indirectly impairs the phosphorylation of Thr44 or Ser49 [44]. The ABCB4-mediated secretion of PC is enhanced by the activation of protein kinase A or C and decreased by the inhibition of these kinases [44].

Itraconazole, an antifungal agent (Figure 5), is known to cause drug-induced cholestasis (DIC). Yoshikado et al. have demonstrated that, in itraconazole-treated rats, biliary phospholipids, rather than bile salts, are drastically decreased and that the ABCB4-mediated efflux of [^{14}C]PC from LLC-PK1 cells is reduced in the presence of itraconazole [45].

6. ABCB4-Mediated Efflux of Drugs

Initially, it was thought that ABCB4 is responsible for exporting various drugs due to its high homology with ABCB1 (Figure 2). However, Schinkel et al. have shown that ABCB4-expressing BRO human melanoma cells exhibit no resistance against a range of drugs including vincristine, colchicine, etoposide, daunorubicin, doxorubicin, actinomycin D, and gramicidin D [5]. Kino et al. have reported that the expression of ABCB4 in yeast confers resistance to aureobasidin A, an antifungal cyclic depsipeptide antibiotic, which is overcome by vinblastine, verapamil, and cyclosporine A [46]. The treatment of ovarian cancer cells with ABCB4 siRNA induces minor reduction in the paclitaxel resistance [47]. Smith et al. have reported that polarized monolayers of ABCB4-expressing LLC-PK1 cells show an increased directional transport of several ABCB1 substrates, such as digoxin, paclitaxel, daunorubicin, vinblastine, and ivermectin, and that the transport rate of these drugs, except for paclitaxel, is lower in ABCB4-expressing cells than in ABCB1-expressing cells [35]. Furthermore, ABCB4-dependent transport of digoxin is inhibited by ABCB1 reversal agents, cyclosporine A, valspodar, and verapamil [35]. Recently, we have demonstrated that the expression of ABCB1 or ABCB4 in HEK293 cells decreases the accumulation of rhodamine 123 and rhodamine 6G and that these reductions are more marked in ABCB1-expressing cells than in ABCB4-expressing cells [42]. The accumulation of BODIPY-verapamil in HEK293 cells is strikingly reduced by ABCB1 expression but is not altered by ABCB4 expression, indicating that BODIPY-verapamil is not a transport substrate of ABCB4 but an inhibitor of the ABCB4-mediated phospholipid efflux [42]. These findings suggest that ABCB4 cannot cause multidrug resistance due to the low rates of ABCB4-mediated export of drugs compared with ABCB1-mediated export. The nonphospholipid substrates may have lower affinities for ABCB4 than ABCB1 and/or compete with membrane PC for binding to ABCB4.

Furthermore, we have shown that the addition of taurocholate has no effect on the ABCB4-mediated efflux of rhodamine 123 and rhodamine 6G, which may be attributed to the sufficient solubility of these substrates in the aqueous medium [42].

7. ATP Hydrolysis by ABCB4

NBFs of all ABC transporters show extensive identity of amino acid sequence and conserved motifs, including the Walker A, Walker B, and signature motif (Figure 1) [48]. It has been assumed that the conformational changes at NBFs as a consequence of ATP binding and/or hydrolysis are transmitted to TMHs, leading to a high-affinity to low-affinity switch at the substrate-binding site. ATP binds at the interface of the two NBFs, which induces the formation of a closed dimer.

Smith et al. have confirmed the specific MgATP binding and the vanadate-dependent, *N*-ethylmaleimide-sensitive

nucleotide trapping activity of ABCB4, using the radiolabeled photoaffinity ATP analog [α-^{32}P]8-azido-ATP and insect Sf9 cell membranes overexpressing ABCB4 [35]. Vanadate replaces inorganic phosphate bound to ABCB4 and inhibits ATP-hydrolysis, which results in the formation of a complex between ADP and ABCB4 that cannot be dissolved by high MgATP concentrations. The nucleotide trapping by ABCB4 in the presence of vanadate is nearly abolished by EDTA, paclitaxel, vinblastine, verapamil, cyclosporine A, and valspodar, whereas the nucleotide trapping by ABCB1 is greatly stimulated by verapamil [35].

More recently, Ishigami et al. have constructed the chimera protein containing TMHs of ABCB1 and NBFs of ABCB4 and have analyzed the features of human ABCB4 NBFs [49]. Similar to ABCB1, the chimera protein confers the resistance against vinblastine and paclitaxel and mediates the calcein AM efflux but not the phospholipid efflux in the presence of taurocholate [49]. In the presence of vanadate, verapamil strongly enhances the ADP trapping of ABCB4 NBFs in the chimera protein as well as that of ABCB1 NBFs [49]. The ATPase activity of the purified chimera protein is stimulated by vinblastine and verapamil. However, the drug-stimulated ATPase activity of the purified chimera protein is much lower than that of purified ABCB1 [49].

8. Subcellular Localization of ABCB4

Fibrates are ligands for peroxisome proliferator-activated receptor α. In HepG2 cells, the treatment with bezafibrate has no effect on the levels of ABCB4 protein but induces the redistribution of ABCB4 into pseudocanaliculi between cells [36]. Ghonem et al. have recently reported that fenofibrate upregulates ABCB4 mRNA and protein expression in primary cultured human hepatocytes [37]. Receptor for activated C-kinase 1 (RACK1) is a 36 kDa cytosolic protein and can bind to various signaling molecules. ABCB4 protein, exogenously expressed in HeLa cells using the recombinant adenoviruses, is located dominantly on the plasma membrane and only a minor portion is observed intracellularly [50]. Downregulation of RACK1 expression by siRNA results in the localization of *ABCB4* in the cytosolic compartment, suggesting that RACK1 is involved in the trafficking of ABCB4 from the Golgi to the plasma membrane [50].

The mutation I541F, located in the first NBF of ABCB4, has been described in a homozygous patient with PFIC3 [19]. ABCB4 is localized at the pseudocanalicular membrane in HepG2 cells or at the apical surface in MDCK cells, whereas the I541F mutant is retained intracellularly [19]. After shifting cells to 27°C, the intracellular traffic of this mutant is restored [19]. In addition, cyclosporine A allows a significant amount of the I541F mutant protein to reach the pseudocanalicular membrane in HepG2 cells [51]. The S320F variant is linked with the development of cholestatic disorders including ICP, LPAC, DIC, and PFIC3, and the A953D mutation is found in heterozygosity with the S320F mutant [52]. The transient expression of the S320F or A953D mutant is low at the plasma membranes in HEK293 cells, but cyclosporine A improved the plasma membrane localization of both mutants [52]. Two mutations, G68H and D459H, have been identified in children with PFIC3 and result in the retention of ABCB4 in endoplasmic reticulum in MDCK cells [53].

Lipid rafts are small (10–200 nm) plasma membrane domains containing high levels of sphingolipids, mainly SM, and cholesterol, which are characterized physicochemically by tight packing and reduced fluidity leading to a liquid-ordered phase surrounded by the bulk liquid-disordered membranes [54–56]. Recently, we have investigated the relationships between the functions of ABCB4 and lipid rafts [42]. To isolate the lipid rafts, we have used Triton X-100 insolubility assay and OptiPrep gradient centrifugation method [57, 58]. In mouse canalicular membranes, Abcb4 is exclusively localized to the nonraft membranes [42]. Likewise, in ABCB4-expressing HEK293 cells, ABCB4 is predominantly distributed into the nonraft membranes [42]. ABCB4 expression leads to significant increases in the contents of PC, PE, and SM in nonraft membranes and to further enrichment of SM and cholesterol in raft membranes [42]. The ABCB4-stimulated efflux of PC, PE, and SM in the presence of taurocholate is completely abolished by BODIPY-verapamil, which partitions hardly into the raft membranes [42]. Collectively, these results have indicated that the taurocholate-stimulated phospholipid efflux is mediated exclusively by ABCB4 located in the nonraft membranes.

9. Molecular Model for ABCB4-Mediated Flopping and Efflux

ABCB1 and ABCB4 are 86% similar in terms of amino acid sequence (Figure 2). The crystal structures of ligand-bound mouse Abcb1a and ADP-bound sav1866, a bacterial homolog of ABCB1, have been published [59, 60]. The nucleotide-free but ligand-bound ABCB1 structure represents the inward-facing conformation, which is formed from two bundles of six TMHs and two separated NBFs [60]. The inward-facing structure has a large internal pocket open to both the cytosol and the inner leaflet and does not allow substrate access from the outer membrane leaflet or the extracellular space. Binding and/or hydrolysis of ATP have been thought to trigger a conformational switch opening the binding pocket to the outer leaflet and external aqueous environment and to reduce the affinity for the ligand [61]. After ATP hydrolysis and dissociation of the ligand, the TMHs reset to the inward-facing conformation.

On the basis of the information inferred from the crystal structures and experimental evidence, we propose the following model for the molecular mechanism of the ABCB4-mediated flopping and efflux (Figure 6). (I) As a first step, a substrate, mainly PC, enters the binding pocket of ABCB4 from the inner leaflet of nonraft membrane through the gaps between TMHs. Following the conformational change induced by ATP binding/hydrolysis, (II-A) in the absence of bile salts, a phospholipid molecule laterally diffuses from the binding pocket of ABCB4 to the outer leaflet of the membrane through the gaps between TMHs; (II-B) in the presence of bile salts, a phospholipid molecule is picked up by bile salt monomers, and then a mixed bile salt/phospholipid micelle is formed in the extracellular space; and (II-C) a nonphospholipid substrate with sufficient aqueous solubility

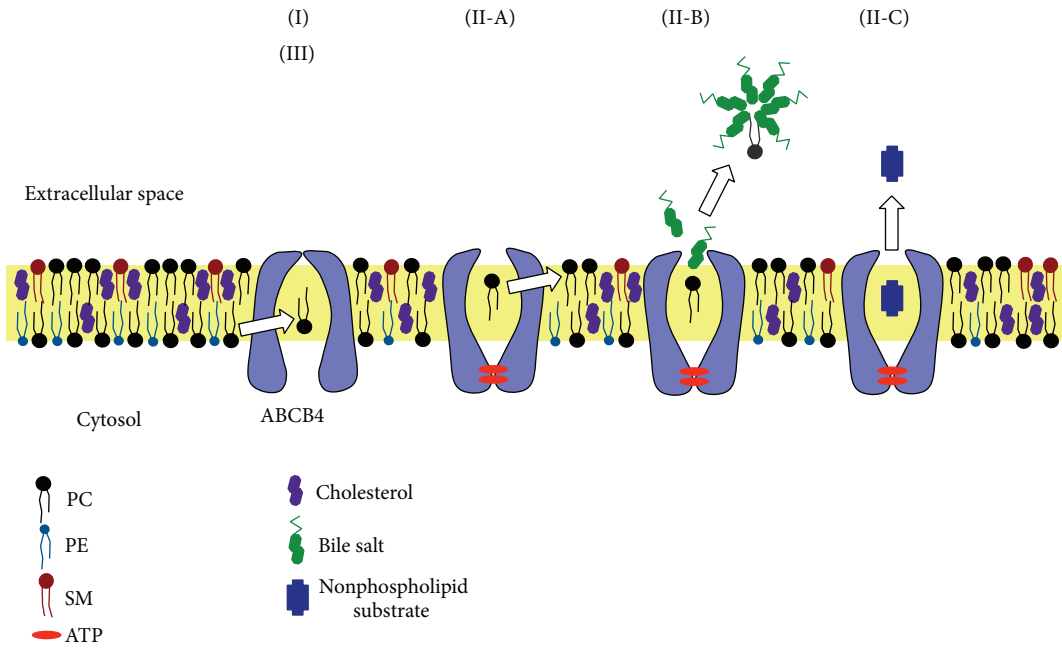

Figure 6: Model of molecular mechanism of ABCB4-mediated transport. (I) A substrate, mainly PC, enters the binding pocket of ABCB4 from the inner leaflet through the gaps between TMHs. (II) Binding and/or hydrolysis of ATP trigger a conformational change opening the binding pocket to the outer leaflet and the extracellular space. (II-A) In the absence of bile salts, a PC molecule laterally diffuses from the binding pocket of ABCB4 to the outer leaflet through the gaps between TMHs, which represents a floppase function of ABCB4. (II-B) In the presence of bile salts, a PC molecule is taken up from the binding pocket of ABCB4 by bile salt monomers, which represents an exporter function of ABCB4, and then a mixed bile salt/PC micelle is formed in the extracellular space. (II-C) A substrate with sufficient aqueous solubility directly diffuses from the binding pocket into the extracellular space regardless of the presence or absence of bile salts. (III) After dissociation of the substrate and ADP molecules, ABCB4 reset to the inward-facing state.

diffuses directly into the extracellular space regardless of the presence or absence of bile salts. Thus, we speculate that ABCB4 has a dual role as a floppase or as an exporter, depending on the presence of bile salts and on the aqueous solubility of the substrate.

ABCB4 has been predicted to be a floppase that translocates phospholipids from the inner leaflet to the outer leaflet of the canalicular membrane [32, 34, 62–65] or to be a transporter that moves phospholipids to be directly extracted by bile salts [33, 38]. However, the floppase activity of ABCB4 for long-chain PC has not been directly observed, although the ABCB4-mediated translocation of fluorescent-labeled short-chain PC analog (C_6-NBD-PC) in yeast secretory vesicles [32, 33] and the release of C_6-NBD-PC from ABCB4-expressing epithelial cells into the apical albumin-containing medium [34, 35] have been demonstrated previously. Meanwhile, Crawford et al. have observed abundant unilamellar vesicles in the bile canaliculi of *Abcb4* (+/+) mice but not *Abcb4* (−/−) mice and proposed that the biliary phospholipids are secreted as vesicles, which are formed by the ABCB4-mediated translocation of PC to the outer leaflet of the canalicular membrane and subsequently by the destabilization of the membrane by bile salts [63]. In contrast, Oude Elferink and Paulusuma have suggested that the biliary PC excretion takes place by the translocation followed by exposition of the PC molecule by ABCB4, which subsequently allows the extraction by bile salt micelles [4].

We consider that taurocholate monomers can access to the substrate-binding pocket of ABCB4 owing to their small size and take up phospholipid molecules directly from the binding pocket. The association of taurocholate monomers with a phospholipid molecule should reduce the activation energy required to move a phospholipid from the binding pocket of ABCB4 to the aqueous environment. It is also possible that taurocholate monomers directly interact with the amino acids lining on the binding pocket of ABCB4 and help the release of a phospholipid molecule because the hydrophilic bile salt, taurocholate, can induce the ABCB4-mediated secretion of phospholipids more efficiently than the hydrophobic bile salt, cholate.

10. Future Directions

In this review, we summarize current knowledge of the molecular properties of ABCB4 and its physiological relevance and discuss possible mechanism for the phospholipid efflux mediated by ABCB4. Clearly, further studies are required to elucidate the molecular mechanism. At present, it is largely unknown how taurocholate molecules support the phospholipid secretion mediated by ABCB4 or why ABCB4 is highly specific for PC, unlike ABCB1. Nonetheless, the lipid transport mechanism of ABCB4 may be less complex than the mechanisms of other ABC lipid transporters, such as ABCA1, ABCA3, ABCA12, ABCG1, and ABCG5/ABCG8.

Accordingly, investigations into the molecular function of ABCB4 will also help to clarify the mechanisms of other ABC lipid transporters.

Abbreviations

ABC: ATP-binding cassette
DIC: Drug-induced cholestasis
ICP: Intrahepatic cholestasis of pregnancy
LPAC: Low-phospholipid-associated cholelithiasis
MDR3: Multidrug resistance 3
NBF: Nucleotide-binding fold
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PFIC3: Progressive familial intrahepatic cholestasis type 3
RACK1: Receptor for activated C-kinase 1
SM: Sphingomyelin
TMH: Transmembrane helix.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Butylidenephthalide Blocks Potassium Channels and Enhances Basal Tension in Isolated Guinea-Pig Trachea

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Butylidenephthalide (Bdph, 30~300 μM), a constituent of *Ligusticum chuanxiong* Hort., significantly enhanced tension in isolated guinea-pig trachea. In this study, we investigate the mechanism(s) of Bdph-induced contraction in the tissue. Isolated trachea was bathed in 5 mL of Krebs solution containing indomethacin (3 μM), and its tension changes were isometrically recorded. Cromakalim (3 μM), an ATP-dependent K^+ channel opener, significantly antagonized the Bdph-induced enhancement of baseline tension. Bdph (300 μM) also significantly antagonized cromakalim-induced relaxation. Bdph (300 μM) did not significantly influence the antagonistic effects of glibenclamide (GBC, 1 μM) and tetraethylammonium (TEA, 8 mM) against the cromakalim-induced relaxation. However, Bdph (300 μM) and 4-aminopiridine (4-AP, 5 mM), a blocker of K_v1 family of K^+ channels, in combination significantly rightward shifted the log concentration-relaxation curve of cromakalim. The antagonistic effect of the combination almost equals the sum of the individual effects of Bdph and 4-AP, suggesting that the antagonistic mechanism of Bdph may be similar to that of 4-AP. All calcium channel blockers influenced neither the baseline tension nor antagonistic effect of Bdph against cromakalim. In conclusion, Bdph may be similar to 4-AP, a blocker of K_v1 family of K^+ channels, to enhance the baseline tension of guinea-pig trachea.

1. Introduction

The rhizomes of *Ligusticum chuanxiong* Hort. (previously named *L. wallichii* Franch.) and *Angelica sinensis* Diels. (Apiaceae) have been used by the Chinese for several thousand years. In ancient medical literature, such as Shen-Nung-Pen-Tsao-Ching, the rhizome of *L. chuanxiong* Hort. was delineated to prevent and restore stroke-induced dyskinesia. We reported that butylidenephthalide (Bdph), a neutral oil constituent of the rhizome, inhibited cyclooxygenase to have antiplatelet effects [1]. Other investigators reported that shimotsu-to, a prescription of traditional Chinese medicine (TCM), had antiproliferative effects in primary cultures of mouse aorta smooth muscle cells [2], mainly due to *Cnidium* rhizome-derived phthalides, such as senkyunolide, ligustilide, and Bdph [3]. Both antiplatelet and antiproliferative effects of these crude drugs benefit to prevent stroke. To recover from stroke-induced dyskinesia the damaged nerve cells need to be repaired mainly by themselves. The vasodilating effects of Bdph [4–6] improve the circulation and may partially benefit this restoration. Recently, Bdph was reported to provide neuroprotection by reducing the release of various proinflammatory molecules from activated microglia [7]. It is also reported to maintain stem cell pluripotency by activating the Jak2/Stat3 pathway and increasing the efficiency of induced pluripotent stem cells generation [8]. These results highlight the ability for these crude drugs to aid in the recovery from dyskinesia. Interestingly, Bdph was also reported to inhibit growth of malignant brain tumor [9], lung adenocarcinoma [10], and glioblastomas [11] with a high therapeutic ratio [12].

Bdph (50~250 μM) was reported to noncompetitively inhibit ACh-, KCl-, and $BaCl_2$-induced contractions in guinea-pig ileum [13]. Bdph (30~300 μM) was also reported

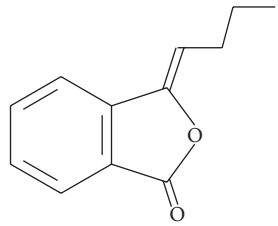


Figure 1: The chemical structure of butylidenephthalide (Bdph, mol. wt. 188.23).

to noncompetitively inhibit phenylephrine- and KCl-induced contractions in rat aortic rings [6]. However, in preliminary test, we found that Bdph (30~300 μM) failed to, except at very high concentrations (600~1000 μM), inhibit histamine (10 μM)-induced contraction in isolated guinea-pig trachea. More surprisingly, we found that Bdph (30~300 μM) alone can significantly induce enhancement of baseline tension in the tissue. Therefore we are interested in investigating the mechanism(s) of Bdph-induced contraction in the tissue.

2. Methods and Materials

2.1. Drugs and Animals. Bdph was synthesized and yielded a light yellow oily substance according to the previously described method [14]. Its purity (99.8%) was analyzed by using high performance liquid chromatography and the structure is shown in Figure 1 [15]. 4-Aminopiridine (4-AP), atropine sulfate, α-chymotrypsin, diltiazem, glibenclamide (GBC), histamine diphosphate, indomethacin, nicardipine, nifedipine (Nif), pyrilamine maleate, tetraethylammonium bromide (TEA), and verapamil (Vrp) were purchased from Sigma-Aldrich, St. Louis, MO. U.S.A. Papaverine was purchased from Narcotics Bureau, Taipei, Taiwan. Cromakalim, methysergide, and FPL 557121 were gifts from SmithKline Beecham Pharmaceutical, U.K., Sandoz, Swiss, and Fisons, U.K., respectively.

Male Hartley guinea-pigs (250~400 g) were obtained from the Animal Center of the National Science Council (Taipei, Taiwan). The animals were housed in ordinary cages at 22 ± 1°C with a humidity of 50%~60% under a constant 12/12-h light/dark cycle and provided with food and water *ad libitum*. Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, the following *in vitro* experiments were performed.

2.2. Tracheal Preparation. The guinea-pigs were sacrificed by cervical dislocation after anesthesia, and their tracheas were removed. Each trachea was cut into six segments. Each segment consisted of three cartilage rings. All segments were cut open opposite the trachealis. The segments were randomized to minimize regional variability. Each segment was tied at one end to a holder via silk sutures, placed in 5 mL of Krebs solution containing indomethacin (3 μM) throughout the entire experiment, bubbled with a 95% O_2 and 5% CO_2 mixture at 37°C, and attached by the other end to a force displacement transducer (Grass FT03) for the isometric recording of tension changes on a polygraph (Gould RS3200). The composition of the Krebs solution was (mM): NaCl 120, KCl 4.7, $MgSO_4$ 0.5, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and dextrose 11.0. The tissues were suspended under an initial tension of 1.5 g and allowed to equilibrate for 1 h with washing at 15-min intervals. After equilibration, the following experiments were performed.

2.3. Bdph Enhanced Baseline Tension but Relaxed Precontraction at High Concentrations. Bdph (30~1000 μM) or its vehicle (0.03~1% ethyl alcohol) was cumulatively added to examine the tension change of baseline. After the histamine (10 μM)-induced precontraction reached steady state, Bdph (30~1000 μM) or its vehicle was cumulatively added to the organ bath. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of Bdph for both baseline tension and relaxation were constructed.

2.4. Cromakalim Antagonized Bdph-Induced Enhancement of Baseline Tension. In order to examine the possible transmitter(s) or mediators which may enhance the tracheal baseline tension, some pharmacological agents, such as 1 μM atropine (a cholinergic antagonist), 1 μM FPL 55712 (a leukotriene receptor antagonist) [16], 1~10 μM pyrilamine (a histamine receptor antagonist), 1 μM methysergide (a serotonin receptor antagonist), and 2 u/mL α-chymotrypsin (a neuropeptidase), were added 30 min prior to the cumulative addition of Bdph (30~300 μM). However, 1~3 μM cromakalim (an ATP-sensitive K^+ channel opener) was pretreated for only 10 min which was enough to reach equilibration [17].

2.5. Bdph Also Antagonized Cromakalim-Induced Relaxation. After preincubation of Bdph (30~300 μM) or its vehicle for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction and then cumulatively added cromakalim (0.1~10 μM). At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of cromakalim in the absence and presence of Bdph were constructed.

2.6. Interaction between Bdph and Other K^+ Channel Blockers to Antagonize Cromakalim-Induced Relaxation. After preincubation of Bdph (100 or 300 μM) and other K^+ channel blockers, such as GBC (1 μM), TEA (8 mM), and 4-AP (5 mM) alone or combination for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction, and then cromakalim (0.1~10 μM) was cumulatively added. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of cromakalim in the absence and presence of drug(s), such as Bdph and other K^+ channel blockers, alone or combination, were constructed.

2.7. Interaction between Bdph and Other Ca^{2+} Channel Blockers to Antagonize Cromakalim-Induced Relaxation. First,

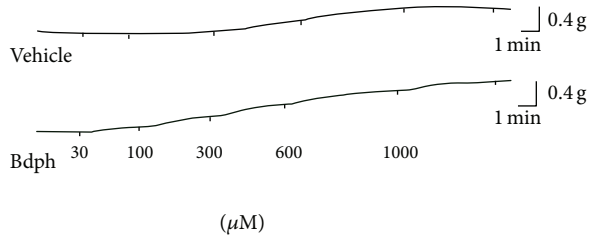


Figure 2: Typical tracing of baseline tension change induced by cumulative butylidenephthalide (Bdph, lower panel) compared to its vehicle (0.03~1% ethyl alcohol, upper panel) in isolated guinea-pig trachea. The vertical line indicates tension change.

Vrp, Nif, diltiazem, nicardipine, or their vehicles were cumulatively added to examine the tension change of baseline in the isolated trachea. Second, after preincubation of Bdph (300 μM) or its vehicle for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction, and then Vrp (0.01~10 μM) or Nif (0.001~1 μM) was cumulatively added. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of Vrp and Nif in the absence and presence of Bdph were constructed. Third, after preincubation of Bdph (300 μM), Vrp (1 μM), and Nif (0.1 μM) alone or combination for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction, and then cromakalim (0.1~10 μM) was cumulatively added. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of cromakalim in the absence and presence of drugs, such as Bdph, Vrp, and Nif, alone or combination were constructed.

2.8. Statistical Analysis. The tracheal contraction was expressed as percentage of maximal contraction (100%), with some exceptions expressed as tension. However, the tracheal relaxation was expressed as percentage of maximal relaxation induced by papaverine (100%) at the end of experiment. All values are expressed as mean ± SEM, n is the number of experiment. Student's unpaired t-test was used for statistical analysis between test and control with P values < 0.05 being regarded as significant.

3. Results

3.1. Effects of Bdph on Baseline and Histamine-Induced Precontraction. The effect of Bdph (30~1000 μM), compared to its vehicle, on the baseline tension in isolated guinea-pig trachea is shown in Figures 2 and 3(b). Bdph (30~300 μM) did not significantly relax the histamine (10 μM)-induced precontraction, except at higher concentrations of 600~1000 μM in the tissue (Figure 3(a)). In contrast, Bdph (30~300 μM) significantly enhanced its baseline tension (Figure 3(b)).

3.2. Cromakalim Antagonized Bdph-Induced Enhancement of Baseline Tension. Atropine (1 μM), FPL 55712 (1 μM),

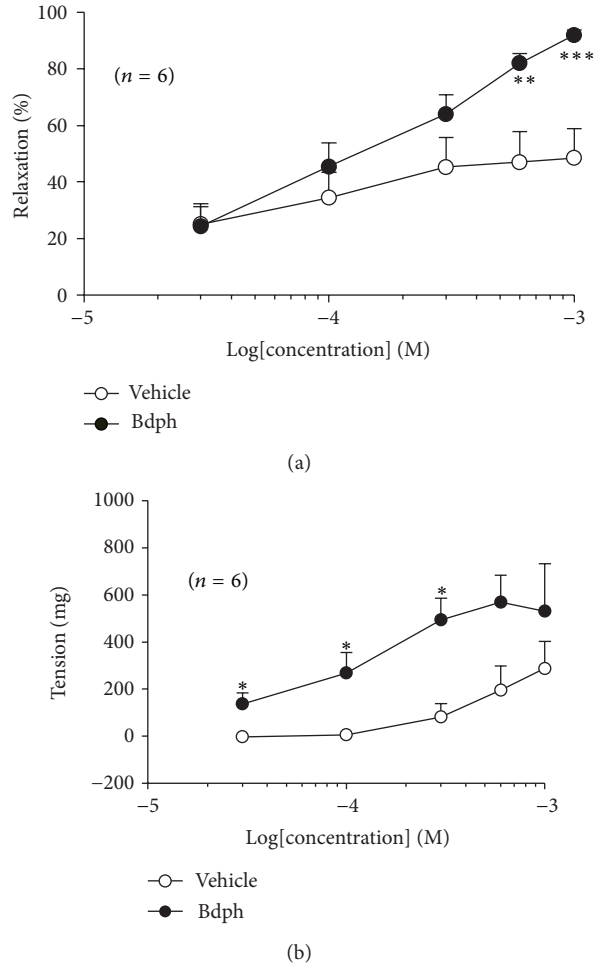


Figure 3: Log concentration relaxant (a) and contractile (b) response curves of butylidenephthalide (Bdph) in isolated guinea-pig trachea. The relaxant and contractile effects of cumulative Bdph (30~1000 μM) on histamine (10 μM)-induced precontraction and on baseline tension were performed as method described, respectively. All values are shown as mean ± SEM, and n is the number of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to its vehicle.

pyrilamine (1 and 10 μM), methysergide (1 μM), or α-chymotrypsin (2 u/mL) did not significantly influence the Bdph-induced enhancement of baseline tension (Figure 4). However, cromakalim (3 μM) significantly antagonized the Bdph-induced enhancement of baseline tension (Figure 5).

3.3. Bdph Also Antagonized Cromakalim-Induced Relaxation. Bdph (300 μM) significantly antagonized cromakalim-induced relaxation (Figure 6).

3.4. Interaction between Bdph and Other K^+ Channel Blockers to Antagonize Cromakalim-Induced Relaxation. Bdph at concentrations of 100 μM and 300 μM did not significantly influence the antagonistic effects of GBC (1 μM) against the cromakalim-induced relaxation (Figure 7). Bdph (300 μM) never influenced the antagonistic effects of TEA at 8 mM

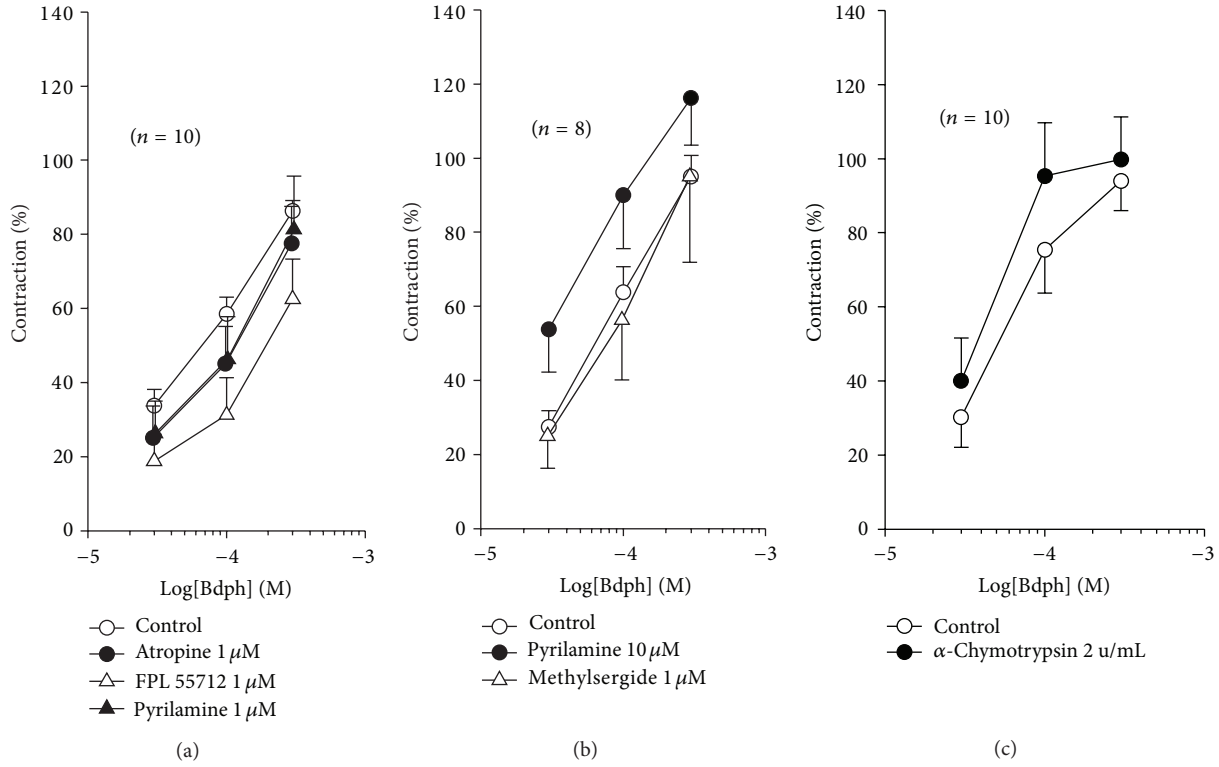


Figure 4: Inhibitory effects of atropine, FPL 55712, or pyrilamine 1 μM (a), as well as pyrilamine 10 μM or methysergide (b) and α-chymotrypsin (c) on cumulative butylidenephthalide- (Bdph-) induced contraction of baseline tension in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. There is no significant difference between test and respective control.

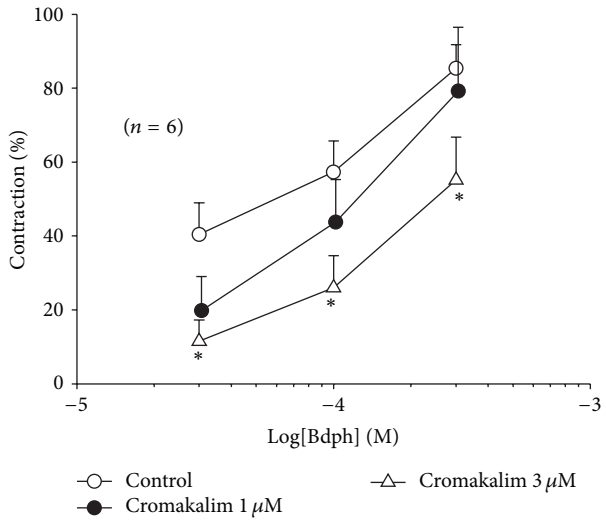


Figure 5: Inhibitory effect of cromakalim on cumulative butylidenephthalide (Bdph)-induced contraction of baseline tension in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. $^*P < 0.05$ compared to its vehicle.

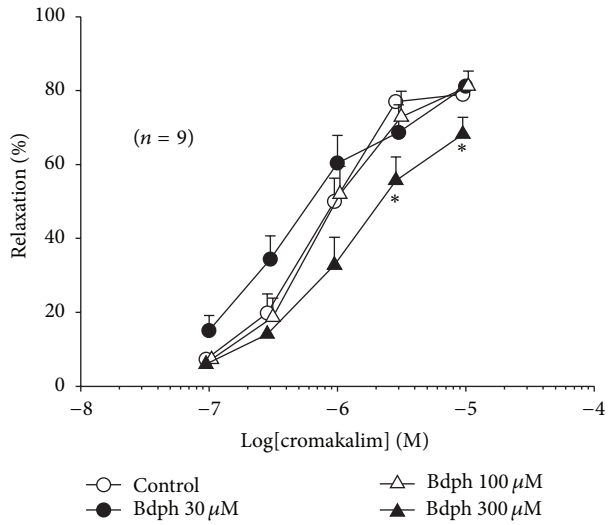
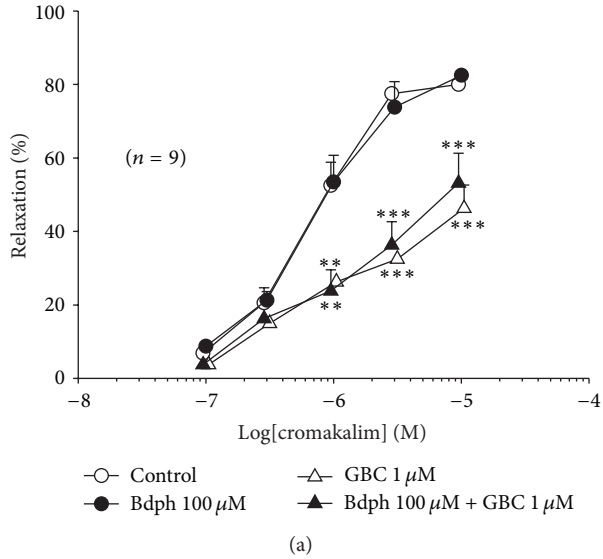
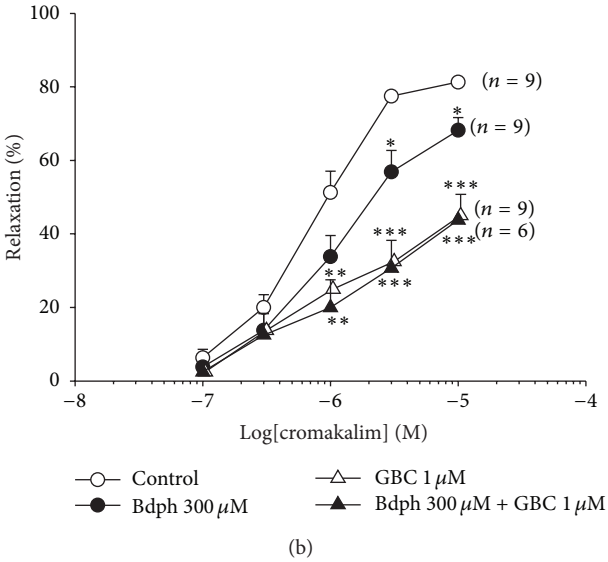


Figure 6: Inhibitory effect of butylidenephthalide (Bdph) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. $^*P < 0.05$ compared to its vehicle.

(Figure 8(a)). However, Bdph (300 μM) and 4-AP (5 mM) in combination significantly antagonized the cromakalim-induced relaxation, compared to the individual effects on the relaxation, and rightward shifted the log concentration-response curve of cromakalim. The antagonistic effect of the

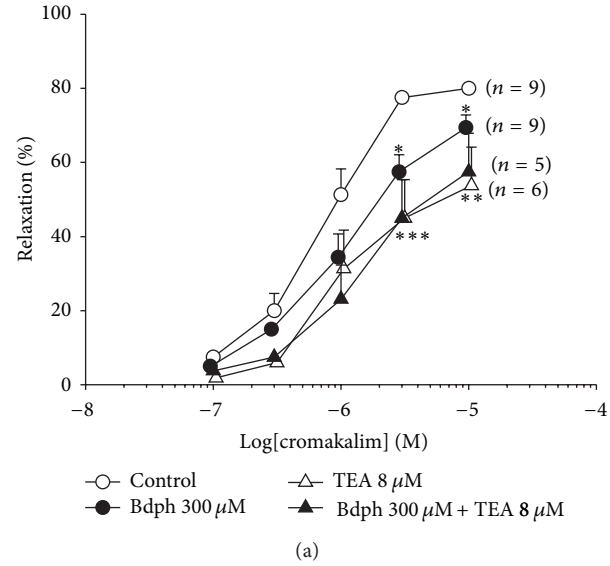


(a)

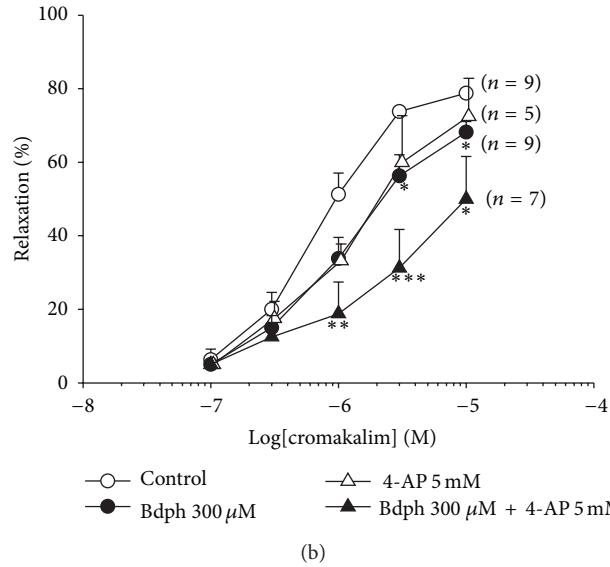


(b)

Figure 7: Influences of glibenclamide (GBC) to the antagonistic effects of butylidenephthalide (Bdph) 100 μM (a) and 300 μM (b) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to its vehicle.



(a)



(b)

Figure 8: Influences of tetraethylamine (TEA, (a)) and 4-aminopyridine (4-AP, (b)) to the antagonistic effect of butylidenephthalide (Bdph) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to its vehicle.

combination is almost equal to the sum of individual effects (Figure 8(b)).

3.5. Interaction between Bdph and Other Ca^{2+} Channel Blockers to Antagonize Cromakalim-Induced Relaxation. All Ca^{2+} channel blockers used did not enhance or reduce the baseline tension of the isolated guinea-pig trachea (data not shown). Bdph did not influence the relaxant effects of Vrp (Figure 9(a)) and Nif (Figure 9(b)) on the histamine-induced precontraction. Vrp (1 μM) and Nif (0.1 μM) also did not influence the antagonistic effect of Bdph (300 μM) against the cromakalim-induced relaxation (Figure 10).

4. Discussion

The present results suggest that the enhancement of basal tension by Bdph is unrelated to the release of cholinergic transmitter, leukotrienes, histamine, serotonin, and neuropeptides [18]. It is also unrelated to the release of prostaglandins, as the experiment was conducted throughout in the presence of indomethacin. However, the enhancement was antagonized by cromakalim (3 μM), an ATP-sensitive K^{+} channel opener [17], which may increase outflux of K^{+} and hyperpolarize the membrane of tracheal smooth muscle cells and cause relaxation. Furthermore, Bdph (300 μM) also antagonized

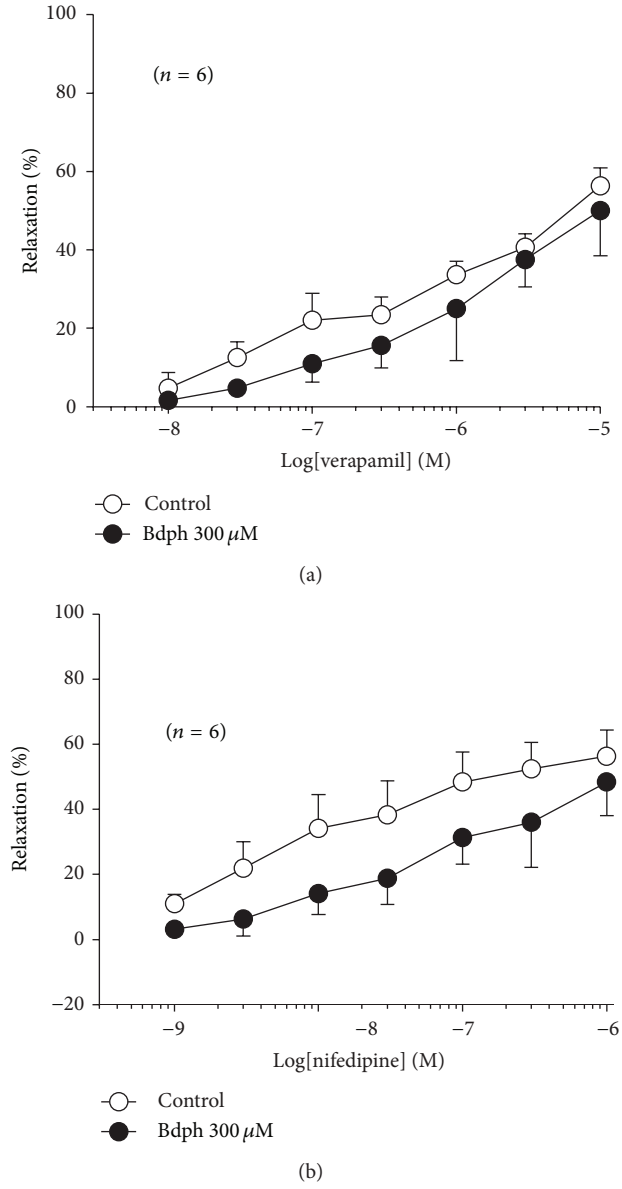


Figure 9: Antagonistic effects of butylidenephthalide (Bdph) on cumulative verapamil-induced (a) and nifedipine-induced (b) relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. There is no significant difference between test and respective control.

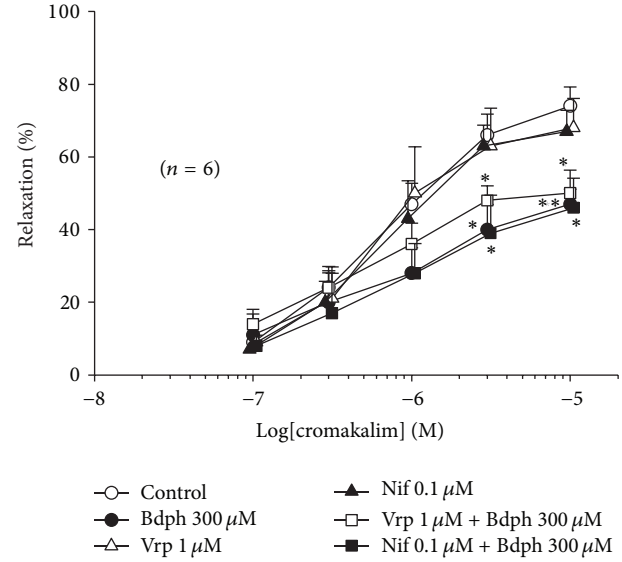


Figure 10: Influences of verapamil (Vrp) and nifedipine (Nif) to the antagonistic effect of butylidenephthalide (Bdph) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean ± SEM, and n is the number of experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to its vehicle.

and rightward shifted the log concentration-relaxation curve of cromakalim on histamine-induced precontraction in the isolated guinea-pig trachea (Figure 6). Thus, Bdph may be a kind of K^+ channels blockers, which have been reviewed to have a potential clinical use for Alzheimer disease [19]. Indeed, Bdph have been reported to reverse the deficits of inhibitory avoidance performance and improve memory in rats [20]. GBC (1 μM), a specific ATP-sensitive K^+ channel blocker [17], effectively antagonized and rightward shifted the curve of cromakalim. Bdph neither at 100 μM nor at 300 μM influenced the antagonistic effect of GBC. Also, Bdph at 300 μM did not affect the antagonistic effect of TEA (8 mM), a nonselective big (BKca) and intermediate (IKca) conductance Ca^{2+}-activated K^+ channels blocker [21]. However, Bdph at 300 μM significantly enhanced the antagonistic effect of 4-AP (5 mM) and rightward shifted the curve in the combination. The antagonistic effect of the combination was almost the sum of individual effects (Figure 8(b)). This result strongly suggests that the mechanism of Bdph may be similar to that of 4-AP to antagonize cromakalim. The mechanism of Bdph was unrelated to Ca^{2+}-dependent K^+ channels, as all Ca^{2+} channel blockers did not influence the antagonistic effect of Bdph against cromakalim.

Episodic ataxia type 2 (EA2) is a form of hereditary neurological disorder caused by cerebellar malfunction and is characterized by interictal ataxia and frequent attacks of dyskinesia, vertigo, and imbalance [22]. Recently, 4-AP was reported to treat EA2 [23, 24]. The targets of 4-AP are K_v1 family of K^+ channels, possibly the $K_v1.5$ subtype [25]. Further investigation is needed to determine whether Bdph is useful in treating EA2.

In conclusion, Bdph (30~300 μM) concentration-dependently evoked an enhancement of baseline tension in isolated guinea-pig trachea. The enhancement was antagonized by cromakalim, and Bdph (300 μM) also antagonized cromakalim-induced relaxation. Furthermore, Bdph (300 μM) and 4-AP (5 mM) in combination rightward shifted the log concentration-response curve of cromakalim and significantly antagonized the cromakalim-induced relaxation. The antagonistic effect of the combination is almost equal to the sum of individual effects. Therefore, Bdph may be similar to 4-AP, a blocker of K_v1 family of

K^+ channels, to enhance the baseline tension of guinea-pig trachea.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

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Research Article

The K–Cl Cotransporter KCC3 as an Independent Prognostic Factor in Human Esophageal Squamous Cell Carcinoma

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The objectives of the present study were to investigate the role of K–Cl cotransporter 3 (KCC3) in the regulation of cellular invasion and the clinicopathological significance of its expression in esophageal squamous cell carcinoma (ESCC). Immunohistochemical analysis performed on 70 primary tumor samples obtained from ESCC patients showed that KCC3 was primarily found in the cytoplasm of carcinoma cells. Although the expression of KCC3 in the main tumor (MT) was related to several clinicopathological features, such as the pT and pN categories, it had no prognostic impact. KCC3 expression scores were compared between the MT and cancer nest (CN), and the survival rate of patients with a CN > MT score was lower than that of patients with a CN ≤ MT score. In addition, the survival rate of patients in whom KCC3 was expressed in the invasive front of tumor was lower than that of the patients without it. Furthermore, multivariate analysis demonstrated that the expression of KCC3 in the invasive front was one of the most important independent prognostic factors. The depletion of KCC3 using siRNAs inhibited cell migration and invasion in human ESCC cell lines. These results suggest that the expression of KCC3 in ESCC may affect cellular invasion and be related to a worse prognosis in patients with ESCC.

1. Introduction

The K–Cl cotransporter (KCC) mediates the coupled movement of K^+ and Cl^- ions across the plasma membrane and is involved in the regulation of cell volume, transepithelial ion transport, and maintenance of intracellular Cl^- concentrations ($[Cl^-]_i$) [1, 2]. Four isoforms of the KCC have been identified and are termed KCC1, KCC2, KCC3, and KCC4 [3]. The four KCC isoforms share a common protein structure with 12 transmembrane regions in a central hydrophobic domain, together with hydrophilic N- and C-termini that may be cytoplasmic [4]. Although the expression of KCC1 is reportedly ubiquitous [5], that of KCC2 is restricted to neurons in the central nervous system [6]. KCC3 is expressed in the muscle, brain, lung, heart, and kidney [7], and KCC4 transcripts are the most abundant in the heart and kidney [4].

Several recent studies described the important roles of KCC in cancer development, tumor invasion, and possibly metastasis [8–11]. KCC3 was found to be important for cell-cycle progression, migration, and invasion in cervical carcinoma, ovarian cancer, breast cancer, and glioma [8, 9, 12, 13]. In addition, the overexpression of KCC3 downregulated the formation of the E-cadherin/β-catenin complex, and the subsequent upregulation of KCl cotransport activity was

shown to benefit cancer cells in the epithelial-mesenchymal transition (EMT) [8]. However, the roles of KCC3 in the invasion of esophageal squamous cell carcinoma (ESCC) cells remain uncertain. Furthermore, the clinicopathological meaning of the expression of KCC3 in human ESCCs has not yet been evaluated.

The objectives of the present study were to investigate the roles of KCC3 in the cell migration and invasion of ESCC. Furthermore, we analyzed the expression of KCC3 in human ESCC samples and determined its relationships with the clinicopathological features and prognosis of ESCC patients. Our results revealed the important role of KCC3 in the tumor progression of ESCC.

2. Materials and Methods

2.1. Patients and Primary Tissue Samples. ESCC tumor samples were obtained from 70 patients with histologically proven primary ESCC who underwent esophagectomy (potentially curative R0 resection) at Kyoto Prefectural University of Medicine (Kyoto, Japan) between 1998 and 2007 and were analyzed retrospectively. These samples were embedded in paraffin 24 h after being fixed in formalin. Patient eligibility criteria included not having developed synchronous tumors or multiple metachronous tumors and not having received preoperative chemotherapy or radiation therapy. We excluded patients with noncuratively resected tumors or nonconsecutive data. All patients gave their written informed consent for inclusion in this study. Relevant clinicopathological and survival data were obtained from the hospital database. Staging was principally based on the seventh TNM staging system [14].

2.2. Immunohistochemistry. Paraffin sections (3 μm thick) of tumor tissue were subjected to immunohistochemical staining for KCC3 using the avidin-biotin-peroxidase method. Briefly, paraffin sections were dewaxed in xylene and hydrated through a graded series of alcohols. Antigen retrieval was performed by heating the samples in Dako REAL Target Retrieval Solution (Glostrup, Denmark) for 40 min at 95°C. Endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% H_2O_2. Sections were incubated for one hour at room temperature with the following antibody: the KCC3 antibody (HPA034563; Atlas Antibodies AB, Stockholm, Sweden). The avidin-biotin-peroxidase complex system (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) was used for color development with diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin. These sections were then dehydrated through a graded series of alcohols, cleared in xylene, and mounted. Control sections of known positive ESCC were included in each antibody run, and negative control sections were produced by omitting the primary antibody.

Immunohistochemical samples stained with KCC3 were graded semiquantitatively by considering both the staining intensity and percentage of positive tumor cells using an immunoreactive score (IRS) [15]. Staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The proportion of positive tumor cells was scored from 0 to 1.0. The score of each sample was calculated as the maximum multiplied product of the intensity and proportion scores (0 to 3.0).

2.3. Cell Culture. The human ESCC cell lines TE5 and TE9 were obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan) [16]. These cells were grown in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (FBS). Cells were cultured in flasks or dishes in a humidified incubator at 37°C under 5% CO_2 in air.

2.4. Small Interfering RNA (siRNA) Transfection. Cells were transfected with 10 nmol/L KCC3 siRNA (Stealth RNAi siRNA #HSS HSS115159, HSS190762, HSS190763; Invitrogen, Carlsbad, CA) using the Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions. The medium containing siRNA was replaced with fresh medium after 24 h. We used three independent KCC3 siRNAs to exclude off target effects. The control siRNA provided (Stealth RNAi siRNA Negative Control; Invitrogen) was used as a negative control.

2.5. Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). Messenger RNA (mRNA) expression was measured by quantitative real-time PCR (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA) with TaqMan Gene Expression Assays (Applied Biosystems), according to the manufacturer's instructions. Expression levels were measured for the following gene: KCC3 (Hs00994548_m1) (Applied Biosystems). Expression was normalized for the KCC3 gene to the housekeeping gene beta-actin (ACTB, Hs01060665_g1; Applied Biosystems). Assays were performed in duplicate.

2.6. Analysis of Cell Migration and Invasion. The migration assay was conducted using a Cell Culture Insert with a pore size of 8 μm (BD Biosciences, Bedford, MA). Biocoat Matrigel (BD Biosciences) was used to evaluate cell invasion potential. Briefly, cells (1.5×10^5 cells per well) were seeded in the upper chamber in serum-free medium 24 h after siRNA transfection. The lower chamber contained medium with 10% FBS. The chambers were incubated for 48 h at 37°C in 5% CO_2, and nonmigrated or noninvaded cells were then removed from the upper side of the membrane by scrubbing with cotton swabs. Migrated or invaded cells were fixed on the membrane and stained with Diff-Quick staining reagents (Sysmex, Kobe, Japan). The migrated or invaded cells on the lower side of the membrane were counted in four independent fields of view at 100x magnification of each insert. Each assay was performed in triplicate.

2.7. Statistical Analysis. Statistical analysis was carried out using Fisher's exact test to investigate correlations between clinicopathological parameters and KCC3 expression.

Survival curves were constructed using the Kaplan-Meier method, and differences in survival were examined using the log-rank test. Multivariate analysis of the factors influencing survival was performed using the Cox proportional hazard model. Multiple comparisons were carried out using Dunnett's test after one-way ANOVA. Differences were considered significant when the associated P value was less than 0.05. All analyses were performed using statistical software (JMP, version 10; SAS Institute Inc., Cary, NC, USA). Correlation analyses were performed by creating Fit Y by X plots using JMP.

3. Results

3.1. KCC3 Protein Expression in Human ESCCs. An immunohistochemical investigation with the KCC3 antibody revealed the expression of KCC3 in the parabasal cell layer of normal esophageal mucosa (Figure 1(a)). We examined the expression of KCC3 in 70 primary tumor samples of human ESCC based on their immunohistochemical reactivity. The KCC3 protein was mostly expressed in the cytoplasm of carcinoma cells (Figure 1(b)). The KCC3 score in the main tumor (MT) varied widely between the tumors. The minimum KCC3 score was 0 while the maximum KCC3 score was 2.4 in MT (median = 0.725; mean ± standard error of the mean (SEM) = 0.780 ± 0.072). Regarding the expression of KCC3 in MT, we divided ESCC patients into 2 groups using the median staining score: a low grade KCC3 expression group with staining scores ≤0.725, $n = 35$, and a high grade KCC3 expression group with staining scores >0.725, $n = 35$. Figures 1(c) and 1(d) show the representative histopathological findings of low or high KCC3 expression samples. Correlations between the expression of KCC3 in MT and various clinicopathological parameters were analyzed (Table 1). We found correlations between the expression of KCC3 in MT and location of the primary tumor, the pT or pN category (Table 1).

We then focused on the expression of KCC3 in the cancer nest (CN) of ESCC (Figure 1(e)) and analyzed the KCC3 score in CN. The minimum KCC3 score was 0, and the maximum KCC3 score was 2.6 in CN (median = 1.000; mean ± SEM = 1.087 ± 0.096). The KCC3 score in CN was positively correlated with the KCC3 score in MT ($R^2 = 0.3388$, $P < 0.0001$) (Figure 2). Regarding the expression of KCC3 in CN, we divided ESCC patients into 2 groups using the median staining score, a low grade KCC3 expression group with staining scores ≤1.000, $n = 36$, and a high grade KCC3 expression group with staining scores >1.000, $n = 34$, and compared their clinicopathological features (Table 1). A correlation was found between the expression of KCC3 in CN and location of the primary tumor (Table 2). Regarding the comparison of KCC3 scores between MT and CN in each sample, we divided ESCC patients into 2 groups, CN > MT, $n = 39$, and CN ≤ MT, $n = 31$, and compared their clinicopathological features (Table 1). A correlation was not found between the comparison of KCC3 scores and clinicopathological features (Table 1).

Furthermore, we analyzed the localization of KCC3 expression in tumors. In 48 cases, the expression of KCC3 was found in the invasive front of the tumor (Figure 1(f)). Regarding the expression of KCC3 in the invasive front of the tumor, we divided ESCC patients into 2 groups, negative ($n = 22$) and positive ($n = 48$), and compared their clinicopathological features (Table 2). A correlation was found between the expression of KCC3 in the invasive front and the MT score, CN score, or their comparison (CN/MT) (Table 2). No correlation was found between the expression of KCC3 in the invasive front and any other clinicopathological parameter (Table 2).

3.2. Prognostic Impact of KCC3 Protein Expression for Patients with ESCC. We determined the prognostic impact of the expression of KCC3 for patients with ESCC. Regarding the expression of KCC3 in MT, no significant difference was observed in the 5-year survival rate between patients with the high grade expression of KCC3 and those with the low grade expression of KCC3 in MT (Figure 3(a)). Similarly, no significant differences were observed in the 5-year survival rate between patients with the high grade expression of KCC3 and those with the low grade expression of KCC3 in CN (Figure 3(b)). Regarding comparisons of KCC3 score, the 5-year survival rate of the patients with CN > MT (55.5%) was lower than that of patients with CN ≤ MT (76.7%) ($P = 0.133$) (Figure 3(c)). The 5-year survival rate of the patients with KCC3 expression in the invasive front (57.1%) was lower than that of the patients without it (81.8%), although there was no statistical difference ($P = 0.089$) (Figure 3(d)). Interestingly, when patients were divided into 2 groups, CN > MT and invasive front positive, $n = 31$, and others, $n = 39$, the 5-year survival rate of patients with CN > MT and invasive front positive (46.1%) was significantly lower than that of other patients (79.0%) ($P = 0.022$) (Figure 3(e)).

Furthermore, we assessed which of the 13 variables studied (age, gender, location of the primary tumor, histological type, tumor size, lymphatic invasion, venous invasion, pT and pN category, KCC3 score in MT, KCC3 score in CN and CN/MT, and KCC3 expression in the invasive front) influenced survival following curative resection of esophageal cancer. Univariate analysis of survival after esophagectomy revealed that lymphatic invasion, venous invasion, and the pT and pN categories were found to be significant prognostic factors ($P = 0.017$, 0.017, 0.002, and 0.003, resp.) (Table 3). Multivariate analysis with variables whose P values were less than 0.100 in univariate analysis demonstrated that lymphatic invasion, pT and pN category, and KCC3 expression in invasive front were independent prognostic factors ($P = 0.044$, 0.015, 0.011, and 0.001, resp.) (Table 4). KCC3 expression in invasive front was the strongest prognostic factor of all clinicopathological features. These findings suggest that the expression of KCC3 might be a valuable prognostic factor for patients with ESCC.

3.3. KCC3 Controlled Cell Migration and Invasion in ESCC Cells. We conducted knockdown experiments with KCC3 siRNA in ESCC cells and analyzed the effects of KCC3 knockdown on cell migration and invasion. We used three independent KCC3 siRNAs to exclude off target effects. All three KCC3 siRNAs effectively reduced KCC3 mRNA levels

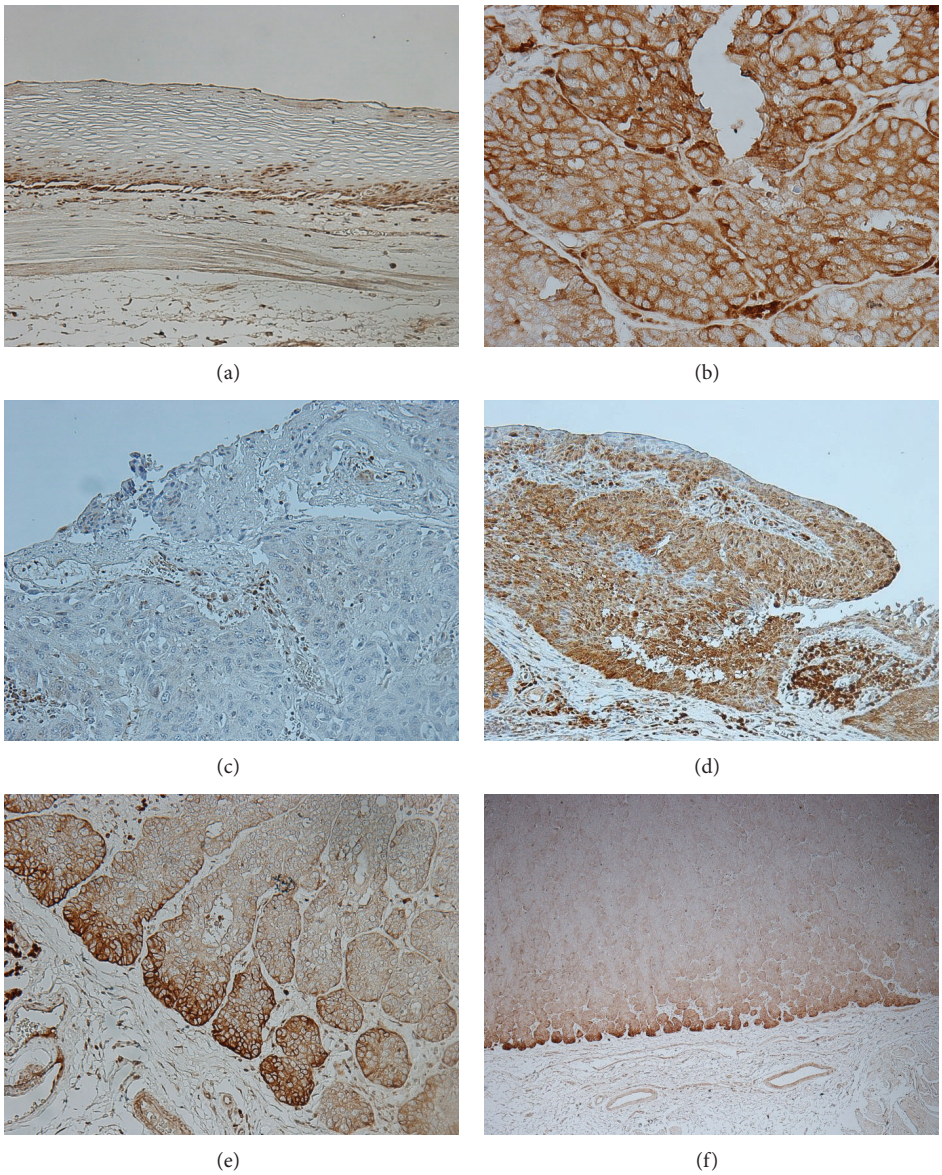


Figure 1: KCC3 protein expression in human esophageal squamous cell carcinoma (ESCC). (a) Immunohistochemical staining of noncancerous esophageal epithelia with the KCC3 antibody. Magnification: ×200. (b) Immunohistochemical staining of primary human ESCC samples with the KCC3 antibody. Magnification: ×400. (c) Immunohistochemical staining of primary human ESCC samples with the low grade expression of KCC3 in the main tumor (MT). Magnification: ×200. (d) Immunohistochemical staining of primary human ESCC samples with the high grade expression of KCC3 in the main tumor (MT). Magnification: ×200. (e) Immunohistochemical staining of primary human ESCC samples with the high grade expression of KCC3 in the cancer nest (CN). Magnification: ×200. (f) Immunohistochemical staining of primary human ESCC samples that expressed KCC3 in the invasive front of the tumor. Magnification: ×40.

in both TE5 and TE9 cells (Figure 4(a)). In TE5 cells, all three KCC3 siRNAs significantly inhibited cell migration and invasion (Figure 4(b)). In TE9 cell, downregulation of KCC3 inhibited cell migration and invasion, too (Figure 4(c)). These results suggest that KCC3 plays an important role in regulating cell migration and invasion in ESCC cells.

4. Discussion

Recent studies have shown that ion channels and transporters play crucial roles in cellular functions, and their roles have been studied in cancer cells [17, 18]. Various types of ion channels, such as voltage-gated K^+ channels, voltage-gated HERG channels, $Ca_2{}^+$ channels, and transient receptor potential channels, have been found to be expressed in gastrointestinal cancer cells and tissues and to regulate tumor behavior [19–22]. Regulators of intracellular pH such as anion exchanger, sodium-hydrogen exchanger, and carbonic anhydrases also related to tumor development of gastrointestinal cancer cells [23–25]. Furthermore, several reports have indicated that Cl^- channels/transporters, such as Cl^- channels, chloride intracellular channel (CLIC), KCC, and NKCC, play crucial

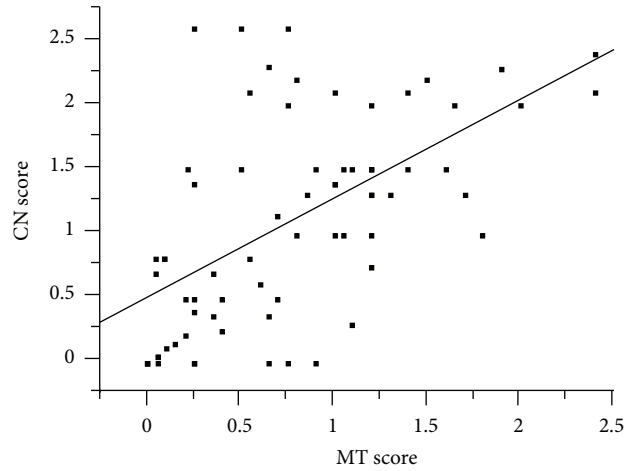


Figure 2: A correlation analysis of the relationship between the KCC3 score in main tumor (MT) and KCC3 score in the cancer nest (CN) was performed by producing Fit Y by X plots. The KCC3 score in CN was positively correlated with the KCC3 score in MT ($R^2 = 0.3388$, $P < 0.0001$).

Table 1: Relationships between the clinicopathological features of esophageal cancer and expression of KCC3 in the main tumor or cancer nest.

Variable	MT			CN			CN/MT		
	Low ($n = 35$)	High ($n = 35$)	P value	Low ($n = 36$)	High ($n = 34$)	P value	CN ≤ MT ($n = 31$)	CN > MT ($n = 39$)	P value
Age									
<60 years	11	11	1.000	11	11	1.000	6	16	0.071
≥60 years	24	24		25	23		25	23	
Gender									
Male	30	29	1.000	30	29	1.000	25	34	0.520
Female	5	6		6	5		6	5	
Location of the primary tumor									
Ut-Mt	18	29	0.0101*	19	28	0.0112*	21	26	1.000
Lt-Ae	17	6		17	6		10	13	
Histological type									
Well/moderately differentiated SCC	22	27	0.297	26	23	0.796	22	27	1.000
Poorly differentiated SCC	13	8		10	11		9	12	
Tumor size									
<50 mm	22	27	0.2968	24	25	0.6069	24	25	0.2967
≥50 mm	13	8		12	9		7	14	
Lymphatic invasion									
Negative	15	18	0.6324	20	13	0.1606	18	15	0.1482
Positive	20	17		16	21		13	24	
Venous invasion									
Negative	19	21	0.8094	20	20	0.8133	18	22	1.000
Positive	16	14		16	14		13	17	
pT									
pT1	10	23	0.0037*	14	19	0.2309	18	15	0.1482
pT2-3	25	12		22	15		13	24	
pN									
pN0	11	22	0.0160*	15	18	0.473	16	17	0.631
pN1-3	24	13		21	16		15	22	

MT: main tumor; CN: cancer nest; Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; SCC: squamous cell carcinoma; pT: pathological T stage; pN: pathological N stage.
* $P < 0.05$: Fisher's exact test.

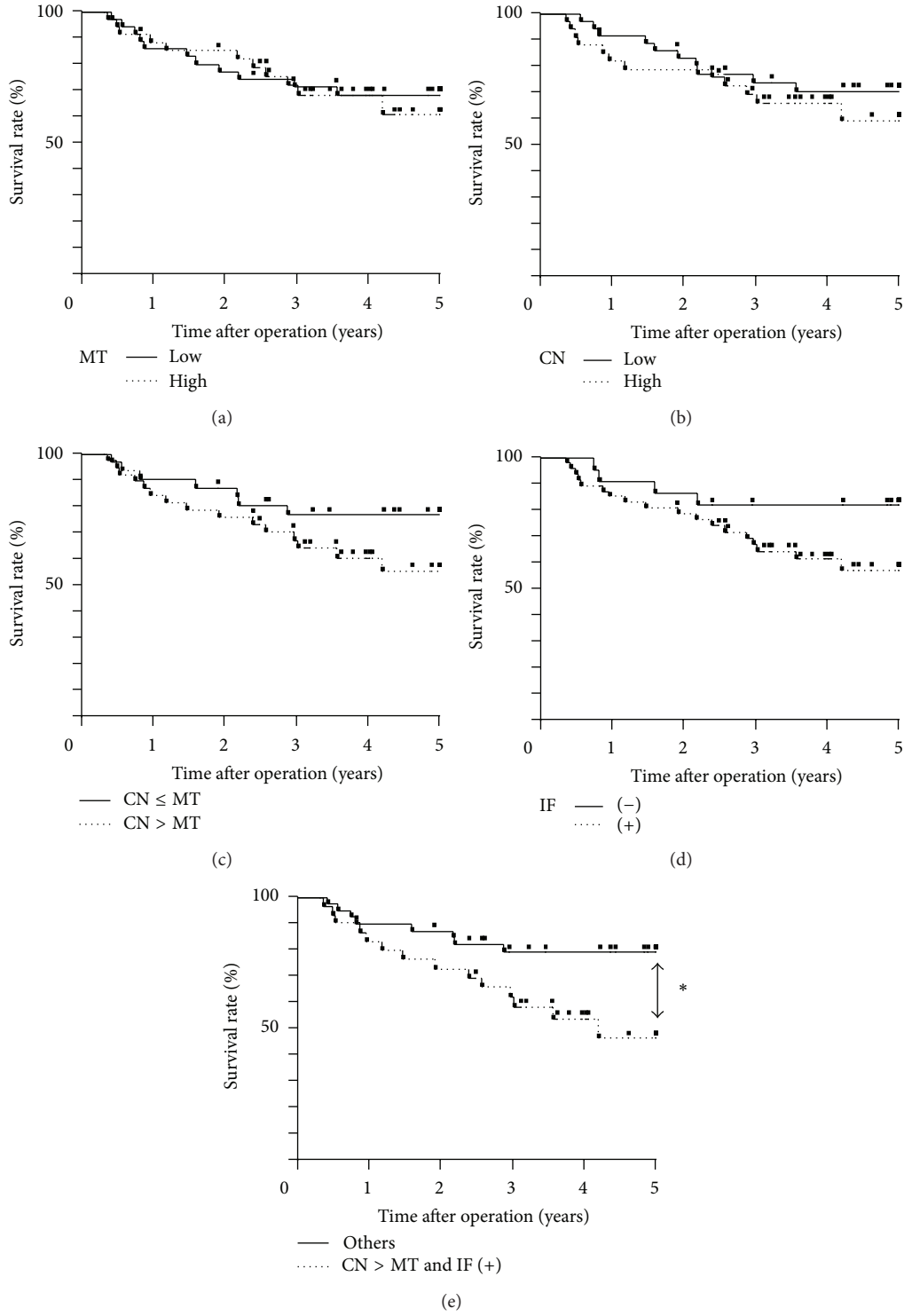
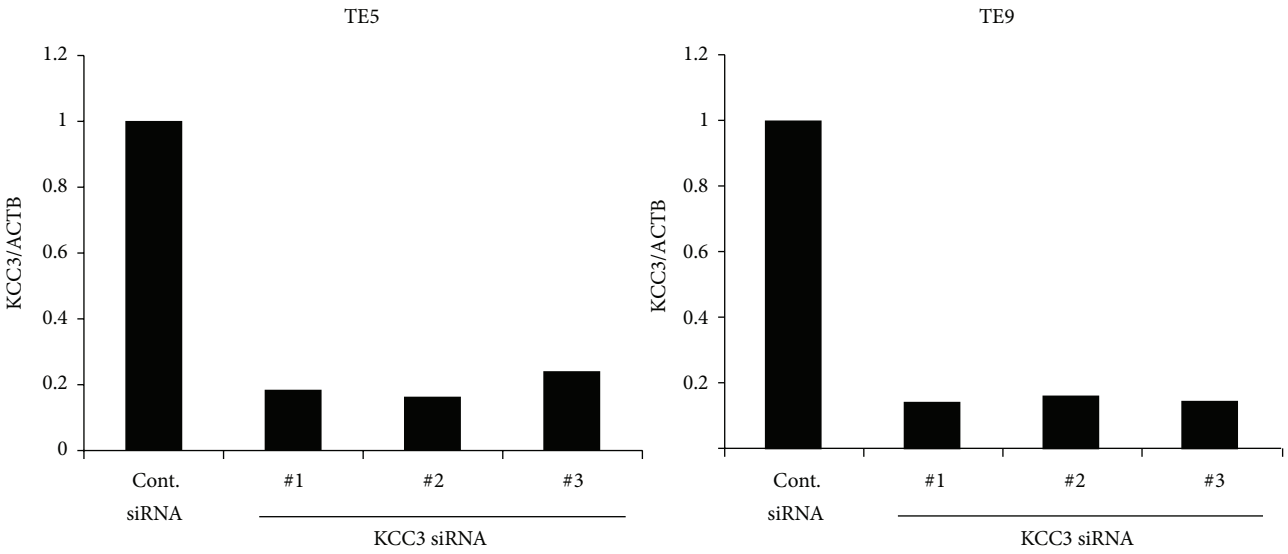
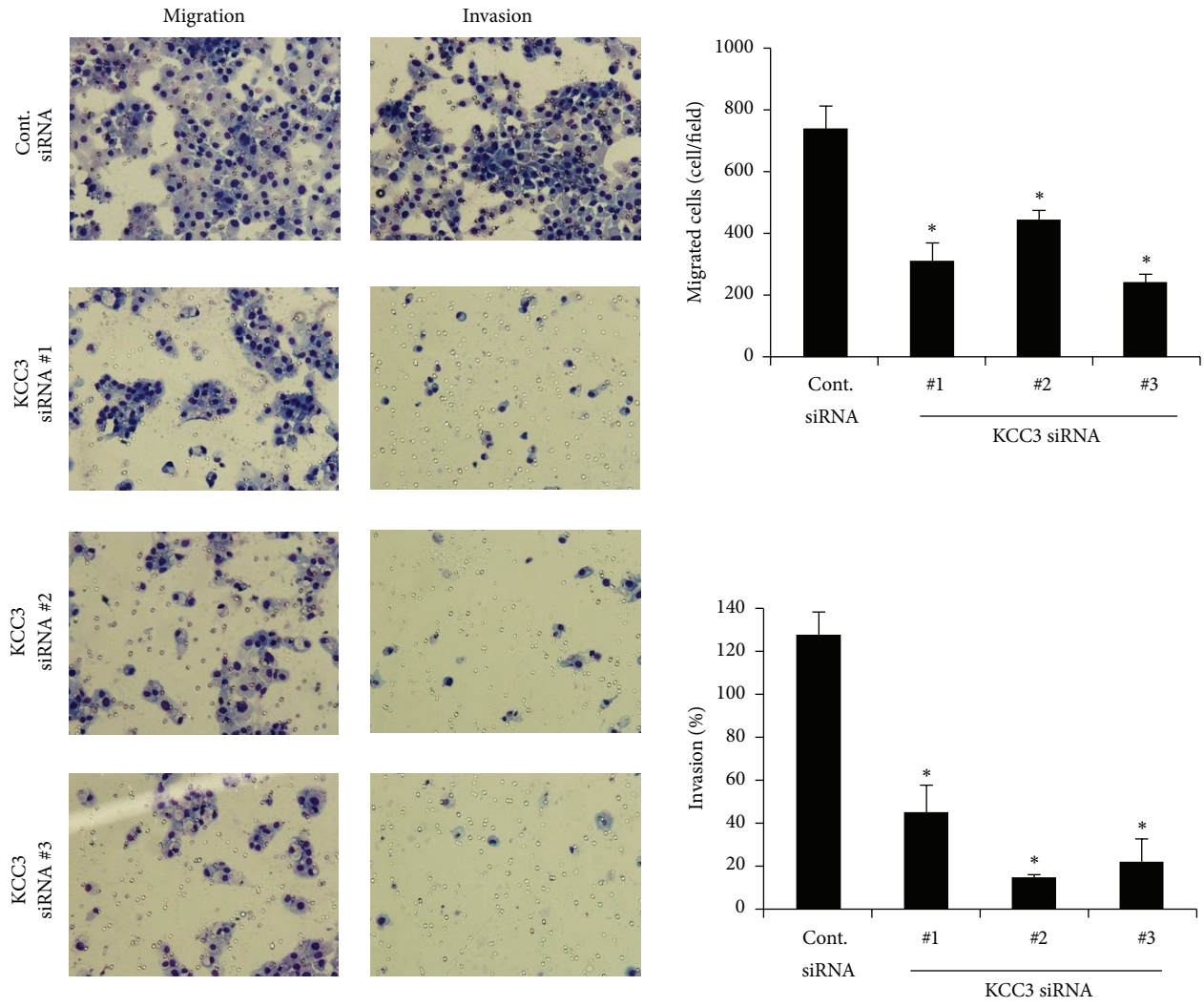


Figure 3: Survival curve of patients after curative resection for esophageal squamous cell carcinoma (ESCC) according to the expression of KCC3. (a) Patients were classified into two groups: low grade expression of KCC3 ($n = 35$) and high grade expression of KCC3 ($n = 35$) in the main tumor (MT). (b) Patients were classified into two groups: the low grade expression of KCC3 ($n = 36$) and high grade expression of KCC3 ($n = 34$) in the cancer nest (CN). (c) Patients were classified into two groups based on comparisons of the KCC3 score: CC ≤ MT ($n = 31$) and CN > MT ($n = 39$) in the cancer nest (CN). (d) Patients were classified into two groups based on the expression of KCC3 in the invasive front of the tumor: negative ($n = 22$) and positive ($n = 48$). (e) Patients were classified into two groups: patients with CN > MT and invasive front positive ($n = 31$) and others ($n = 39$). $^*P < 0.05$: log-rank test.



(a)



(b)

Figure 4: Continued.

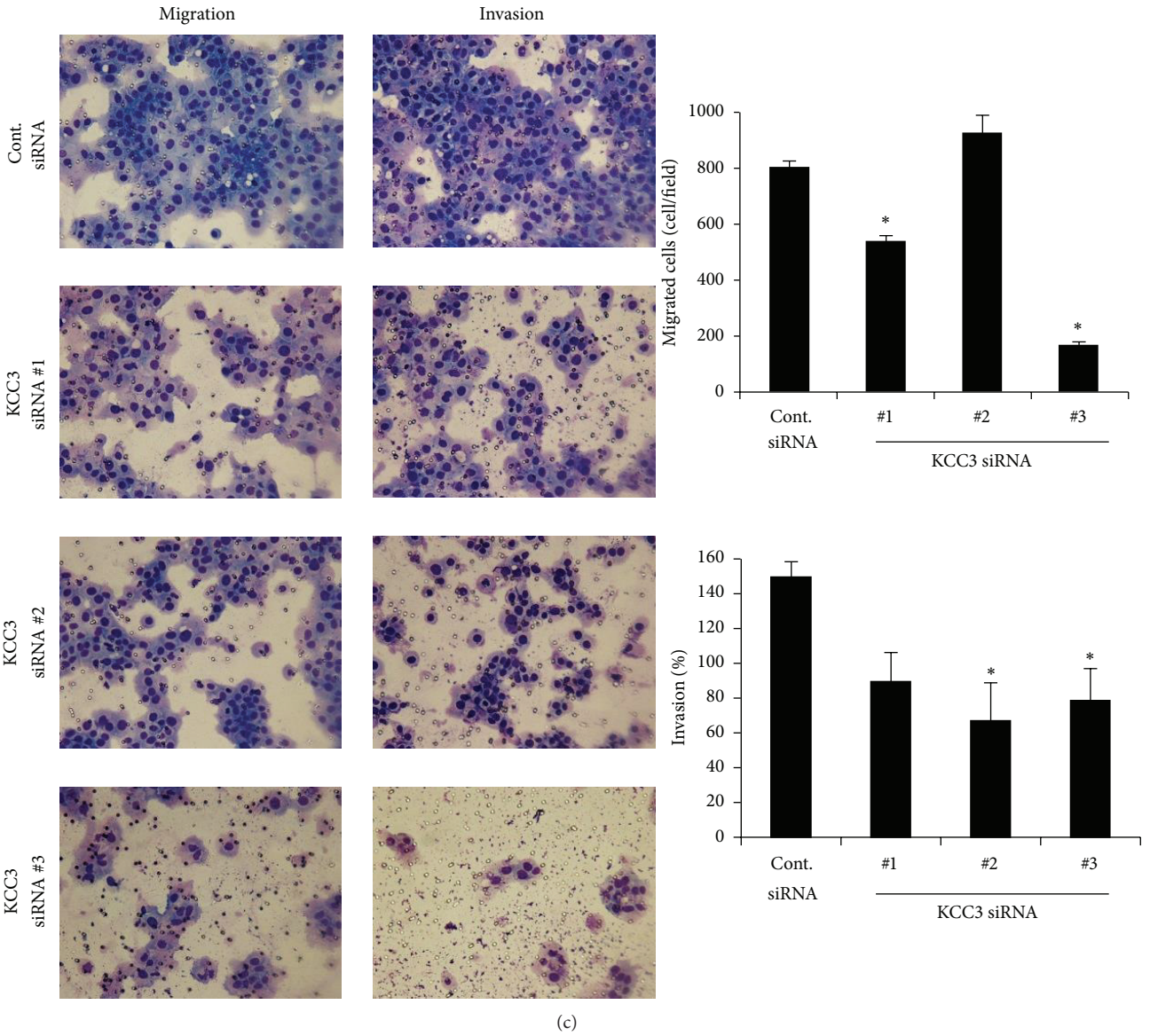


Figure 4: KCC3 controlled the cell migration and invasion of esophageal squamous cell carcinoma (ESCC) cells. (a) KCC3 siRNA effectively reduced KCC3 mRNA levels in both TE5 and TE9 cells. Three independent KCC3 siRNAs were investigated to exclude off target effects. (b) The downregulation of KCC3 significantly inhibited cell migration and invasion in TE5 cells. Cell migration and invasion were determined by the Boyden chamber assay. Mean ± SEM; $n = 3$. $^{*}P < 0.05$: Dunnett's test (ANOVA: migration; $P < 0.0001$, invasion; $P < 0.0001$). (c) The downregulation of KCC3 inhibited cell migration and invasion in TE9 cells. Cell migration and invasion were determined by the Boyden chamber assay. Mean ± SEM; $n = 3$. $^{*}P < 0.05$: Dunnett's test (ANOVA: migration; $P < 0.0001$, invasion; $P = 0.0210$).

roles in the tumorigenesis of colorectal, gastric, cervical, breast, lung, and prostate cancer cells [17, 18, 26]. We have also focused on and investigated transepithelial Cl^- transport in various types of cancer cells [11, 27–29].

In the present study, we investigated the KCC3 expression in ESCC and determined its relationships with clinicopathological features and prognosis. To the best of our knowledge, this is the first report examining KCC3 expression in human ESCC tissue. Our results showed that KCC3 expression in MT related to several clinicopathological features, such as the pT and pN categories. However, the expression of KCC3 in MT itself did not have a prognostic impact. Although these results may not be persuasive because of the limitation of a small sample size, they showed that KCC3 was expressed in MT of ESCC from an early stage. Regarding the expression of KCC in cancer tissue, previous studies demonstrated that KCC3 was abundant in cervical carcinoma and CN invaded deeply into stromal tissues whereas KCC4 was abundant in metastatic cervical and ovarian cancer tissues [8, 10]. Furthermore, both the progression-free and overall survival rates of patients with the high grade expression of KCC4 were significantly poorer than those of patients with the low grade expression of KCC4 in cervical cancer [10], which suggested a relationship between the expression pattern of KCC and

Table 2: Relationships between the clinicopathological features of esophageal cancer and expression of KCC3 in the invasive front of the tumor.

Variable	Invasive front		P value
	Negative ($n = 22$)	Positive ($n = 48$)	
Age			
<60 years	6	16	0.783
≥60 years	16	32	
Gender			
Male	21	38	0.154
Female	1	10	
Location of the primary tumor			
Ut-Mt	12	35	0.172
Lt-Ae	10	13	
Histological type			
Well/moderately differentiated SCC	15	34	1.000
Poorly differentiated SCC	7	14	
Tumor size			
<50 mm	14	35	0.575
≥50 mm	8	13	
Lymphatic invasion			
Negative	10	23	1.000
Positive	12	25	
Venous invasion			
Negative	13	27	1.000
Positive	9	21	
pT			
pT1	8	25	0.3035
pT2-3	14	23	
pN			
pN0	8	25	0.3035
pN1-3	14	23	
MT			
Low	20	15	<0.0001*
High	2	33	
CN			
Low	19	17	<0.0001*
High	3	31	
CN/MT			
CN ≤ MT	14	17	0.0385*
CN > MT	8	31	

Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; SCC: squamous cell carcinoma; pT: pathological T stage; pN: pathological N stage; MT: main tumor; CN: cancer nest.
* $P < 0.05$: Fisher's exact test.

Table 3: Five-year survival rate of patients with esophageal cancer according to various clinicopathological parameters.

Variables	5-year survival rate (%)	P value
Age		
<60 years	53.13	0.1179
≥60 years	72.2	
Gender		
Male	65.35	0.8597
Female	68.57	
Location of the primary tumor		
Ut-Mt	73.63	0.0564
Lt-Ae	50.82	
Histological type		
Well/moderately differentiated SCC	71.94	0.2301
Poorly differentiated SCC	53.43	
Tumor size		
<50 mm	68.68	0.2809
≥50 mm	59.52	
Lymphatic invasion		
Negative	78.4	0.0168*
Positive	54.78	
Venous invasion		
Negative	78.18	0.0169*
Positive	48.7	
pT		
pT1	82.22	0.0024*
pT2-3	50.68	
pN		
pN0	82.72	0.0029*
pN1-3	49.68	
MT		
Low	68.21	0.7838
High	60.56	
CN		
Low	70.48	0.4151
High	59.25	
CN/MT		
CN ≤ MT	76.74	0.1329
CN > MT	55.48	
Invasive front		
Negative	81.82	0.0887
Positive	57.07	

Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; SCC: squamous cell carcinoma; pT: pathological T stage; pN: pathological N stage; MT: main tumor; CN: cancer nest.
* $P < 0.05$: log-rank test.

clinical outcome. Therefore, we focused on the distribution of KCC3 in tumors and analyzed its expression in CN or the invasive front of the tumor. Although the expression of KCC3 in CN itself had no prognostic impact, the 5-year survival rate of patients with a CN > MT score was slightly lower than that of patients with a CN ≤ MT score. Furthermore, the 5-year survival rate of patients in whom KCC3 was expressed in the invasive front was lower than that of patients without it, and multivariate analysis revealed that

Table 4: Prognostic factors of esophageal cancer according to multivariate analysis.

Variables	Risk ratio	95% CI	*P* value
Location of the primary tumor			
Ut-Mt	Ref.	0.861452–2.20629	0.1813
Lt-Ae	1.37267		
Lymphatic invasion			
Negative	Ref.	1.012886–2.725895	0.0437*
Positive	1.605417		
Venous invasion			
Negative	Ref.	0.954510–2.395284	0.08
Positive	1.483897		
pT			
pT1	Ref.	1.122531–3.250204	0.0146*
pT2-3	1.834223		
pN			
pN0	Ref.	1.152392–3.450263	0.0110*
pN1-3	1.911249		
Invasive front			
Negative	Ref.	1.357757–4.524146	0.0014*
Positive	2.332559		

Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; pT: pathological T stage; pN: pathological N stage; Ref.: referent.
*$P < 0.05$: Cox's proportional hazards model; 95% CI: 95% confidence interval.

the expression of KCC3 in the invasive front was the strongest prognostic factor of all clinicopathological features. These results suggest the role of KCC3 in cancer invasion as well as the importance of its distribution in tumors as a prognostic predictor. We have previously identified several prognostic biomarkers in human ESCC, such as Ki-67, antiphosphohistone H3, p21, and E2F5 [30–33]. The expressions of these cell-cycle related proteins were mainly analyzed in MT. On the other hand, we focused on the distribution of KCC3 in the present study and showed its prognostic impact via cellular invasion.

Recent studies have indicated the importance of KCC in the cell migration and invasion of glioma, cervical, ovarian, and breast cancer cells [8–10, 12, 13]. Regarding the mechanism by which KCC regulates tumor invasion, KCC3 was previously shown to downregulate the formation of the E-cadherin/β-catenin complex in order to promote EMT, which is important for cervical cancer cell invasiveness [8]. In addition, a previous study reported that the motor protein-dependent membrane trafficking of KCC4 was important for cancer cell invasion [10]. Our *in vitro* study also demonstrated the important roles of KCC3 in cell migration and invasion in ESCC cells. One possible mechanism by which KCC regulates the malignant behavior of cancer cells may be through the regulation of $[Cl^-]_i$ [11, 13]. Recent studies have shown that $[Cl^-]_i$ is a critical signal mediator for the regulation of various cellular functions [34–36]. For instance, we showed that $[Cl^-]_i$ could act as an important signal to control the gene expression of the epithelial Na^+ channel via a tyrosine kinase in renal epithelial A6 cells [36]. We also previously reported that $[Cl^-]_i$ controlled cell-cycle progression in gastric and prostate cancer cells [27–29, 37, 38]. Shen et al. showed that an alteration in the $[Cl^-]_i$ concentration affected the activity of the retinoblastoma protein and cdc2 kinase, two key cell-cycle regulators that control progression from the G_1 into the S phase and from the G_2 into the M phase, respectively [13]. We considered KCC to be one of the important transporters that regulates $[Cl^-]_i$ in the steady state and previously showed that the blockage of KCC decreased $[Cl^-]_i$ in breast cancer cells [11]. Although this mechanism should be verified in more detail in further studies, these findings suggest that the changes induced in $[Cl^-]_i$ by KCC3 may be a critically important messenger that regulates cellular invasiveness in ESCC cells.

In summary, we found that KCC3 played a role in the cell migration and invasion of ESCC cells. An immunohistochemical analysis revealed that the expression of KCC3 in the invasive front of tumors was the strongest prognostic factor in patients with ESCC. A deeper understanding of the role of KCC3 may lead to its use as a crucial biomarker of tumor progression and/or a new therapeutic target for ESCC.

Conflict of Interests

None of the authors have any conflict of interests or financial ties to disclose.

Authors' Contribution

Atsushi Shiozaki and Kenichi Takemoto contributed equally to this work.

Acknowledgments

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Research Article

Sensitization of Cancer Cells through Reduction of Total Akt and Downregulation of Salinomycin-Induced pAkt, pGSk3β, pTSC2, and p4EBP1 by Cotreatment with MK-2206

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MK-2206 is an inhibitor of Akt activation. It has been investigated as an anticancer drug in clinical trials assessing the potential of pAkt targeting therapy. The purpose of this study was to identify conditions that increase the sensitivity of cancer cells to MK-2206. We found that the treatment of cancer cells with a high concentration of salinomycin (Sal) reduced total Akt protein levels but increased activated Akt levels. When cancer cells were cotreated with MK-2206 and Sal, both pAkt and total Akt levels were reduced. Using microscopic observation, an assessment of cleaved PARP, FACS analysis of pre-G1 region, and Hoechst staining, we found that Sal increased apoptosis of MK-2206-treated cancer cells. These results suggest that cotreatment with MK-2206 and Sal sensitizes cancer cells via reduction of both pAkt and total Akt. Furthermore, cotreatment of cancer cells with Sal and MK-2206 reduced pp70S6K, pmTOR, and pPDK1 levels. In addition, Sal-induced activation of GSK3β, TSC2, and 4EBP1 was abolished by MK-2206 cotreatment. These results suggest that cotreatment using MK-2206 and Sal could be used as a therapeutic method to sensitize cancer cells through targeting of the PI3K/Akt/mTOR pathway. Our findings may contribute to the development of MK-2206-based sensitization therapies for cancer patients.

1. Introduction

MK-2206, an oral small molecule and allosteric Akt inhibitor, binds to the Akt protein through a site located in the pleckstrin-homology domain. The binding of MK-2206 induces a conformational change of Akt that prevents its localization to the plasma membrane, thus inhibiting its subsequent activation [1–5]. MK-2206 is a first-in-class highly selective inhibitor of all Akt isoforms, which is active in several human cancer models through a number of possible mechanisms, including the induction of autophagy and apoptosis in glioma cells [1–5]. As an anticancer agent, MK-2206 is being tested in adult tumors [6–12] and in a spectrum of pediatric tumors [13] both in vitro and in vivo. The effect of MK-2206 against glioma cells has been confirmed in vitro [14]. In addition, a recent clinical trial investigated the use of MK-2206 in patients with advanced solid tumors [15]. A more complete understanding of the mechanisms governing MK-2206 sensitization is required to facilitate its therapeutic use in patients with cancer. Identifying the mechanism(s) underlying cell sensitization to MK-2206 would be an important step in the development of new treatment methods for pharmacological cancer.

Salinomycin (Sal) was originally used to eliminate bacteria, fungi, and parasites [16, 17]. More recently, this drug has been exploited to inhibit the growth of tumor stem cells and chemoresistant cancer cells [18–20]. Sal also functions as an efflux pump p-glycoprotein (P-gp) inhibitor [21, 22] and is considered to be a potential anticancer drug for cancer chemoprevention. Sal, a polyether ionophore antibiotic isolated from *Streptomyces albus*, has been shown to kill cancer stem cells in different types of human cancers [23]. The ionophore involves various mechanisms, including inhibition of ABC transporters and oxidative phosphorylation [23]. In addition, Sal can overcome radiation resistance via inhibition of the Wnt/beta-catenin signaling pathway [23]. Sal can promote both cytoplasmic and mitochondrial potassium efflux and stimulate the differentiation of cancer stem cells

[23]. Additionally, Sal sensitizes cancer cells to doxorubicin, etoposide, radiation, and antimitotic drugs [22, 24, 25]. Various Sal-sensitization mechanisms for cancer have also been investigated [26–28].

In the present study, we investigated whether cotreatment of Sal would sensitize cancer cells to MK-2206. We further analyzed whether the cotreatment influenced the activation status or levels of various signaling proteins of the PI3K/Akt/mTOR pathway.

2. Materials and Methods

2.1. Reagents. Sal was purchased from Sigma-Aldrich (St. Louis, MO). MK-2206 was supplied by Selleckchem (Houston, TX). LY294002 was supplied by Calbiochem (Bellerica, MA).

2.2. Antibodies. Antibodies against Akt, phosphorylated Akt, PI3K, phosphorylated PDK1, phosphorylated TSC2, phosphorylated GSK3β, phosphorylated p70S6K, phosphorylated 4EBP1, mTOR, PTEN, FOXO1, PCNA, and cleaved poly ADP ribose polymerase (C-PARP) were from Cell Signaling Technology (Danvers, MA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), survivin, CDK4, and pRb were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated mTOR and phosphorylated PTEN were from Abcam (Cambridge, UK). Antibody against Cyclin D1 was from Biosource (Camarillo, CA).

2.3. Cell Culturing. Hs578T breast cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea) and were previously used [22, 24–27, 29]. Human oral squamous carcinoma KB cell line was previously described [26, 30]. All cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (WelGENE, Daegu, South Korea).

2.4. Western Blot Analysis. Total cellular proteins were extracted using a previously described trichloroacetic acid (TCA) method [22, 24–27]. Briefly, cells grown in 60 mm dishes were washed three times with 5 mL PBS. Next, 500 μL of 20% trichloroacetic acid (TCA) was added to each plate. The cells were then dislodged by scraping and were transferred to Eppendorf tubes. Proteins were pelleted by centrifugation for 5 min at 3000 rpm and resuspended in 1 M Tris-HCl (pH 8.0) buffer. The total protein concentrations were estimated. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis as previously described [22, 24–27].

2.5. Fluorescence-Activated Cell Sorting (FACS) Analysis. FACS analysis was performed as previously described [22, 24–27]. Cells were grown in 60 mm dishes and treated with the indicated drugs for the prescribed times. The cells were then dislodged by trypsin and pelleted by centrifugation. The pelleted cells were washed thoroughly with PBS, suspended in 75% ethanol for at least 1 h at 4°C, washed again with PBS, and resuspended in a cold propidium iodide (PI) staining solution (100 μg/mL RNase A and 50 μg/mL PI in PBS) for 40 min at 37°C. The stained cells were analyzed for relative DNA content using a FACSCalibur flow cytometry system (BD Bioscience, Franklin Lakes, NJ). We performed more than two independent tests.

2.6. Hoechst Staining. The tests were used to identify nuclear disruption, an indicator of apoptosis. Briefly, cells in 6-well plates were treated with the indicated drugs and incubated for 24 h, 48 h, or 72 h at 37°C. Cells were then incubated with 9.4 μM Hoechst 33258 (Sigma-Aldrich, St. Louis, MO) for 30 min in the dark at 37°C before image acquisition. The medium was removed, and the cells were washed twice with PBS. Stained cells were subsequently examined using an inverted fluorescence microscope. We performed more than two independent tests.

3. Results

3.1. Higher Concentration of Sal Reduced Both pAkt and Total Akt in MK-2206-Treated Cells. The potential for Sal to sensitize MK-2206-treated Hs578T breast cancer cells has been investigated. As shown in Figure 1(a), Akt activation was increased by Sal, while increasing concentrations of Sal induced a reduction in total Akt protein levels. In contrast, increasing concentrations of MK-2206 did not reduce total Akt protein levels, but it reduced pAkt levels (Figure 1(a)). The effect of MK-2206 and Sal cotreatment on pAkt and total Akt was then tested in Hs578T breast cancer cells. As shown in Figure 1(b), cotreatment with Sal and MK-2206 reduced both Sal-induced pAkt and total Akt protein levels, suggesting that combining MK-2206 and Sal treatments may reduce both pAkt and total Akt levels.

Dose and time dependence of the cotreatment effect on both pAkt and total Akt levels were further analyzed. As described in Figure 1(c), a low dose of MK-2206 can induce the reduction of both pAkt and total Akt levels in Sal-treated cells. Furthermore, the effect observed after 48 h of cotreatment was similar to the effect observed after 24 h of cotreatment (Figure 1(d)). C-PARP production was increased by MK-2206 and Sal cotreatment (Figure 1(d)), suggesting that the sensitization involved apoptosis. A reduction of pRb levels by the cotreatment was also observed, suggesting that the sensitization involved other cell cycle-related proteins. Collectively, our results indicated that Sal treatment can increase the sensitivity of cancer cells to MK-2206 by reducing total Akt protein levels.

3.2. Cotreatment with Sal and MK-2206 Increased Apoptosis. Cotreatment with Sal and MK-2206 increased pre-G1 regions in a dose-dependent manner (Figure 2), suggesting that the cotreatment with Sal led to an increase in the apoptosis of MK-2206-treated cells. In order to test whether the sensitization effect of the cotreatment was time dependent, we tested the time dependency of C-PARP production. As shown in Figure 3(a), when compared to the single treatments with

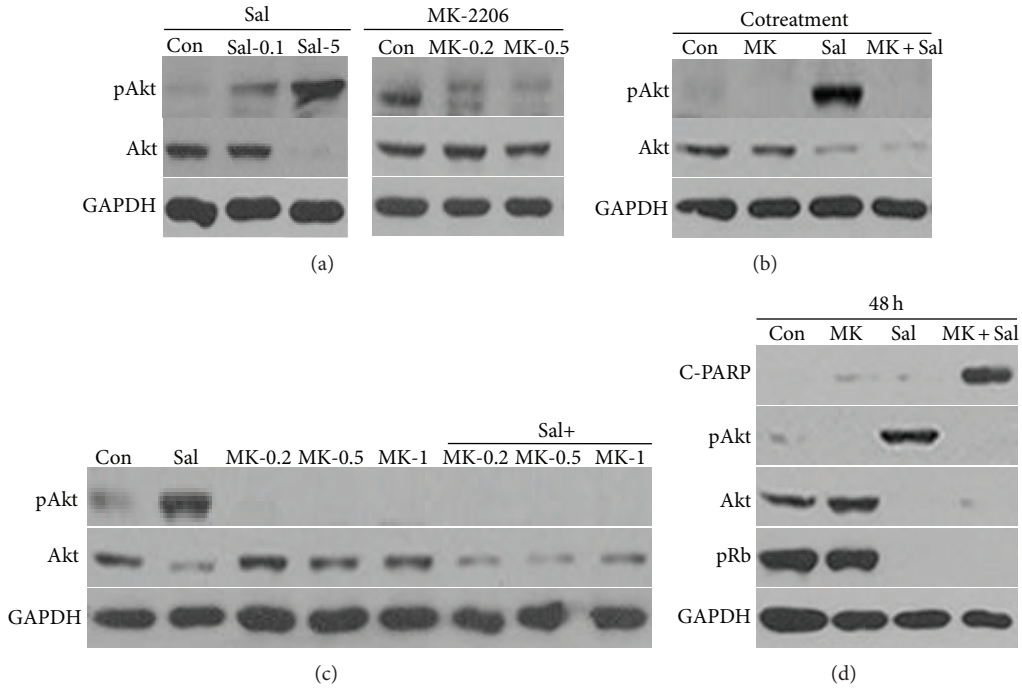

Figure 1: High concentration of Sal reduced pAkt and total Akt levels in MK-2206-treated cells. (a) Hs578T cell extracts were collected at 24 h after treatment with 0.1 μM Sal (Sal-0.1), 5 μM Sal (Sal-5), 0.2 μM MK-2206 (MK-0.2), 0.5 μM MK-2206 (MK-0.5), or DMSO (Con). (b) Hs578T cell extracts were collected at 24 h after treatment with 0.5 μM MK-2206 (MK), 5 μM Sal (Sal), 0.5 μM MK-2206 with 5 μM Sal (MK + Sal), or DMSO (Con). (c) Hs578T cell extracts were collected at 24 h after treatment with 5 μM Sal (Sal), 0.2 μM MK-2206 (MK-0.2), 0.5 μM MK-2206 (MK-0.5), 1 μM MK-2206 (MK-1), 5 μM Sal with 0.2 μM MK-2206 (Sal + MK-0.2), 5 μM Sal with 0.5 μM MK-2206 (Sal + MK-0.5), 5 μM Sal with 1 μM MK-2206 (Sal + MK-1), or DMSO (Con). (d) Hs578T cell extracts were collected at 48 h after treatment with 1 μM MK-2206 (MK), 5 μM Sal (Sal), 1 μM MK-2206 with 5 μM Sal (MK + Sal), or DMSO (Con). The cells were used for Western blot analyses using antibodies against pAkt, Akt, C-PARP, pRb, and GAPDH.

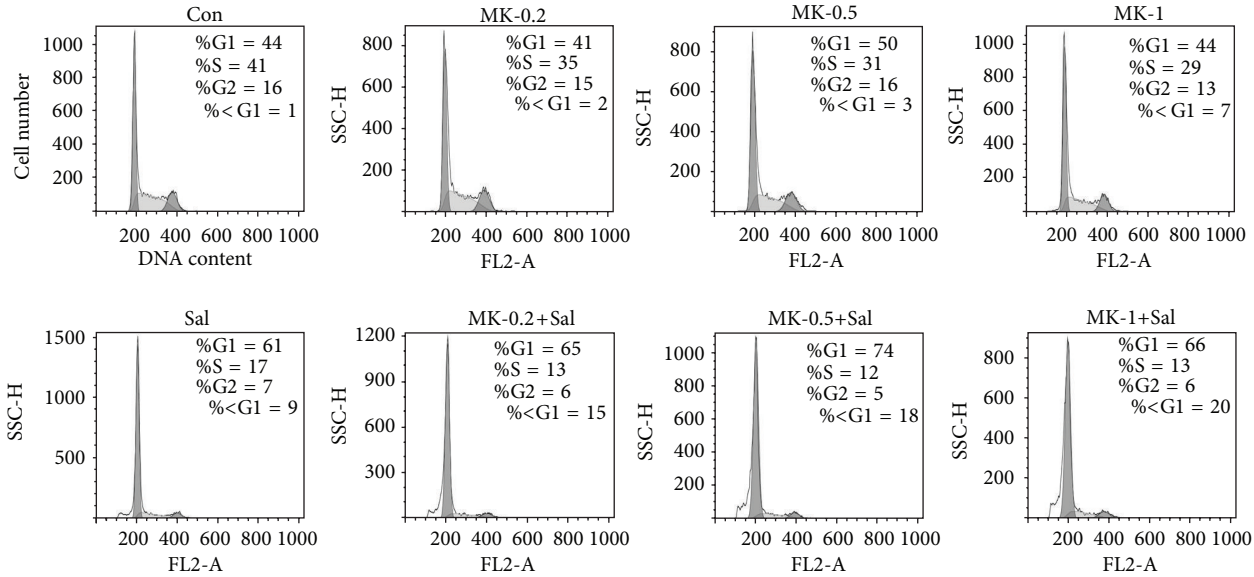

Figure 2: Cotreatment with MK-2206 and Sal increased pre-G1 regions in a dose-dependent manner. Hs578T cells were grown on 60 mm diameter dishes and treated with 0.2 μM MK-2206 (MK-0.2), 0.5 μM MK-2206 (MK-0.5), 1 μM MK-2206 (MK-1), 5 μM Sal (Sal), 0.2 μM MK-2206 with 5 μM Sal (MK-0.2 + Sal), 0.5 μM MK-2206 with 5 μM Sal (MK-0.5 +Sal), 1 μM MK-2206 with 5 μM Sal (MK-1 + Sal), or DMSO (Con). After 72 h, FACS analysis was performed as described in "Materials and Methods."

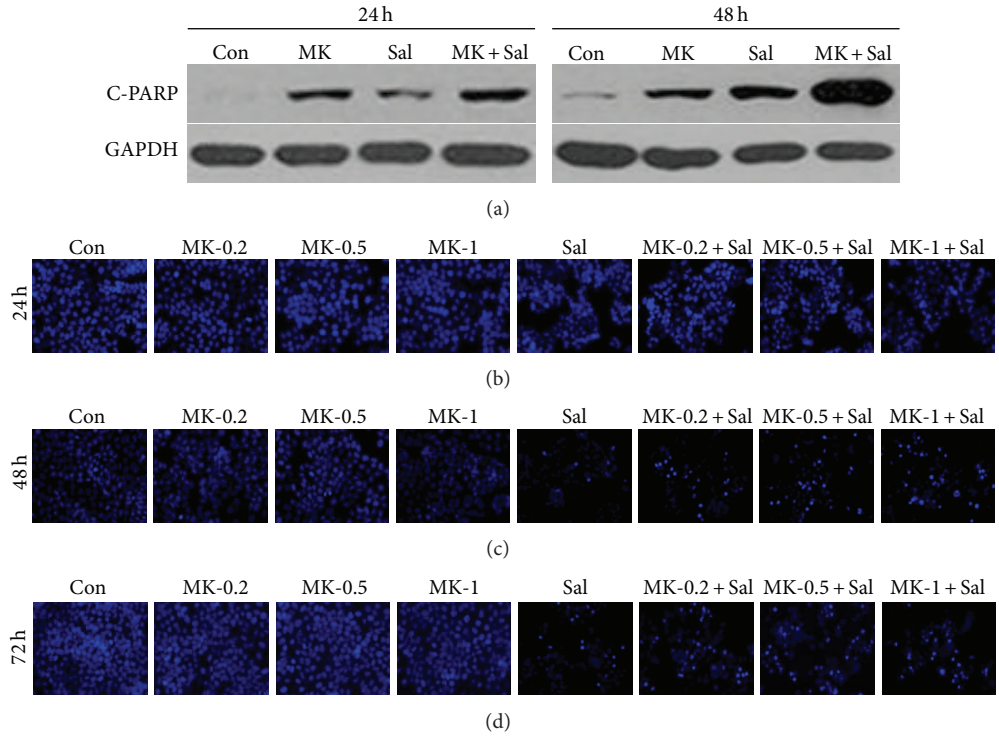

Figure 3: Cotreatment with MK-2206 and Sal increased apoptosis in a time-dependent manner. (a) Hs578T cell extracts were collected at 24 h or 48 h after treatment with 0.5 μM MK-2206 (MK), 5 μM Sal (Sal), 0.5 μM MK-2206 with 5 μM Sal (MK + Sal), or DMSO (Con). The cells were used for Western blot analyses using antibodies against C-PARP and GAPDH. (b–d) Hs578T cells were grown on 6-well plates and treated with 0.2 μM MK-2206 (MK-0.2), 0.5 μM MK-2206 (MK-0.5), 1 μM MK-2206 (MK-1), 5 μM Sal (Sal), 0.2 μM MK-2206 with 5 μM Sal (MK-0.2 + Sal), 0.5 μM MK-2206 with 5 μM Sal (MK-0.5 + Sal), 1 μM MK-2206 with 5 μM Sal (MK-1 + Sal), or DMSO (Con). After 24 h, 48 h, or 72 h, all cells were then stained with Hoechst as described in "Materials and Methods." The stained cells were subsequently examined using an inverted fluorescence microscope with a 32x objective lens.

MK-2206 or Sal, C-PARP production increased in a time-dependent manner when the cancer cells were cotreated with MK-2206 and Sal. To confirm these results, we performed Hoechst staining, which revealed marked morphological changes in cotreated cancer cells, consistent with apoptosis such as condensation of chromatin and nuclear fragmentation (Figures 3(b)–3(d)). Collectively, the data indicated that cotreatment with Sal increased the apoptosis of MK-2206-treated cancer cells in a dose- and time-dependent fashion.

3.3. Cotreatment with MK-2206 Reduced Sal-Activated GSk3β, TSC2, and 4EBP1. We further analyzed whether the cotreatment influenced the activation status or levels of the signaling proteins that function upstream and downstream of the Akt pathway. In this study, the major proteins of the PI3K/Akt/mTOR pathway, mTOR, p70S6K, PDK1, PI3K, GSK3β, TSC2, 4EBP1, and PTEN were tested [26, 31, 32]. MK-2206 single treatment did not affect pmTOR, pPDK1, PI3K, mTOR, and PTEN levels but reduced both pp70S6K and pPTEN (Figures 4(a) and 4(b)). Phospho-mTOR, pp70S6K, pPDK1, PI3K, pPTEN, and PTEN levels were further reduced in cancer cells cotreated with Sal and MK-2206, when compared to cancer cells treated with either MK-2206 or Sal alone (Figures 4(a) and 4(b)). Interestingly, as previously observed for pAkt (Figure 1(a)), the high concentration of Sal also increased pGSK3β, pTSC2, and p4EBP1 (Figure 4(c)). This effect of Sal was reduced or abolished by MK-2206 cotreatment (Figure 4(c)), suggesting that the activation of the PI3K/Akt/mTOR signaling pathway can be effectively reduced by MK-2206 cotreatment. Similar results were observed in the presence of high concentration of MK-2206 (Figure 4(d)). In conclusion, the cotreatment sensitization mechanism involved an effective reduction of various activated proteins belonging to the PI3K/Akt/mTOR pathway.

Since the PI3K/Akt/mTOR pathway is involved in proliferation and survival signals [26, 31, 32], we also tested whether the level of cell cycle- and proliferation-related proteins (FOXO1, CDK4, Cyclin D1, PCNA, and pRb) was reduced. MK-2206 single treatment did not affect these proteins (Figures 4(a) and 4(b)), whereas Sal treatment induced a reduction in most of them (Figures 4(a) and 4(b)). Cotreatment with Sal and MK-2206 had similar effects as Sal single treatment on cell cycle- and proliferation-related proteins (Figures 4(a) and 4(b)). It suggests that Sal cotreatment induced the reduction of cell cycle- and proliferation-related proteins in MK-2206-treated cells. In case of survivin, MK-2206 single treatment reduced protein levels in a manner similar to Sal single treatment (Figure 4(b)).

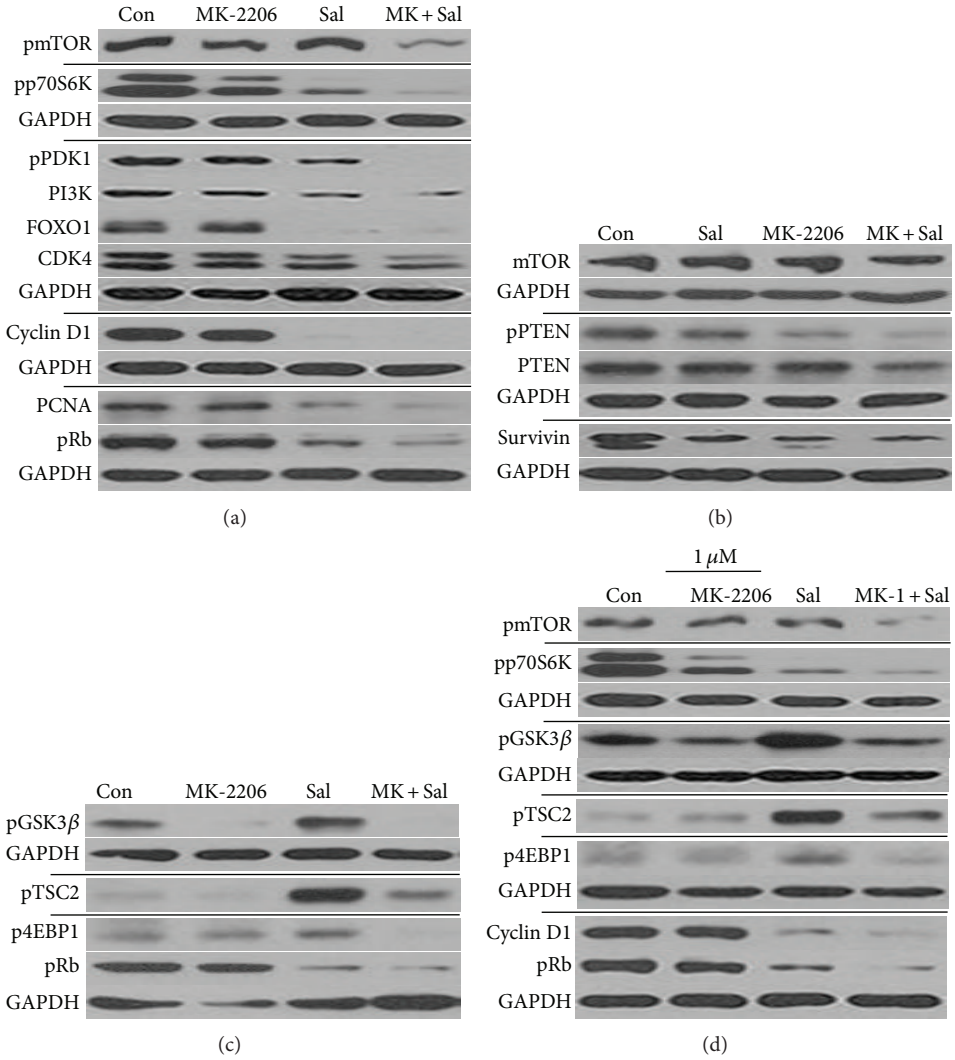

Figure 4: Cotreatment with MK-2206 reduced the levels of p70S6K, mTOR, and PDK1 activated forms and reduced Sal-activated GSk3β, TSC2, and 4EBP1. (a–c) Hs578T cell extracts were collected at 24 h after treatment with 0.5 μM MK-2206, 5 μM Sal (Sal), 0.5 μM MK-2206 with 5 μM Sal (MK + Sal), or DMSO (Con). The cells were used for Western blot analyses using antibodies against phosphorylated mTOR, mTOR, phosphorylated p70S6K, phosphorylated PDK1, phosphorylated GSK3β, phosphorylated TSC2, phosphorylated 4EBP1, phosphorylated PTEN, PTEN, PI3K, FOXO1, Survivin, Cyclin D1, CDK4, PCNA, pRb, and GAPDH. (d) Hs578T cell extracts were collected at 24 h after treatment with 1 μM MK-2206, 5 μM Sal (Sal), 1 μM MK-2206 with 5 μM Sal (MK-1 + Sal), or DMSO (Con). The cells were used for Western blot analyses using antibodies against phosphorylated mTOR, phosphorylated p70S6K, phosphorylated GSK3β, phosphorylated TSC2, phosphorylated 4EBP1, Cyclin D1, pRb, and GAPDH.

Other cell lines were tested to assess whether a similar sensitization mechanism could be observed. KB cell line presented an increase in C-PARP production when cotreated with MK-2206 and Sal (Figure 5(a)). As observed in Hs578T cells, pTSC2, pGSK3β, pAkt, and total Akt proteins were also reduced in KB cells cotreated with Sal and MK-2206 (Figure 5(a)), suggesting that Sal and MK-2206 cotreatment sensitization mechanism is also conserved in KB cancer cell line. However, future studies are warranted to determine whether this sensitization effect is observed in other cancer cell types.

3.4. Cotreatment with LY294002 Reduced Sal-Activated GSk3β, TSC2, and 4EBP1. Since MK-2206 is known to be an Akt inhibitor [1–5], we tested whether another Akt inhibitor LY294002 [26, 31, 32] also had similar effects. As shown in Figure 5(b), LY294002 and Sal cotreatment increased C-PARP production, suggesting that the cotreatment with LY294002 sensitized Sal-treated cells. In addition, as with MK-2206 cotreatment, the sensitization mechanism induced by LY294002 cotreatment involved the reduction of pAkt, total Akt, pGSK3β, pTSC2, and p4EBP1 (Figures 4(a)–4(c) versus Figure 5(b)). LY294002 single treatment reduced pRb

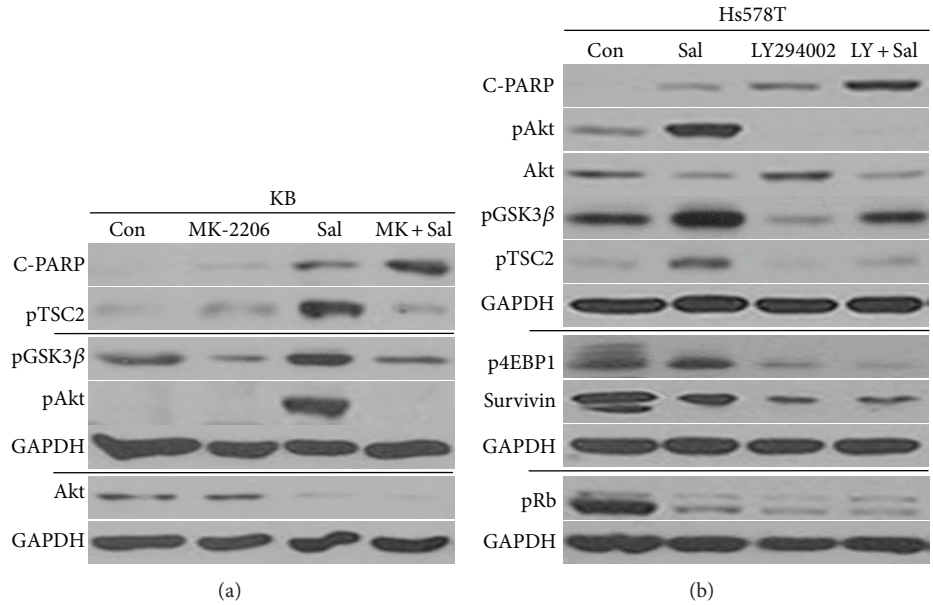

Figure 5: Sal and MK-2206 cotreatment sensitization mechanism is also conserved in KB cancer cell line. (a) KB cell extracts were collected at 24 h after treatment with 0.5 μM MK-2206, 5 μM Sal (Sal), 0.5 μM MK-2206 with 5 μM Sal (MK + Sal), or DMSO (Con). (b) Cotreatment with LY294002 reduced Sal-activated GSk3β, TSC2, and 4EBP1. Hs578T cell extracts were collected at 24 h after treatment with 5 μM Sal (Sal), 20 μM LY294002, 5 μM Sal with 20 μM LY294002 (Sal + LY), or DMSO (Con). The cells were used for Western blot analyses using antibodies against C-PARP, phosphorylated TSC2, phosphorylated GSK3β, phosphorylated Akt, Akt, phosphorylated 4EBP1, Survivin, pRb, and GAPDH.

levels (Figure 5(b)), whereas MK-2206 single treatment did not (Figures 4(a) and 4(c)), suggesting that MK-2206 is a more specific Akt inhibitor than LY294002. Collectively, our results indicated that the sensitization mechanism observed when Sal is combined with MK-2206 can be observed when combined with other Akt inhibitors.

4. Discussion

MK-2206 is a recently developed drug that targets Akt activation [1–5]. In this study, we attempted to identify ways to sensitize MK-2206-treated cells or ways to overcome MK-2206-resistance in cancer cells. We hypothesized that MK-2206-treated cancer cells can be sensitized if total Akt protein levels are reduced. We determined that both total Akt and pAkt levels could be reduced by Sal and MK-2206 cotreatment. We also demonstrated that a relatively low dose of MK-2206 is enough to reduce both pAkt and total Akt levels in Sal and MK-2206 cotreated cancer cells. This finding suggests that MK-2206 toxicity can be reduced by combining the treatment with Sal in future clinical trials. Sensitization of cancer cells to various anticancer drugs and radiation by cotreatments with Sal has been demonstrated [22, 24, 25]. However, the sensitization of cancer cells to a specific molecular-targeting drug using Sal has yet to be determined. To the best of our knowledge, our work is the first report of the sensitization of MK-2206-treated cells by Sal cotreatment. This suggests that Sal may also be useful in combination with various specific molecule-targeting drugs. Furthermore, Sal was shown to reduce total Akt protein levels. In conclusion, high concentrations of Sal can be used to reduce total Akt levels or prevent the generation of its activated form, pAkt. Further studies are warranted to understand the mechanism(s) by which Sal reduces total Akt protein levels. These future studies include measuring Akt mRNA levels, Akt protein stability, and Akt protein translation. The signaling pathways involved in the reduction of total Akt levels by Sal should also be investigated. Since Sal has been shown to sensitize resistant cancer cells or cancer stem cells, cotreatments using MK-2206 and Sal could also be applied to these cell types.

We further analyzed the activation status or levels of the PI3K/mTOR/Akt pathway signaling proteins. Sal reduced pmTOR, pp70S6K, pPDK1, PI3K, pPTEN, and PTEN protein levels in MK-2206-treated cells. Interestingly, we found that high concentration of Sal increased pAkt, pGSK3β, pTSC2, and p4EBP1. The increased activation of these proteins by Sal was reduced by MK-2206 cotreatment, suggesting that various Sal-activated PI3K/Akt/mTOR pathway proteins are effectively reduced by MK-2206 cotreatment. In addition, Sal treatment reduced levels of proliferation- and survival-related proteins such as FOXO1, CDK4, Cyclin D1, PCNA, and pRb in MK-2206-treated cells. Therefore, cotreatment with Sal and MK-2206 allows the reduction of two types of protein families: reduction of Sal-activated proteins by MK-2206 and reduction of cell cycle- and proliferation-related proteins by Sal.

Our data indicated that cotreatment-induced sensitization mechanism involves apoptosis, since an increase in

C-PARP production and pre-G1 region was detected and confirmed by Hoechst staining. The apoptotic effect of the cotreatment was observed for a long period of time after cotreatment, suggesting that the initial treatment could be effective for long periods of time. It also suggests that the number of cotreatments could be reduced when used in clinical trials. The cotreatment with MK-2206 and Sal sensitized two different cancer cell lines, Hs578T breast cancer and KB oral squamous cancer cell lines, suggesting that the sensitization mechanism of the cotreatment is generally conserved in different cancer cell lines. However, future studies are warranted to determine whether this sensitization effect is observed in other cancer cell types. LY294002, another Akt inhibitor [26, 31, 32], was also found to reduce total Akt protein as well as activated Akt, pGSK3β, pTSC2, and p4EBP1 in Sal-treated cells. The fact that the sensitization effect is observed when combining Sal with either LY294002 or MK-2206 suggests that this effect may be conserved among Akt inhibitors. These results suggest that various Akt inhibitors could be combined with Sal for sensitization.

In summary, our results could help determine the potential clinical use of Sal for MK-2206-treated cancer patients. The present study also enhances our understanding of Sal-sensitization mechanisms. Our findings may contribute to the development of MK-2206-based therapies for patients.

Abbreviations

Sal:	Salinomycin
MK:	MK-2206
LY:	LY294002
PI3K:	Phosphatidylinositol 3-kinase
C-PARP:	Cleaved poly ADP ribose polymerase
DMSO:	Dimethylsulfoxide
FACS:	Fluorescence-activated cell sorting
FBS:	Fetal bovine serum
TCA:	Trichloroacetic acid
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
RT:	Room temperature
mTOR:	Mammalian target of rapamycin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Efficacy of a Hypotonic Treatment for Peritoneal Dissemination from Gastric Cancer Cells: An *In Vivo* Evaluation

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The aim of the present study was to determine the efficacy of a hypotonic treatment for peritoneal dissemination from gastric cancer cells using an *in vivo* model. We firstly evaluated the toxicity of a peritoneal injection of distilled water (DW) (2 mL for 3 days) in mice. Macroscopic and microscopic examinations revealed that the peritoneal injection of DW did not severely damage the abdominal organs of these mice. MKN45 gastric cancer cells preincubated with NaCl buffer or DW for 20 minutes *in vitro* were then intraperitoneally injected into nude mice, and the development of dissemination nodules was analyzed. The total number, weight, and volume of the dissemination nodules were significantly decreased by the DW preincubation. We then determined whether the peritoneal injection of DW inhibited the establishment of peritoneal dissemination. After a peritoneal injection of MKN45 cells into nude mice, NaCl buffer or DW was injected into the abdominal cavity for 3 days. The total volume of dissemination nodules was significantly lower in DW-injected mice than in NaCl-injected mice. In conclusion, we demonstrated the safeness of a peritoneal injection of DW. Furthermore, the development of dissemination nodules from gastric cancer cells was prevented by a preincubation with or peritoneal injection of DW.

1. Introduction

Gastric cancer is a leading cause of cancer-related deaths worldwide, and peritoneal dissemination is the most common form of recurrence in patients with gastric cancer [1, 2]. Peritoneal metastasis is associated with a poor prognosis and, therefore, the management of dissemination in the peritoneal cavity is important in the treatment of gastric cancer [3, 4]. However, there is currently no effective treatment for peritoneal dissemination from gastric cancer. On the other hand, the roles of ion and water channels/transporters have recently been examined in cancer cells, and cellular physiological approaches are expected as novel therapeutic strategies [5–9]. The regulation of extracellular osmolality is a promising method, with previous studies demonstrating the cytocidal effects of hypotonic treatments on cancer cells [10–12]. We recently examined changes in the cellular morphology and volume of gastric cancer cells subjected to hypotonic shock using several unique methods and apparatus, such as a differential interference contrast microscope connected to a high-speed digital video camera and a high-resolution flow cytometer [13]. Our findings confirmed the cytocidal effects of hypotonic shock on gastric cancer cells *in vitro* [13]. However, the effects of hypotonic treatments on the development of peritoneal dissemination from gastric cancer and their safeness *in vivo* have not yet been fully evaluated.

In the present study, we determined, using an *in vivo* model, the toxicity and therapeutic effects of a peritoneal injection of distilled water (DW) for the treatment of peritoneal dissemination from gastric cancer. We showed that the peritoneal injection of DW was not toxic to mice. Furthermore, the development of dissemination nodules from gastric cancer cells was prevented by a preincubation with or peritoneal injection of DW. These results support the efficacies of peritoneal lavage with DW during surgery and the peritoneal injection of DW against dissemination from gastric cancer.

2. Materials and Methods

2.1. Cell Culture and Materials. The poorly differentiated human gastric adenocarcinoma cell line MKN45 was used in the present study. Cells were grown in plastic culture flasks (Corning Incorporated, NY, USA) and maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The flasks were kept in a humidified incubator at 37°C under 5.0% CO_2 in air.

The 140 mM isotonic NaCl solution (NaCl buffer) contained 140 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM glucose, and 10 mM HEPES.

2.2. In Vivo Experiments. Four-week-old female BALB/c mice were used to evaluate the toxicity of the peritoneal injection of DW. Four-week-old female BALB/c nude mice were used as the peritoneal dissemination model and were purchased from SHIMIZU Laboratory Supplies Co., Ltd. (Kyoto, Japan) and maintained under pathogen-free barrier conditions. Mice were provided with sterile food and water and housed in cages. Ambient light was controlled to provide regular 12 h light-dark cycles. All animal protocols were approved by the institutional guidelines of the Kyoto Prefectural University of Medicine, Kyoto, Japan.

To evaluate the toxicity of the peritoneal injection of DW, 2 mL of NaCl buffer or DW was injected into the abdominal cavities of 4-week-old female BALB/c mice ($n = 3$, each group) for 3 days (Figure 1(a)). The volume of buffer was decided, referring to the circulating blood volume of mouse. At a defined time point of 1 week after the start of the peritoneal injections, all mice were sacrificed, and intra-abdominal findings were investigated. Abdominal organs, including the small intestine, peritoneum, and liver, were fixed in 10% formaldehyde in PBS, paraffin embedded, and stained with hematoxylin and eosin.

To examine the establishment of peritoneal dissemination from gastric cancer cells exposed to hypotonic shock, MKN45 cells grown in culture flasks were detached and centrifuged. A total of 1.0×10^6 pelleted cells were then suspended in 5 mL DW or isotonic NaCl buffer as a control, and each cell was incubated for 20 min. The incubation time was decided, referring to our previous report [13]. Thereafter, the suspension was centrifuged, and pelleted cells were suspended in 0.3 mL PBS and then intraperitoneally injected into 4-week-old female nude mice ($n = 5$, each group) (Figure 2(a)). All mice were sacrificed at a defined time point of 2 weeks after the intraperitoneal injection of MKN45 cells, and the degree of peritoneal dissemination was evaluated macroscopically. Tumors more than 0.5 mm in diameter were resected and counted, and the weights of the resected tumors were measured. Tumor diameters were measured with a caliper and tumor volumes were calculated using the formula [14]:

$$\text{Tumor volume} = \text{length} \times \text{width}^2 \times 0.5. \quad (1)$$

To examine the efficacy of the peritoneal injection of DW on the establishment of peritoneal dissemination, MKN45 cells grown in culture flasks were detached and centrifuged. A total of 1.0×10^6 pelleted cells were suspended in 0.3 mL PBS and then intraperitoneally injected into 4-week-old female nude mice ($n = 6$, each group) on day 1. Two milliliters of NaCl buffer or DW was injected into the abdominal cavities of nude mice from day 2 to day 4 (for 3 days) (Figure 3(a)). All mice were sacrificed at a defined time point of 2 weeks after the intraperitoneal injection of MKN45 cells, and the degree of peritoneal dissemination was evaluated macroscopically. Tumors more than 0.5 mm in diameter were resected and counted, and their weights and volumes were analyzed.

2.3. Statistical Analysis. Statistical analysis was carried out using Student's t-test. Differences were considered significant when the P value was <0.05. Statistical analyses were performed using JMP ver. 5 from SAS in Cary, NC, USA.

3. Results

3.1. Evaluation of the Toxicity of the Peritoneal Injection of DW. To determine the toxicity of the peritoneal injection of DW *in vivo*, 2 mL of NaCl buffer or DW was injected into the abdominal cavities of 4-week-old female BALB/c mice for 3 days (Figure 1(a)). All mice in both groups were alive ($n = 3$, each group), and no abnormal findings were detected following the peritoneal injections. All mice were sacrificed 1 week after the start of the peritoneal injections and intra-abdominal findings were investigated. No significant differences were observed in the macroscopic findings of abdominal organs, including the gastrointestinal tract, liver, and peritoneum, between the NaCl and DW groups (Figure 1(b)). The microscopic findings of the small intestine, peritoneum, and liver revealed no histological damage or inflammation by DW injection or NaCl injection (Figure 1(c)). These results suggested that the peritoneal injection of DW for 3 days did not cause severe toxicity in mice.

3.2. Inhibition of Establishment of Peritoneal Dissemination after Preincubation of Gastric Cancer Cells with DW. We previously reported the cytocidal effects of hypotonic shock induced by DW on MKN45 cells [13]. To examine these effects in an *in vivo* model, MKN45 cells incubated with NaCl buffer or DW for 20 minutes *in vitro* were intraperitoneally injected into nude mice, and the development of dissemination nodules was analyzed (Figure 2(a)). Many dissemination nodules were established 2 weeks after the intraperitoneal

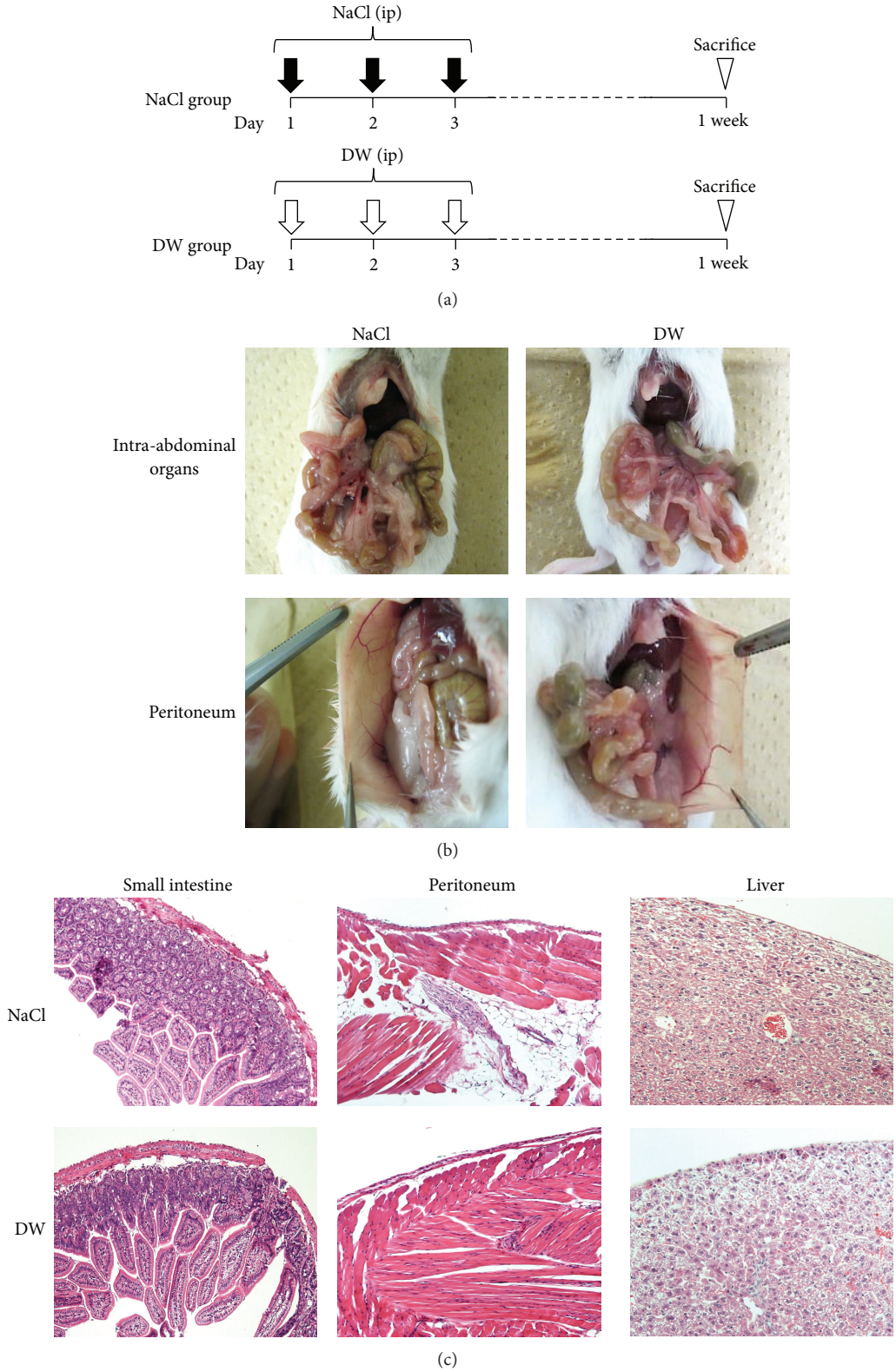

FIGURE 1: Evaluation of the toxicity of the peritoneal injection of DW. (a) Two milliliters of NaCl buffer or DW was injected into the abdominal cavities of 4-week-old female BALB/c mice for 3 days. At a defined time point of 1 week after the start of the peritoneal injections, all mice were sacrificed, and intra-abdominal findings were investigated. $n = 3$. (b) No significant differences were observed in the representative macroscopic findings of abdominal organs, including the gastrointestinal tract, liver, and peritoneum, between the NaCl and DW groups. (c) No significant differences were observed in the representative histopathological findings of the small intestine, peritoneum, or liver between the NaCl and DW groups. Magnification: 100x. DW: distilled water.

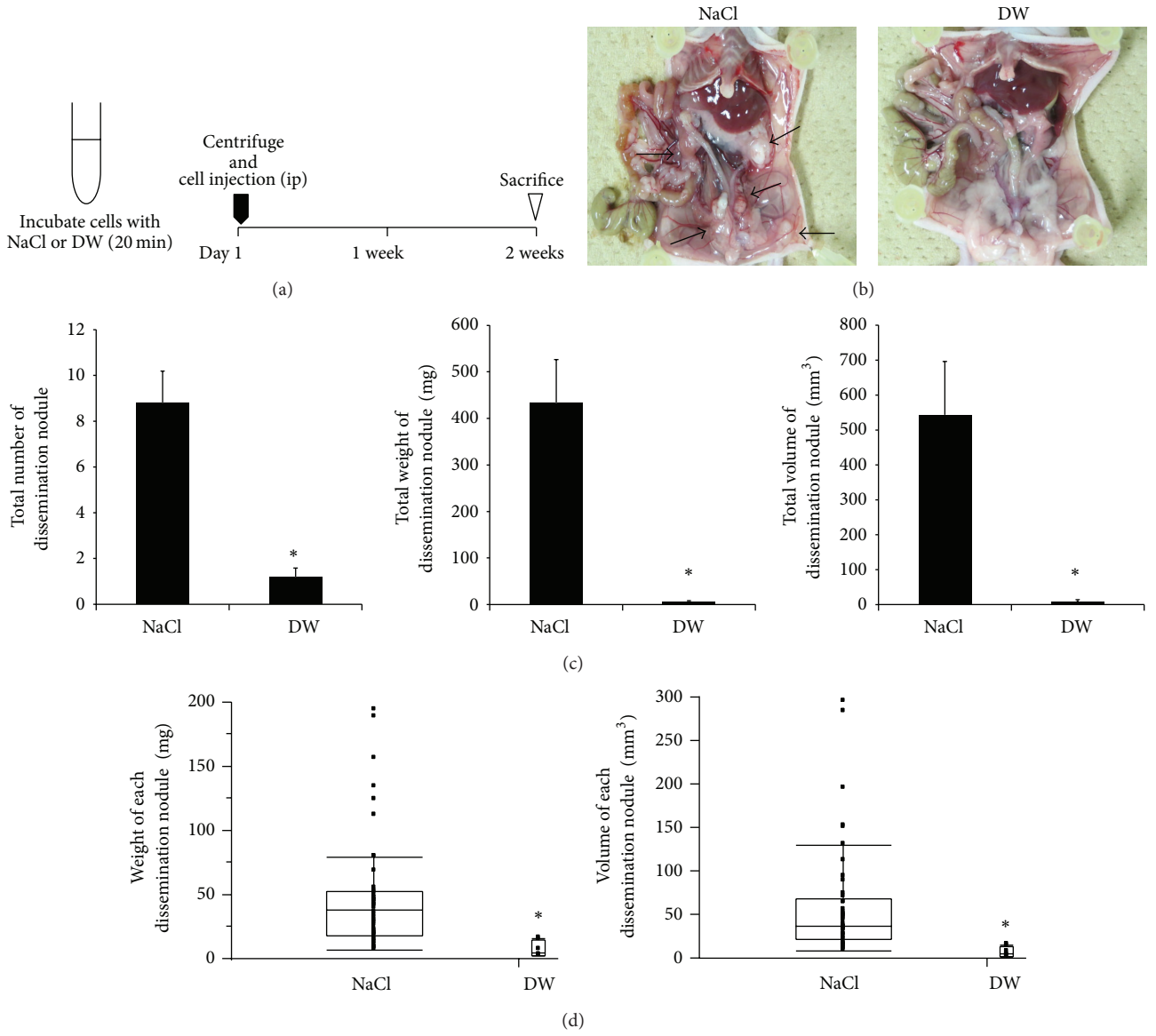

Figure 2: Inhibition of the establishment of peritoneal dissemination after the preincubation of gastric cancer cells with DW. (a) A total of 1.0×10^6 pelleted MKN45 cells were suspended in 5 mL NaCl buffer or DW, and each cell was incubated for 20 min. Thereafter, the suspension was centrifuged, and pelleted cells were suspended in 0.3 mL PBS and then intraperitoneally injected into 4-week-old female nude mice. At a defined time point of 2 weeks after the intraperitoneal injection of cells, all mice were sacrificed, and the degree of peritoneal dissemination was evaluated macroscopically. $n = 5$. (b) Representative macroscopic findings of the abdominal cavity. Many dissemination nodules were established in NaCl preincubated mice, whereas very few tumors were observed in DW preincubated mice. Arrows indicate dissemination nodules. (c) The total number, weight, and volume of dissemination nodules were significantly decreased by the preincubation with DW. The results are presented as the mean ± SEM ($n = 5$) $^*P < 0.05$. (d) Box plots of dissemination nodules in both groups. The weight and volume of each dissemination nodule were markedly lower in the DW group than in the NaCl group. $^*P < 0.05$. DW: distilled water.

injection of MKN45 cells preincubated with NaCl, whereas very few tumors were observed in mice injected with MKN45 cells preincubated with DW (Figure 2(b)). Data from 5 mice for each group were analyzed (Figure 2(c)). The total number of dissemination nodules was significantly decreased by the DW treatment (NaCl group: 8.8 ± 1.4; DW group: 1.2 ± 0.8; mean ± standard error of the mean (SEM)). The total weight and volume of dissemination nodules were markedly lower in the DW group (5.8 ± 2.7 mg, 9.1 ± 5.2 mm^3) than in the NaCl group (433.0 ± 93.5 mg, 544.1 ± 151.9 mm^3). Each dissemination nodule in both groups was analyzed (Figure 2(d)). A total of 44 dissemination nodules were detected in the NaCl group. The weights of these nodules in the NaCl group ranged from 7.2 to 193.8 mg (median: 37.8 mg; mean ± SEM: 49.2 ± 6.6 mg), and their volumes ranged from 9.4 to 295.3 mm^3 (median: 38.0 mm^3; mean ± SEM: 61.8 ± 9.9 mm^3). On the other hand, only 6 dissemination nodules were detected in the DW group. The weights of these nodules

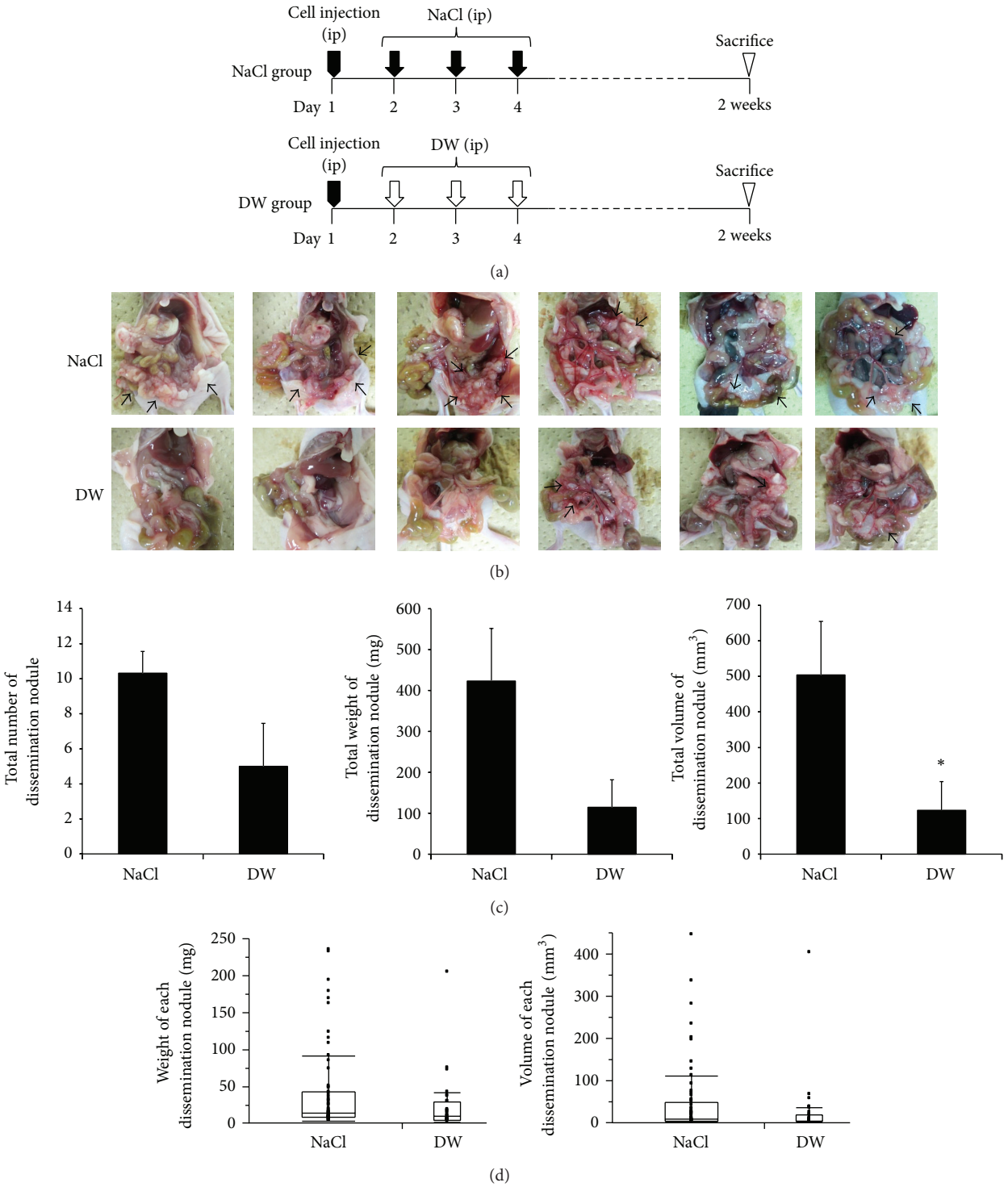

Figure 3: Inhibition of the establishment of peritoneal dissemination by the peritoneal injection of DW. (a) A total of 1.0×10^6 pelleted MKN45 cells were suspended in 0.3 mL PBS and then intraperitoneally injected into 4-week-old female nude mice on day 1. Two milliliters of NaCl buffer or DW was then injected into the abdominal cavities of nude mice from day 2 to day 4 (for 3 days). At a defined time point of 2 weeks after the intraperitoneal injection of cells, all mice were sacrificed, and the degree of peritoneal dissemination was evaluated macroscopically. $n = 6$. (b) Representative macroscopic findings of the abdominal cavities of all mice. Many dissemination nodules were established in all 6 mice injected with NaCl, whereas several nodules were only found in 3 mice injected with DW. Arrows indicate dissemination nodules. (c) The total number, weight, and volume of dissemination nodules in both groups. The total volume of dissemination nodules was significantly lower in the DW group than in the NaCl group. The results are presented as the mean ± SEM ($n = 6$) $^*P < 0.05$. (d) Box plots of dissemination nodules in both groups. No significant differences were observed in the weight or volume of each dissemination nodule in the two groups. $^*P < 0.05$. DW: distilled water.

in the DW group ranged from 2.9 to 15.6 mg (median: 5.2 mg; mean ± SEM: 7.8±2.5 mg), and their volumes ranged from 2.3 to 15.8 mm^3 (median: 5.5 mm^3; mean ± SEM: 7.6 ± 2.4 mm^3). The weight and volume of each dissemination nodule were markedly lower in the DW group than in the NaCl group. These results suggest that the preincubation of gastric cancer cells with DW may markedly inhibit the development of dissemination nodules in nude mice.

3.3. Inhibition of Establishment of Peritoneal Dissemination by the Peritoneal Injection of DW. We examined whether the peritoneal injection of DW inhibited the establishment of peritoneal dissemination. MKN45 cells were intraperitoneally injected into nude mice on day 1, and 2 mL of NaCl buffer or DW was injected into the abdominal cavities of nude mice from day 2 to day 4 (for 3 days) ($n = 6$ in each group). The development of dissemination nodules was analyzed 2 weeks after the cancer cell injection (Figure 3(a)). As shown in Figure 3(b), many dissemination nodules were established in all 6 mice injected with NaCl buffer, whereas several nodules were found in only 3 mice injected with DW. The total number of dissemination nodules was 50% less with the peritoneal injection of DW than with that of NaCl (NaCl group: 10.3 ± 1.2; DW group: 5.0 ± 2.5; $P = 0.081$) (Figure 3(c)). The total weight of dissemination nodules was slightly less in the DW group (114.8 ± 67.2 mg) than in the NaCl group (423.2 ± 128.6 mg) ($P = 0.059$) (Figure 3(c)). The total volume of dissemination nodules was significantly lower in the DW group (123.8 ± 80.0 mm^3) than in the NaCl group (504.7 ± 150.2 mm^3) (Figure 3(c)). We then analyzed each dissemination nodule in both groups (Figure 3(d)). A total of 62 dissemination nodules were detected in the NaCl group. The weights of these nodules in the NaCl group ranged from 1.9 to 235.9 mg (median: 13.6 mg; mean ± SEM: 41.0 ± 7.5 mg), and their volumes ranged from 0.8 to 445.5 mm^3 (median: 8.8 mm^3; mean ± SEM: 48.8 ± 11.1 mm^3). Thirty-one dissemination nodules were detected in the DW group. The weights of these nodules ranged from 0.5 to 205.3 mg (median: 9.1 mg; mean ± SEM: 22.2 ± 7.0 mg), and their volumes ranged from 0.8 to 405.0 mm^3 (median: 5.0 mm^3; mean ± SEM: 25.4 ± 13.0 mm^3). No significant differences were observed in the weight or volume of each dissemination nodule between the two groups (Figure 3(d)). These results suggested the restrictive efficacy of the peritoneal injection of DW in the initial stage of the establishment of peritoneal dissemination from gastric cancer.

4. Discussion

Although recent advances in surgical techniques, adjuvant therapy, chemoradiotherapy, and molecular targeted therapy have improved the prognosis of patients with gastric cancer, the long-term outcomes of these patients remain poor, especially for those with advanced disease. Peritoneal dissemination is the most frequently observed pattern of metastasis and recurrence in gastric cancer patients [3, 4]. Although limited success with treatment methods, such as intraperitoneal chemotherapy, has been reported [15, 16], novel strategies for the treatment of peritoneal dissemination from gastric cancer need to be developed to achieve better results. An improvement in the treatment of peritoneal dissemination from gastric cancer depends on a deeper understanding of the molecular mechanisms regulating tumorigenesis and progression of the disease.

Peritoneal dissemination is considered to be caused by free peritoneal cancer cells exfoliated from serosally invasive tumors, and previous studies have reported the cytocidal effects of hypotonic stress on cancer cells [10–12]. We previously investigated changes in the cellular morphology and volume of gastric cancer cells subjected to hypotonic shock using several unique methods and apparatus [13]. Video recordings using a high-speed digital camera demonstrated that hypotonic shock with DW induced swelling and then rupture in MKN28, MKN45, and Kato-III cells. Measurements of cell volume changes using a high-resolution flow cytometer indicated that severe hypotonicity with DW increased the number of broken fragments of these gastric cancer cells within 5 min. Furthermore, we reincubated these cells after they had been exposed to DW and found that the decrease observed in the number of cells in each of the three gastric cancer cell lines depended on the time for which they had been exposed to DW. Similar findings have been reported in esophageal and pancreatic cancer cells [17, 18]. These results suggest that hypotonic shock could be applied for the treatment of dissemination from gastric cancer by using a peritoneal injection of hypotonic solution. However, to the best of our knowledge, the toxicity of a peritoneal injection of hypotonic solution *in vivo* has not yet been examined in detail. In the present study, we analyzed both macroscopic and microscopic findings and found that the peritoneal injection of DW (2 mL, for 3 days) did not cause severe toxicity in mice. Regarding effects on noncancerous normal cells, we previously showed that severe hypotonic shock also induced cell rupture in human fibroblast WI38 cells [18]. However, the pathological findings of the present study revealed that the effect of hypotonic shock did not appear in the single layer peritoneal cells.

Exfoliated cancer cells were detected in the abdominal cavity following the resection of gastric cancer, and several studies have shown that peritoneal lavage fluid cytology is a significant independent prognostic factor in gastric cancer patients [19–21]. Exfoliated cancer cells from the primary tumor may be viable and tumorigenic; therefore, effective peritoneal lavage is clinically important at the time of the initial surgery. Peritoneal lavage with DW has been performed during surgery for various cancers. Lin et al. reported that peritoneal lavage with DW improved the survival rate of patients with spontaneously ruptured hepatocellular carcinoma [10]. Huguet and Keeling described the optimal method for peritoneal lavage with DW during colorectal cancer surgery [11], and Mercill et al. found that exposure to distilled water reduced the number of surviving gastric cells [12]. In the present study, we intraperitoneally injected gastric cancer cells preincubated with DW into nude mice and analyzed the development of dissemination nodules in order to determine the efficacy of peritoneal lavage with DW during surgery. Our results showed that the total number,

weight, and volume of dissemination nodules were significantly decreased by the DW preincubation. Furthermore, the weight and volume of each dissemination nodule were markedly lower in the DW pretreated group than in the NaCl pretreated group. These results suggested that peritoneal lavage with DW during surgery for gastric cancer may be effective at disrupting exfoliated cancer cells and preventing the development of dissemination nodules.

We also determined the efficacy of the peritoneal injection of DW for inhibiting the development of peritoneal dissemination nodules *in vivo* as a future treatment for patients with peritoneal dissemination from gastric cancer. Our results showed that the total volume of dissemination nodules was significantly lower in DW-injected mice than in NaCl-injected mice. These results confirmed the efficacy of the peritoneal injection of DW for preventing peritoneal dissemination from gastric cancer. However, its inhibitory effects appeared to be weaker than those observed in the DW preincubation model, as described above. One reason may be the increase in osmolarity by the DW injection due to the contamination of existing intraperitoneal secretions and many types of cells. We previously reported that very severe hypotonicity was needed to disrupt gastric cancer cells into fragments [13], and, therefore, we investigated only DW condition for *in vivo* model instead of different solutions containing different concentration of solute in the present study. We previously showed that the osmolarity of the fluid collected after peritoneal lavage with DW during surgery for gastric cancer was approximately 50 mosmol/kgH_2O due to the contamination of disrupted cells [13]. To keep the intra-abdominal osmolality as low as possible, the maximal dose of DW needed to be administered to mice. Therefore, we decided to inject 2 mL of buffer, referring to the circulating blood volume of mouse in the present study. The persistent perfusion of the abdominal cavity with DW may overcome this phenomenon and become an effective technique in clinical practice; however, it is difficult to establish an *in vivo* experimental model with mice.

Our results revealed that no significant differences in the weight and volume of each dissemination nodule existed between the DW-injected and NaCl-injected groups, whereas a prominent inhibitory effect was found in the DW preincubated model. These results suggested that the peritoneal injection of DW may not have enough effect for already established dissemination, whereas the cytocidal effects of hypotonic shock were sufficient for isolated single cells. Therefore, the hypotonic solutions used in peritoneal injections should be modified to enhance their inhibitory effects. The roles of ion and water channels/transporters have recently been reported in cancer cells [5–9] and are expected to become novel therapeutic targets. Various types of transporters have been found in gastric cancer, and we previously investigated the roles of Cl^- channels/transporters [22–25]. We showed that a treatment with 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), a Cl^- channel blocker, increased cell volume by inhibiting regulatory volume decrease (RVD) and enhanced the cytocidal effects of the hypotonic solution in MKN45 cells [13]. RVD was shown to occur after hypotonicity-induced cellular swelling and has been attributed to the activation of ion channels and transporters, which cause K^+, Cl^-, and H_2O effluxes, ultimately leading to cell shrinkage [26, 27]. Similar phenomena have been reported in esophageal and pancreatic cancer cells [17, 18]. We could not use NPPB, which blocks multiple types of chloride channels, in the present study because of its neurotoxicity *in vivo*. However, the development of a more specific chloride ion channel blocker or novel siRNA delivery system *in vivo* will make it possible to enhance the effects of the peritoneal injection of DW in the treatment of dissemination from gastric cancer. Furthermore, a hypotonic intraperitoneal cisplatin treatment with DW at the time of gastric resection was tolerated well by patients with gastric cancer [28], which suggested that the intraperitoneal DW injection may increase the uptake of anticancer drugs and enhances antitumor effects. Our results together with previous findings show the importance and possible application of these cellular physiological approaches for patients with dissemination from gastric cancer.

In conclusion, we demonstrated the safeness of a peritoneal injection of DW in an *in vivo* model. Our results indicated that the development of dissemination nodules *in vivo* was prevented by the preincubation of gastric cancer cells with DW or peritoneal injection of DW. A deeper understanding of the underlying molecular mechanisms may lead to the application of this cellular physiological approach, such as the regulation of osmolality, as a novel therapeutic strategy for dissemination from gastric cancer.

Conflict of Interests

None of the authors have any conflict of interests or financial ties to disclose.

Acknowledgments

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Research Article

Sodium Is Not Required for Chloride Efflux via Chloride/Bicarbonate Exchanger from Rat Thymic Lymphocytes

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Sodium-dependent Cl^-/HCO_3^- exchanger acts as a chloride (Cl^-) efflux in lymphocytes. Its functional characterization had been described when Cl^- efflux was measured upon substituting extracellular sodium (Na^+) by N-methyl-D-glucamine (NMDG). For Na^+ and Cl^- substitution, we have used D-mannitol or NMDG. Thymocytes of male Wistar rats aged 7–9 weeks were used and intracellular Cl^- was measured by spectrofluorimetry using MQAE dye in bicarbonate buffers. Chloride efflux was measured in a Cl^--free buffer (Cl^- substituted with isethionate acid) and in Na^+ and Cl^--free buffer with D-mannitol or with NMDG. The data have shown that Cl^- efflux is mediated in the absence of Na^+ in a solution containing D-mannitol and is inhibited by H_2DIDS. Mathematical modelling has shown that Cl^- efflux mathematical model parameters (relative membrane permeability, relative rate of exchanger transition, and exchanger efficacy) were the same in control and in the medium in which Na^+ had been substituted by D-mannitol. The net Cl^- efflux was completely blocked in the NMDG buffer. The same blockage of Cl^- efflux was caused by H_2DIDS. The study results allow concluding that Na^+ is not required for Cl^- efflux via Cl^-/HCO_3^- exchanger. NMDG in buffers cannot be used for substituting Na^+ because NMDG inhibits the exchanger.

1. Introduction

Lymphocyte intracellular chloride ($[Cl^-]_i$) is regulated by the relative activities of plasma membrane chloride (Cl^-) influx and Cl^- efflux pathways [1]. Literature data show that rat thymocytes (thymic lymphocytes) possess Na-dependent and Na-independent Cl^-/HCO_3^- exchangers ([1, 2]; Figure 1). So far, two classes of Cl^-/HCO_3^- exchangers have been described in mammalian cells. The first—the band 3 family of Cl^-/HCO_3^- exchanger (AE1, AE2, and AE3 isoforms)—is Na-independent [3]. It acts as a Cl^- influx mechanism (the intracellular HCO_3^- exchanger for extracellular Cl^-) [4, 5]. The second exchanger has been identified as a Na-dependent Cl^-/HCO_3^- exchanger acting as a Cl^- efflux mechanism [1, 2, 4]. The description of functional characterization of Na-dependent Cl^-/HCO_3^- exchangers in lymphocytes has been limited when evaluating fluorimetric intracellular pH change measurements [2]. Later, the same method was used while repeatedly evaluating fluorimetric $[Cl^-]_i$ data [1] in the way the Na-dependent Cl^-/HCO_3^- exchanger was described after cell Cl^- efflux had been measured upon substituting extracellular Na^+ by N-methyl-D-glucamine (NMDG).

In the present study, we examined Cl^- efflux by using the fluorimetric Cl^- dye N(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) to determine rat thymocyte $[Cl^-]_i$ changes during acute exposure to Cl^--free media. For Na^+ and Cl^- substitution, we used D-mannitol or NMDG. We show that Cl^- efflux is mediated in the absence of Na^+ in a solution containing D-mannitol and is totally inhibited by H_2DIDS.

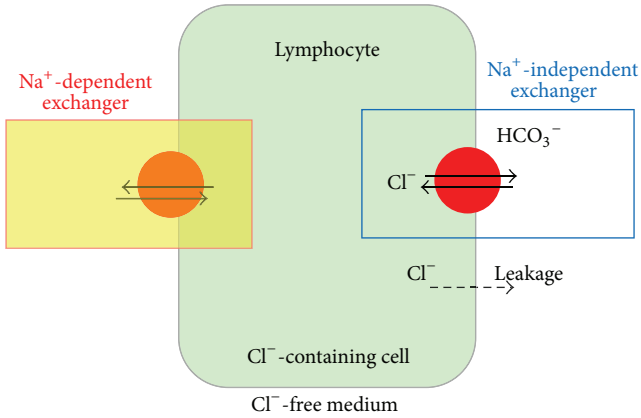

FIGURE 1: Schematic representation of a thymocyte contained in chloride-free and either sodium *containing* or sodium-*free* medium and pathways and mechanisms of chloride fluxes. Only the pathways and mechanisms of the main concern considered relevant for the present investigation are depicted here. The continuous arrows symbolize the controlled ion flux and the broken one, the uncontrolled flux (leakage), of the ions from the cell. Chloride fluxes (and its efflux) are believed to depend on sodium presence in the medium, that is, on the exchangers of both types (Na^+-dependent and Na^+-independent) depicted on both sides of the cell. No contribution of the sodium-dependent exchanger to chloride efflux from rat thymocytes could be observed in this investigation.

2. Material and Methods

Thymocytes were isolated from the *glandula thymus* of male Wistar rats aged 7–9 weeks. Experiments were performed in compliance with the relevant laws and institutional guidelines. The permissions of the State Food and Veterinary Service of Lithuania to use experimental animals for research were obtained (25/07/2013). All bicarbonate-containing buffers were preequilibrated with 5% CO_2, 20% O_2, and 75% N_2 and were kept at 37°C. The composition of buffers is as follows: a standard 100% Cl^- buffer (solution 1), a Cl^--free buffer (solution 2), a Na^+- and Cl^--free buffer with D-mannitol (solution 3), and Na^+- and Cl^--free buffer containing N-methyl-D-glucamine (solution 4) (Table 1).

$[Cl^-]_i$ was measured by the spectrofluorimetric method using MQAE dye as described [1]. Chloride efflux in control thymocytes was measured ($n = 8$) in a buffer in which Cl^- had been substituted with isethionate acid (solution 2), in a Na^+- and Cl^--free buffer containing D-mannitol ($n = 8$; solution 3), in thymocytes pretreated with 125 μM H_2DIDS in a Na^+- and Cl^--free buffer containing D-mannitol ($n = 4$) and in a buffer containing NMDG ($n = 4$; solution 4). The buffer isotonicity was calculated according to NaCl equivalents [6]. Fluorescence measurements were performed with a Perkin Elmer 50B spectrofluorometer (excitation wavelength 352 nm and emission wavelength 450 nm). $[Cl^-]_i$ was calculated by using a procedure as described [1, 7]. MQAE, H_2DIDS, and all other chemicals were purchased from Sigma, Sigma-Aldrich, Fluka, AppliChem, BioEKSMA.

For modelling and model fitting, standard software was used. The models are based on the scheme presented in Figure 1. The systems of simple differential equations were solved with *Maple*. Data processing was carried out using *Microsoft Excel* [8].

3. Results

3.1. General Considerations, Supposed Relationships, and Modeling. The $[Cl^-]_i$ level of thymocytes after their acute exposure to different buffers shows no statistically significant difference ($P > 0.05$): 22.6 ± 2.7 mM in solution 2 (see Table 1), 22.1 ± 3.2 in the same solution pretreated with H_2DIDS, 21.0 ± 2.9 in solution 3, 21.5 ± 2.4 in the same solution pretreated with H_2DIDS, and 19.7 ± 1.3 mM with NMDG (solution 4). In the presence of Cl^- and HCO_3^- in thymocytes, acute exposure of the cells to an isotonic Cl^--free solution and Na^+- and Cl^--free solutions with a D-mannitol substitute resulted in a rapid decline of $[Cl^-]_i$.

The current understanding of the mechanisms of Cl^- efflux from thymocytes is presented in the scheme depicted in Figure 1. The efflux from the cells in which the activity of the exchanger is inhibited has to be assigned to a noncontrollable leakage of chloride anions through the membrane. The rate of the leakage (F_{leak}), presumably, can be expressed as follows:

$$F_{leak} = -\alpha y, \tag{1}$$

where α is relative permeability of cellular membrane and y is transmembrane difference of Cl^- concentration or just Cl^- concentration in the cytoplasm (if the medium is Cl^--free). The solution of the above equation is

$$y = y_0 \exp(-\alpha t), \tag{2}$$

y_0 being initial Cl^- concentration in the cytoplasm. The model is depicted in Figure 2.

Experimental data of cytoplasmic Cl^- concentrations in the absence of Cl^-/HCO_3^- exchange inhibition (Figure 2) suggest the decline to proceed with the relative rate different from constant. At the beginning it is slow, then fast, and slow again, keeping in mind that the observed decline in Cl^- concentration results from both noncontrollable Cl^- leakage via the cellular membrane and its efflux via the exchanger. The total efflux rate can be presented as follows [8]:

$$\frac{dy}{dt} = -\alpha y - A\frac{\lambda\mu}{\lambda-\mu}\left(\exp(-\mu t) - \exp(-\lambda t)\right), \tag{3}$$

where A symbolizes the efficacy of the exchanger and λ and μ stand for relative rates of rise and decline of its activity, the parameters λ and μ being interchangeable. The solution of the above equation is

$$y = y_0 \exp(-\alpha t) - A \exp(-\alpha t)\,\lambda\mu \times \left(\frac{(\mu-\alpha)\exp(-(\lambda-\alpha)t)}{(\lambda-\alpha)(\mu-\alpha)(\lambda-\mu)} - \frac{(\lambda-\alpha)\exp(-(\mu-\alpha)t) + (\lambda-\mu)}{(\lambda-\alpha)(\mu-\alpha)(\lambda-\mu)}\right). \tag{4}$$

Table 1: Solution compositions (mM).

Ingredient	Solution 1 (100% Cl^-)	Solution 2 (Cl^--free)	Solution 3 (Cl^-- and Na^+-free with D-mannitol)	Solution 4 (Cl^-- and Na^+-free with NMDG)
Glucose	5	5	5	5
HEPES	5	5	5	5
$MgSO_4$	0.8	0.8	0.8	0.8
NaH_2PO_4	1	1	0	0
KH_2PO_4	0	0	1	1
Ca acetate	1.8	1.8	0	0
NaCl	96	0	0	0
Na isethionate	16.4	117.3	0	0
D-mannitol	0	0	185	0
KCl	5.3	0	0	0
N-methyl-D-glucamine	0	0	0	185
K gluconate	0	0	4.3	0
$KHCO_3$	0	5.3	0	4.3
$NaHCO_3$	22	16.7	0	0
Choline HCO_3	0	0	22	22

Table 2: Conditions of the experiments and model parameters estimated by fitting (2) and (4) to experimental data.

Parameter	Notation	Estimate	
		Na^+-containing medium (solution 2)	Na^+-free medium (solution 3)
Initial chloride concentration in cytoplasm, mM	$y_{cytoplasm}$	22.6	21.0
Relative membrane permeability, s^{-1}	α	0.000533	0.000774
A rate constant of the exchanger transition, s^{-1}	λ	0.000547	0.00547
Another rate constant of the exchanger transition, s^{-1}	μ	0.0169	0.0169
Exchanger efficacy, mM	A	12.1	12.2

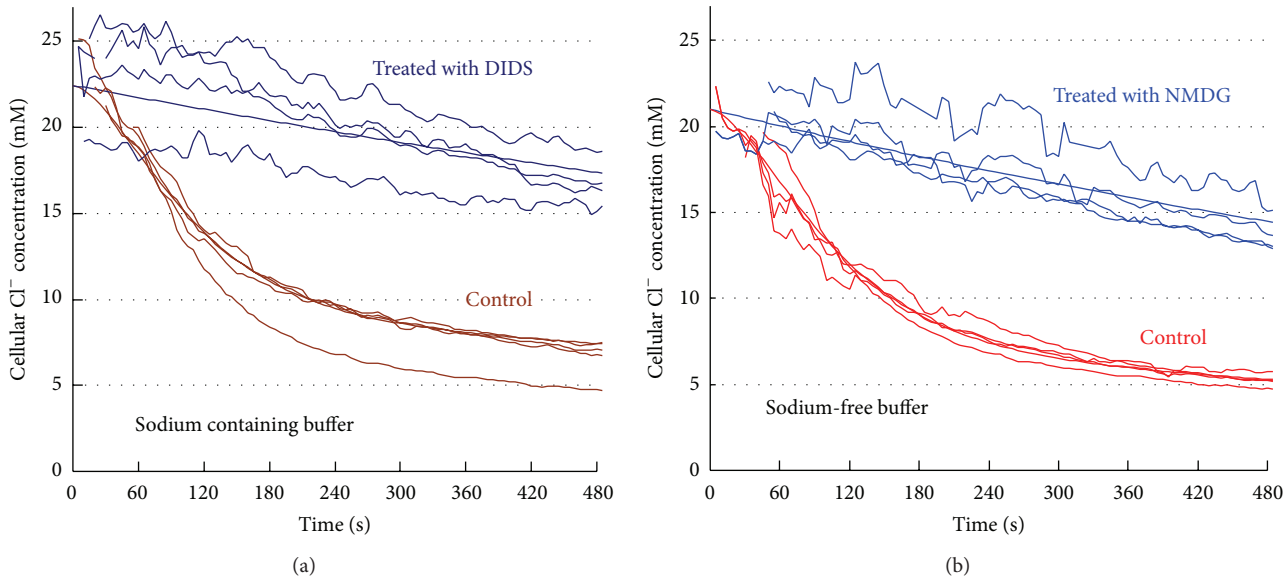

Figure 2: Chloride efflux from thymocytes (treated either with H_2DIDS ($n = 4$) or with NMDG ($n = 4$)) into chloride-free, bicarbonate-*containing*, and either sodium-*containing* or sodium-*free* medium. The smooth curves correspond to model (2) (noncontrollable chloride efflux) or model (4) (taking into account the contribution of the exchanger) with the parameter values presented in Table 2. The controls were Na isethionate (a) and D-mannitol (b). The data (zigzagged lines) are representative of $n = 8$ (a) and $n = 8$ experiments (b).

3.2. Comparison of Models and Experimental Data. The data together with the models are presented in Figure 2. The models seem quite acceptable suggesting the plausibility of the initial assumptions; together with the parameters contained in Table 2 they are discussed in detail in the next section.

4. Discussion

It is commonly accepted that in rat thymocytes the intracellular Cl^- level is regulated by Na-independent and Na-dependent Cl^-/HCO_3^- exchangers and Na-K-2Cl cotransporter [1, 2]. Two classes of Cl^-/HCO_3^- exchangers related to $[Cl^-]_i$ level regulation were identified in mammalian cells: band 3 family (AE1, AE2, and AE3) was found to be Na-independent [3]. It normally acts as a Cl^- influx mechanism [2, 9]. Lymphocytes express the AE2 isoform which shows a lower affinity for DIDS than does AE1 [4, 9, 10]. The Cl^-/HCO_3^- exchanger, which was described as a Na-dependent Cl^-/HCO_3^- one, acts as a Cl^- efflux mechanism in experiments of external Cl^- removal [2, 11]. The Na-dependent Cl^-/HCO_3^- exchanger in thymocytes was described when it was evaluated by measuring intracellular pH changes [2]. Later, this exchanger was evaluated by measuring $[Cl^-]_i$ in the experimental conditions identical to those reported earlier [1]. Evidence for the existence of a Na-dependent Cl^-/HCO_3^- exchanger comes from studies examining the effects of DIDS and Na^+ removal on net Cl^- efflux, in which Na^+ and Cl^- were substituted by NMDG. Such experimental data show that the net Cl^- efflux is completely blocked by the acute removal of Na^+ from the external medium, and this fact led to the conclusion that Cl^-/HCO_3^- exchange was due to a Na-dependent mechanism. The nature of the "blockade" mechanism is not clear, nor has the Na-dependent Cl^-/HCO_3^- exchanger been isolated and cloned to date.

The study was undertaken to examine the Na^+ involved in Cl^- efflux from rat thymocytes, using another Cl^- and Na^+ substitute D-mannitol. Examination of the Cl^- efflux pathway was performed by acutely exposing cells to Na-Cl^--free media and determining changes in the $[Cl^-]_i$ level. The basal $[Cl^-]_i$ level in the study of thymocytes was similar to $[Cl^-]_i$ levels as found by others for different cell types such as rat thymocytes [1], vascular smooth muscle cells [7, 12], and astrocytes [13].

In the presence of Cl^- and HCO_3^- in the thymocytes, acute exposure of the cells to an isotonic Cl^--free solution and Cl^-- and Na^+-free solutions with a D-mannitol substitute resulted in a rapid decline of $[Cl^-]_i$. The parameters of the mathematical model (relative membrane permeability, relative rate of exchanger transition, and exchanger efficacy) show that Cl^- efflux in a bicarbonate buffer in which Cl^- is substituted with Na isethionate is the same as in the Na^+-Cl^--free buffer in which these ions are substituted isotonically with D-mannitol.

We stress that the buffer for experiments examining Na-dependent Cl^- efflux where Na^+ and Cl^- are substituted by NMDG cannot be used for evaluating Cl^- efflux. The NMDG properties listed below should be estimated while investigating a Na-dependent Cl^-/HCO_3^- exchanger. The use of NMDG to substitute monovalent cations requires pH adjustment [14–16]. Following the reconstitution with deionised water, the pH of 185 mM of the NMDG solution was 11.4 due to the alkaline properties of NMDG (its molecule contains a charged methylamine head group responsible for alkalinity), as NMDG has the ionisation equilibrium constant $K_b = 3.98 \cdot 10^{-5}$ [17]. Adjustment of the pH of its solution requires large quantities of acids: 125 mM of H_2SO_4 has to be added to adjust the pH of the solution to 7.3. The osmolarity of the solution was 6.2 atm before pH adjustment and 17.4 atm after it (own calculation).

Historically, NMDG has been used for several decades for the substitution of monovalent cations, assuming that it does not cross the cell membrane. However, today our knowledge of NMDG permeability through ion channels has undergone essential changes. NMDG permeation has been reported in ion channels such as ATP-gated P2X [18], epithelial Ca^{2+} channel ECaC [19], glutamate receptor [20], mechanosensitive channels [21], and mutant Na^+ channels [22]. Some transient receptor potential family cation channels display partial permeability to NMDG [23]. In the absence of K^+, significant NMDG currents were recorded in human kidney cells expressing Kv3.1/Kv3.2b and Kv1.5 R487Y/V channels. Inward currents were much stronger because of the blockade of the outward currents by intracellular Mg^{2+}, resulting in a strong inward rectification [16]. NMDG rapidly blocks Ca^{2+}-activated K^+ channels from the inside of the membrane [24]. Extracellular NMDG causes a partial block of outward currents in the TRPC3 member of the transient receptor potential family cation channels [25], and the intracellular NMDG modifies the properties of the Ca^{2+} L-type channel in guinea pig cardiac myocytes by increasing the overall duration of the Ca-dependent slow action potential 6-fold at 0 mV [26]. NMDG increases the intracellular Ca^{2+} and K^+ in Ehrlich Lettre ascite cells, changing the intracellular pH [27].

When using NMDG for Na^+ substitution, the solution hypertonicity, alkaline features of the substitute, and an additional high concentration of anions appearing after buffer adjustment with acids could change the functioning of the exchanger. AE2 studies have also shown that it is capable of transporting a number of different anions working in a number of different modes of exchange such as Cl^-/Cl^- and sulfate/chloride [28]. Band 3 protein may also be able to exchange Cl^-/OH^- [29]; besides, it has been shown to transport sulphate [30, 31] and phosphate anions [32].

The study data show that there is no Cl^- efflux from thymocytes in a bicarbonate buffer in which Na^+ and Cl^- are substituted by NMDG. However, Cl^- efflux through Cl^-/HCO_3^- exchanger occurs when the neutral substitute D-mannitol is used instead of NMDG. This means that extracellular Na^+ is not required for Cl^- efflux in rat thymocytes. This could be in agreement with studies indicating that in experimental conditions of external Cl^- removal the AE2 antiporter can be reversed; that is, it works at both sides of a lymphocyte membrane [4, 33]. Pretreatment of cells with DIDS before exposure to a Cl^--free solution inhibited the decline in $[Cl^-]_i$, suggesting that the Cl^-/HCO_3^- exchange

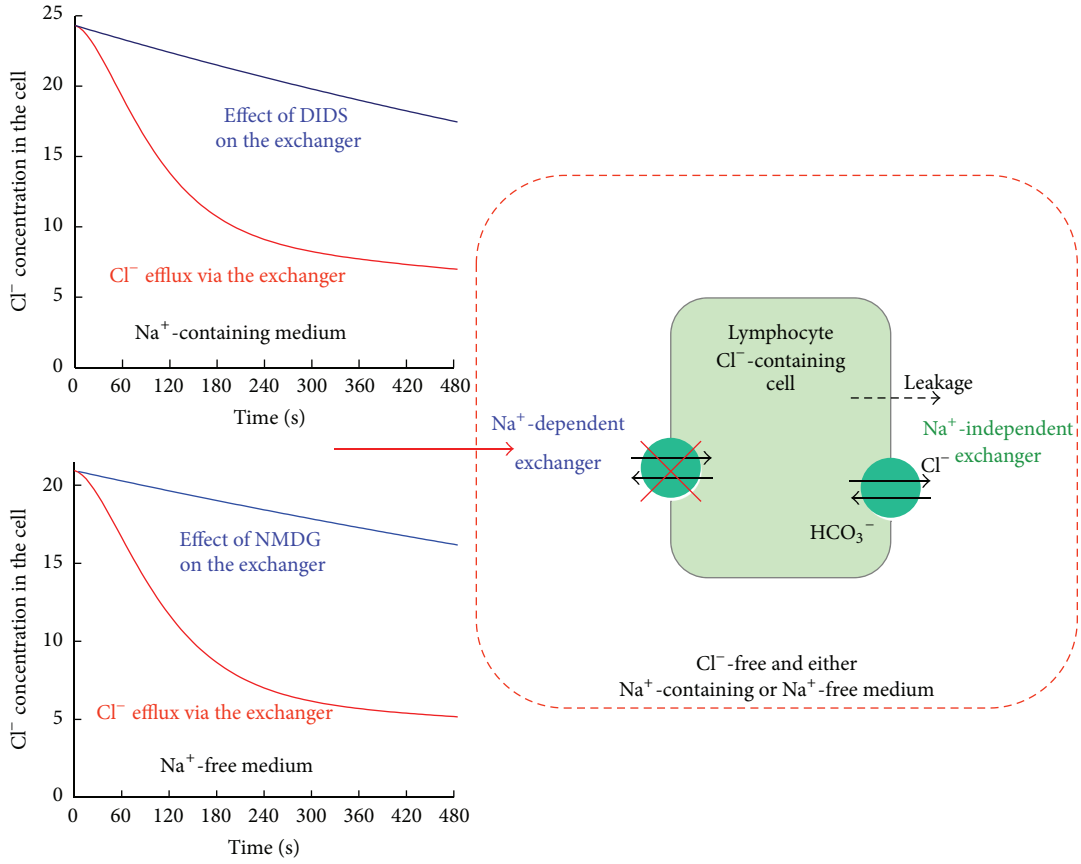

Figure 3: The summarized results of the Cl^- efflux study.

mechanism is responsible for Cl^- efflux in these cells. The stilbene compound (DIDS, SITS) binding site is located on the outer surface of the membrane rather than buried within the pocket formed by the tertiary complex of the protein [34, 35]. No contribution of Na dependence of Cl^-/HCO_3^- exchanger has been observed in the study. The main data of the study are summarized in Figure 3.

Hence, it follows that the presence or absence of Na^+ in the extracellular medium is not relevant for Cl^- efflux from thymocytes, implying that Cl^- efflux in experimental conditions is related to Cl^-/HCO_3^- exchanger (band 3) in thymocytes. Sodium substitution with NMDG leads to an inhibition of the Cl^-/HCO_3^- exchanger. The higher estimate of noncontrolled leakage (α, see Table 2) suggests an incomplete inhibition at the NMDG concentration used in the study.

5. Conclusions

The main results of this work can be summarized as follows:

(i) Na^+ is not required for Cl^- efflux via Cl^-/HCO_3^- exchanger from rat thymocytes;

(ii) no Na-dependent Cl^-/HCO_3^- exchanger is present in rat thymocytes;

(iii) NMDG leads to exchanger inhibition.

Conflict of Interests

Authors of this research have no conflict of interests regarding this paper.

Acknowledgment

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Review Article

Nanotechnology-Applied Curcumin for Different Diseases Therapy

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Curcumin is a lipophilic molecule with an active ingredient in the herbal remedy and dietary spice turmeric. It is used by different folks for treatment of many diseases. Recent studies have discussed poor bioavailability of curcumin because of poor absorption, rapid metabolism, and rapid systemic elimination. Nanotechnology is an emerging field that is potentially changing the way we can treat diseases through drug delivery with curcumin. The recent investigations established several approaches to improve the bioavailability, to increase the plasma concentration, and to enhance the cellular permeability processes of curcumin. Several types of nanoparticles have been found to be suitable for the encapsulation or loading of curcumin to improve its therapeutic effects in different diseases. Nanoparticles such as liposomes, polymeric nanoparticles, micelles, nanogels, niosomes, cyclodextrins, dendrimers, silvers, and solid lipids are emerging as one of the useful alternatives that have been shown to deliver therapeutic concentrations of curcumin. This review shows that curcumin's therapeutic effects may increase to some extent in the presence of nanotechnology. The presented board of evidence focuses on the valuable special effects of curcumin on different diseases and candidates it for future clinical studies in the realm of these diseases.

1. Introduction

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione, is a lipophilic molecule that rapidly permeates cell membrane [1]. Typical extract of *Curcuma longa* L. contains the structures I to III: (I) diferuloylmethane/curcumin (curcumin I, 75%), (II) demethoxycurcumin (curcumin II, 20%), and (III) bisdemethoxycurcumin (curcumin III, 5%) [2, 3] (Figure 1). Curcumin is an active ingredient in the herbal remedy and dietary spice turmeric [4]. It has a long history of administration by different folks of China, India, and Iran for the treatment of many diseases such as diabetes, liver diseases, rheumatoid diseases, atherosclerosis, infectious diseases, cancers, and digestive disorders such as indigestion, dyspepsia, flatulence, and gastric and duodenal ulcers [5, 6]. Many researchers have worked on curcumin due to its various therapeutic effects on different diseases. Shortly, curcumin has received attention mostly due to its antioxidant, anti-inflammatory, antitumoral, apoptosis-inducing, and antiangiogenesis effects, which were reported in many investigations. It acts on multiple targets in cellular pathways making this agent able to perform multiple actions [7]. The simple molecular structure along with the relative density of functional groups in curcumin provides researchers with an outstanding target for structure-activity relationship and lead optimization studies. The structural analogues of curcumin have been reported to enhance the rate of absorption with a peak plasma half-life [8–10]. Recent investigations have considered curcumin a lead compound for designing new chemotherapeutic agents for treatment of cancers including colon cancers [11], prostate cancers [12], and other conditions with indication of chemotherapy [13, 14].

Curcumin is remarkably well tolerated, but its bioavailability is poor. It does not appear to be toxic to animals [15] or humans [16], even at high doses. Recent studies have

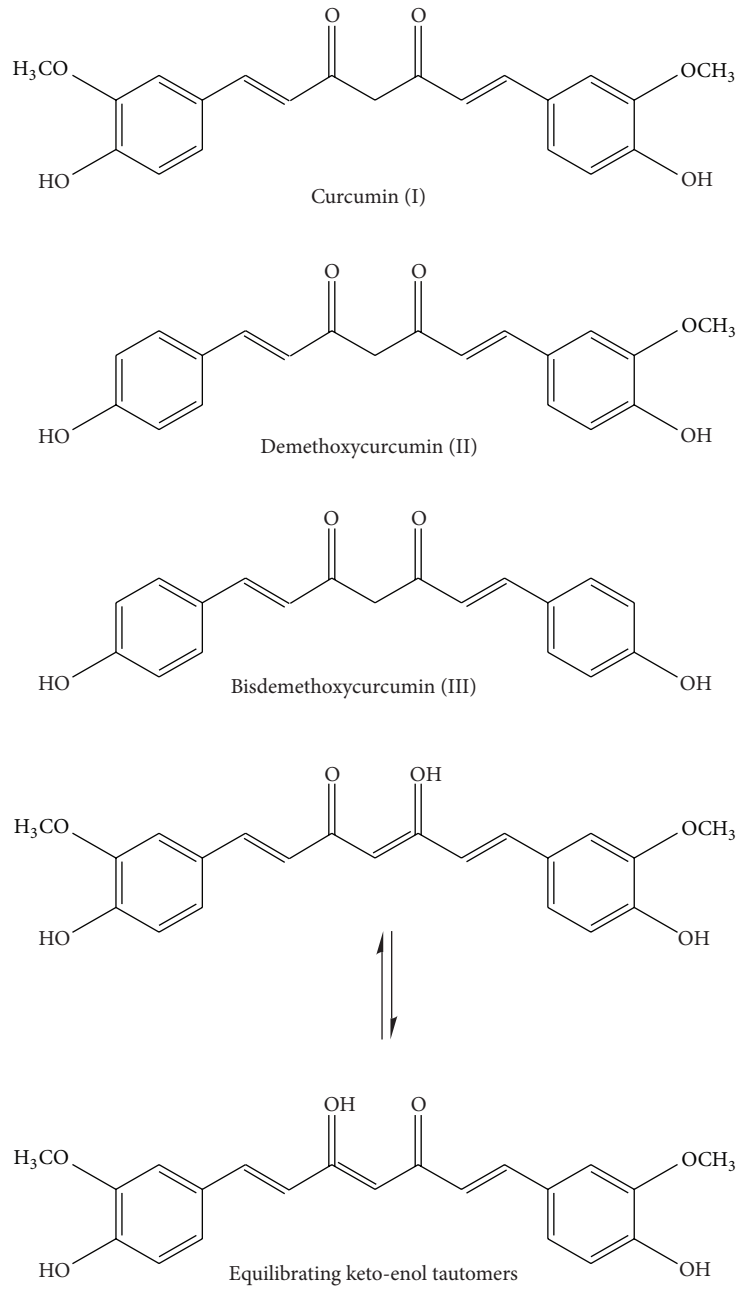


Figure 1: Curcumin I, II, and III (curcumin, demethoxycurcumin, and bisdemethyoxycurcumin) and curcumin keto-enol tautomers.

discussed poor bioavailability of curcumin because of poor absorption, rapid metabolism, and rapid systemic elimination [17, 18]; however, comprehensive pharmacokinetic data are still missing. In a study done by Yang et al. [19], they reported 1% bioavailability for oral administration of curcumin in rats. On the elimination of curcumin, an investigation in rat model demonstrated that after oral administration of 1 g/kg of curcumin, more than 75% was excreted in feces and negligible amount of curcumin was detected in urine [20]. Additionally, FDA has declared curcumin as "generally safe." Although curcumin showed a wide variety of useful pharmacological effects and has been found to be quite safe in both animals and humans, there are some studies concerning its toxicity [21]. In spite of these advantages, curcumin has poor water solubility; as a consequence, it reveals solubility-limited bioavailability, which makes it a class II drug in the biopharmaceutics classification system [22]. Additionally, due to its rapid intestinal and hepatic metabolism, about 60% to 70% of an oral dose of curcumin gets eliminated by the feces [23].

As mentioned above, curcumin has been proven to be effective in treatment of different diseases with low toxicity to human and animals. It is extremely safe upon oral administration even at very high doses; however, it is limited due to its poor bioavailability, stability, low solubility, and rapid degradation and metabolism. Overcoming these problems

has been the main goal of many studies over the past three decades. Since curcumin was demonstrated to have poor bioavailability and selectivity [17, 24], numerous analogues of this material have been introduced and tested in order to evaluate their activities against known biological targets and to also improve their bioavailability, selectivity, and stability [25–28]. In addition, several approaches were introduced to improve the bioavailability, to increase the plasma concentration, and to enhance the cellular permeability and resistance to metabolic processes of curcumin. Using nanoparticles for targeting drug delivery appeared to provide curcumin with longer circulation, better permeability, and stronger resistance to metabolic processes.

2. Nanotechnology Approaches for Curcumin

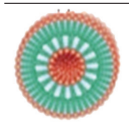
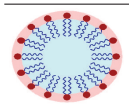
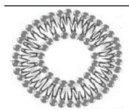
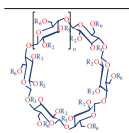
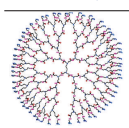
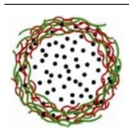
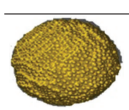
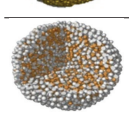
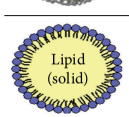
Nanotechnology is increasingly considered to be the technology of the future. Among the wide applications of nanotechnology is the use of nanoparticles for enhancing the bioavailability and the solubility of lipophilic compounds such as curcumin in drug delivery systems. Therefore, applying nanoparticles gained immense popularity in the last decade due to their potential to improve the therapeutic effects of the encapsulated drugs by protecting drugs from enzymatic degradation, providing their controlled release and prolonged blood circulation, changing their pharmacokinetics, decreasing their toxicity, and limiting their nonspecific uptake [29]. Over a period of time, numerous emphases have been given to develop the biodistribution of natural curcumin, but it is only just recently that the application of the field of nanotechnology has considerably enhanced its therapeutic effects. Nanoparticles such as liposomes, polymeric nanoparticles, micelles, nanogels, niosomes, cyclodextrins, dendrimers, silvers, and solid lipids are emerging as one of the useful alternatives that have been shown to deliver therapeutic concentrations of curcumin. The use of the above nanoparticle has improved main problems of curcumin such as low solubility, instability, poor bioavailability, and rapid metabolism in cancers, wound healing, Alzheimer's disease, epilepticus, ischemia diseases, inflammatory diseases, and so on (Table 1).

3. Liposomes

Liposomes are synthetic vesicles with globular character that can be produced from natural phospholipids [82]. They are self-assembling closed colloidal constructions composed of lipid bilayers, and they have a spherical shape in which an outer lipid bilayer surrounds a central aqueous space [83]. The liposome diameter varies from 25 nm to 2.5 mm (Table 1). They are stated to act as immunological adjuvants and drug carriers. Liposomes can encapsulate drugs with widely varying solubility or lipophilicity, entrapped either in the aqueous core of the phospholipid bilayer or at the bilayer interface [84]. Moreover, they are able to deliver drugs into cells by fusion or endocytosis, and practically any drug, irrespective of its solubility, can be entrapped into liposomes (Figure 2). In this regard, to enhance the solubility of curcumin, Rahman et al. [30] prepared β-cyclodextrin-curcumin inclusion complexes that entrapped both native curcumin and the complexes separately into liposomes. All curcumin-containing formulations were effective in inhibiting cell proliferation in in vitro cell culture. In another study, Shi et al. [31] developed a water-soluble liposomal curcumin to examine curcumin's preventive effects on lung fibrosis via intravenous administration in mice by using enzyme-linked immunosorbent assay method (ELISA). Results showed that liposomal curcumin can effectively diminish radiation pneumonitis and fibrosis of lung and sensitize LL/2 cells to irradiation. These data suggest that the systemic administration of liposomal curcumin with enhanced solubility is safe and deserves to be investigated for further clinical application.

Some studies showed that the drugs encapsulated in liposomes are expected to be transported without rapid degradation and result in minimum side effects and show more signs of stability in the recipients. In this regard, to assess curcumin tissue distribution, Matabudul et al. [32] questioned whether different durations of intravenous infusions of Lipocurc can alter curcumin metabolism and its tissue distribution and whether treating necropsied tissues of Beagle dogs with phosphoric acid prior to measuring curcumin and its metabolite (tetrahydrocurcumin) can stabilize the compounds allowing for accurate analytical measurements. Results demonstrated that the addition of liposomes may inhibit or saturate a putative reductase enzyme that converts curcumin to tetrahydrocurcumin and stabilizes the levels of curcumin. Tetrahydrocurcumin in some tissues (lung, spleen, and liver), but not all the examined tissues (lung, spleen, liver, pancreas, kidney, and urinary bladder), raised issues of tissue-specific curcumin and tetrahydrocurcumin stability via a transporter-dependent mechanism that elevated tissue concentrations of curcumin. Additionally, to obtain better understanding of curcumin interaction mechanisms with lipid membranes and improve the stability of curcumin, Karewicz et al. [33] banded curcumin to egg yolk phosphatidylcholine, dihexadecyl phosphate and cholesterol, then in order to determine curcumin binding constant to liposomes they used absorption and fluorescence techniques. The egg yolk phosphatidylcholine/dihexadecyl phosphate/cholesterol liposomal bilayer curcumin stabilized the system proportionally to its content, while the egg yolk phosphatidylcholine/dihexadecyl phosphate system destabilized upon drug loading. The three-component lipid composition of the liposome seems to be the most promising system for curcumin delivery. Furthermore, an interaction of free and liposomal curcumin with egg yolk phosphatidylcholine and mixed monolayers was also studied by using Langmuir balance measurements. Condensing effects of curcumin on egg yolk phosphatidylcholine and egg yolk phosphatidylcholine/dihexadecyl phosphate monolayers and loosening influence on egg yolk phosphatidylcholine/dihexadecyl phosphate/cholesterol ones were observed. It was also demonstrated that curcumin-loaded egg yolk phosphatidylcholine liposomes are more stable upon interaction with the model lipid membrane than the unloaded ones. In another study, Chen et al. [85] reported the effects of different liposomal formulations on curcumin stability in phosphate buffered saline, human blood, plasma, and culture medium.

Table 1: Nanoparticles-conjugated curcumin characterization for different diseases treatment.

	Type of nanoparticles	Form	Size (nm)	Used models	Methods	Results	Reference
	Liposome	Globular	25–205	(i) Breast cancer (ii) Melanoma (iii) Renal ischemia (iv) Malaria	In vitro In vivo (dog and mice)	(i) Increased solubility, tissue distribution, and stability (ii) Enhanced antitumor and antiangiogenesis effects (iii) Showed antimelanoma, anti-inflammatory, and antimalarial effects	[30–33] [34–37] [38, 39]
	Micelle	Spherical	10–100	(i) Lung tumor (ii) Breast cancer	In vitro In vivo (mice)	(i) Increased solubility and bioavailability (ii) Improved antioxidative and antitumor effects (iii) Prolonged circulation time (iv) Enhanced fluorescence effect	[40] [41] [42] [43] [44] [45] [46]
	Noisome	Lamellar	190–1140	(i) Albino rat skin (ii) Cancerous cells	In vitro In vivo (snake and mice)	(i) Increased skin penetration (ii) Prolonged delivery system (iii) Anti-infection and anticancer effects (iv) Enhanced fluorescence intensity	[47] [48] [49]
	Cyclodextrin	Cyclic	150–500	(i) Bowel disease (ii) Breast, lung, pancreatic, and prostate cancer	In vitro In vivo (rat and mice)	(i) Improved solubility (ii) Enhanced antiproliferation effects (iii) Increased anticancer and anti-inflammatory effects (iv) Developed bioavailability	[30] [50–56]
	Dendrimer	Globular polymer	15–150	(i) Breast cancer (ii) Colon cancer	In vitro In vivo (mice)	(i) Improved stability (ii) Increased antitumor and antiproliferative effects	[57, 58] [59–63]
	Nanogel	Cross-linked polymer network	10–200	(i) Melanoma (ii) Breast and pancreatic cancer cells	In vitro	(i) Increased stability (ii) Enhanced fluorescence effects (iii) Developed bioavailability (iv) Improved anticancer effects (v) Get better controlled release (vi) Prolonged half-life (vii) Enhanced treatment of melanoma	[64] [65]
	Chitosan	Linear polysaccha-ride composed	100–250	(i) Wounds (ii) Melanoma tumors	In vitro In vivo (rat and mice)	(i) Improved chemical stability (ii) Showed wound healing effects (iii) Increased antitumor effects (iv) Improved antioxidant effects (v) Prolonged blood circulation	[66–71]
	Gold	Globular	200–250	Cancerous cells	In vitro	(i) Improved solubility (ii) Enhanced antioxidant and anticancer effects	[72] [73]
	Silver	Film layer	~15	(i) Infections (ii) Skin wounds	In vitro	(i) Showed antimicrobial effects (ii) Improved wound healing (iii) Increased antiviral and anticancer effects	[74] [75]
	Solid lipid	Spherical	50–1000	(i) Cerebral ischemia (ii) Colitis (iii) Allergy (iv) Breast cancer	In vitro In vivo (rat and mice)	(i) Prolonged circulation of blood (ii) Increased anti-inflammatory effects (iii) Improved brain delivery	[76–78] [79–81]

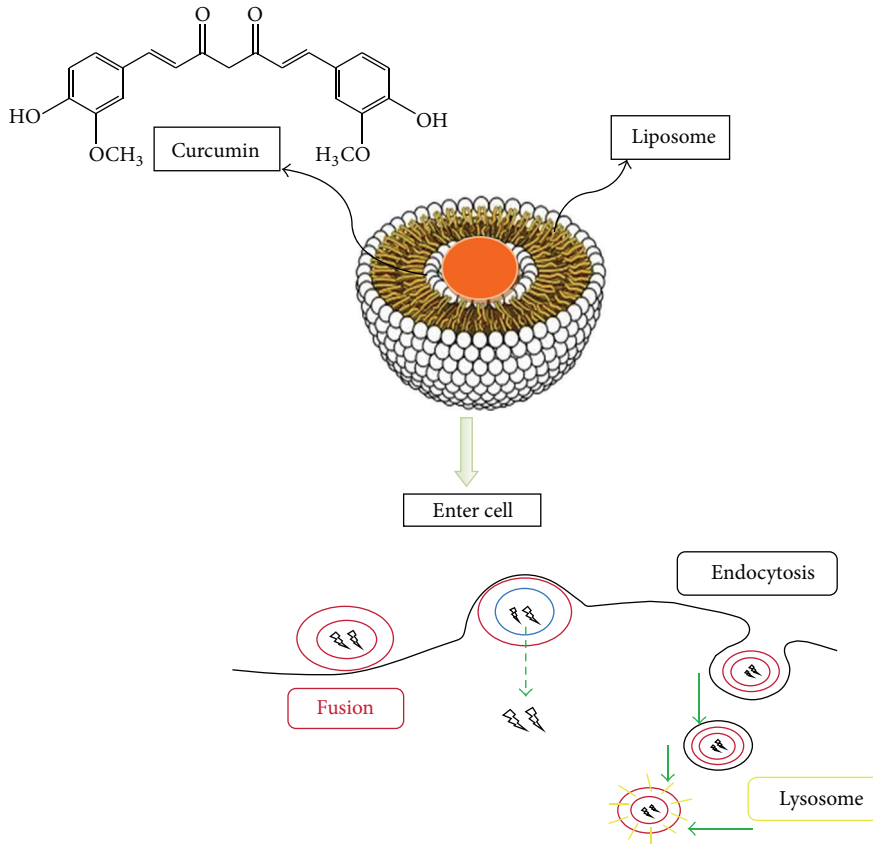


Figure 2: A schematic figure of how curcumin is located in liposomes and enters into cells. Curcumin is encapsulated inside the liposomal container and covalently bound to liposome, so it is protected from destruction on the way to the target. The liposome membrane is usually made of phospholipids, which constitute biological membranes and can deliver curcumin into cells by two different ways: fusion and endocytosis.

Liposomal curcumin showed a higher stability than free curcumin in phosphate buffered saline (PBS). Liposomal and free curcumin showed similar stability in human blood plasma and culture medium. In addition, results on the toxicity of concanavalin-A showed that dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol were toxic on lymphoblastoid cell lines. However, addition of cholesterol to the lipids at dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol/cholesterol almost completely eliminated the lipid toxicity to these cells. Liposomal curcumin had similar or even stronger inhibitory effects on concanavalin-A-stimulated human lymphocyte, splenocyte, and lymphoblastoid cell proliferation. They concluded that liposomal curcumin may be useful for intravenous administration to improve the bioavailability and efficacy, facilitating the in vivo studies that could ultimately lead to clinical application of curcumin.

In addition, liposomal curcumin's potential was evaluated against cancer models of osteosarcoma and breast cancer by Dhule et al. [34] with curcumin-loaded γ-cyclodextrin liposomal nanoparticles. The results showed promising anticancer potential of liposomal curcumin both in vitro and in vivo against osteosarcoma and breast cancer cell lines via the caspase cascade that leads to apoptotic cell death. The efficiency of the liposomal curcumin nanoparticles was also confirmed by using a xenograft osteosarcoma model in vivo. Li et al. [9] encapsulated curcumin in a liposomal delivery system for intravenous administration. They also showed the liposome-encapsulated curcumin effects on proliferation, apoptosis, signaling, and angiogenesis by using human pancreatic carcinoma cells in vitro and in vivo. Liposome-encapsulated curcumin suppressed pancreatic carcinoma growth in murine xenograft models and inhibited tumor angiogenesis in vivo. It also downregulated the NF-κB pathway, suppressed growth, and induced apoptosis of human pancreatic cells in vitro and showed antitumor and antiangiogenesis effects in vivo [35, 36]. Chen et al. [37] studied in vitro skin permeation and in vivo antineoplastic effects of curcumin by using liposomes as the transdermal drug-delivery system. Curcumin-loaded liposomes exhibited ability to inhibit the growth of melanoma cells. A considerable effect on antimelanoma action was detected with curcumin-loaded liposomes. These results, similar to the results of other studies, suggest that liposomes would be a hopeful delivery service for curcumin in cancer management [30, 86, 87]. These data indicate a significant liposomal curcumin potential as delivery vehicles for the treatment of different cancers (Table 1).

Rogers et al. [38] also administered liposomes containing curcumin to target delivery to renal tubular epithelial and antigen-presenting cells in mice renal ischemia model. Liposomal curcumin significantly improved serum creatinine, reduced histological injury and cellular apoptosis, and lowered toll-like receptor-4, heat shock protein-70, and tumor necrosis factor alpha (TNF-α) mRNA expression, and it also decreased neutrophil infiltration and inflammatory interleukins expression. In this regard, Basnet et al. [39] developed vaginal administration of liposomal curcumin. Liposomal curcumin was found to be twofold to sixfold more potent than corresponding free curcumin. Results showed that liposomal delivery systems enhance anti-inflammatory properties of curcumin. Also, evaluation of liposomal curcumin cytochrome P450 inhibition was conducted by Mach et al. [88] in liver tissues. Results demonstrated that there is low potential for CYP450 mediated drug interactions at physiologic serum concentrations of liposomal curcumin. It will not interact with other chemotherapy agents that are metabolized and/or eliminated via the primary drug metabolizing cytochrome P450 pathways [88].

The therapeutic efficacies of novel liposomal delivery systems based on artemisinin or artemisinin-based combination therapy with curcumin have been investigated and reported by Isacchi et al. [89]. They reported that artemisinin alone began to decrease parasitaemia levels only 7 days after the start of the treatment, and it appears to have a fluctuant trend in blood concentration which is reflected in the antimalarial effectiveness. By contrast, treatments with artemisinin loaded with liposomal delivery systems appeared to have an immediate antimalarial effect which cured all malaria-infected mice within the same postinoculation period of time. In particular, artemisinin loaded with liposomal curcumin seems to give the most pronounced and statistically significant therapeutic effect in this murine model of malaria. The enhanced permanency in blood of artemisinin loaded with liposomal curcumin suggests application of these nanosystems as suitable passive targeted carriers for parasitic infections [89]. This strong effect of formulation is added up to the mechanism of action of artemisinin which acts in the erythrocyte cycle stage of human host as a blood schizonticide. Agarwal et al. [90] also assessed the acute effects of liposome-entrapped curcumin on increasing current electroshock seizures, pentylenetetrazole-induced seizures, and status epilepticus in mice. Liposome-entrapped curcumin demonstrated significant increase in seizure threshold current and latency to myoclonic and generalized seizures increasing current electroshock and pentylenetetrazole-induced seizures, respectively. It also increased the latency to the onset and decreased the duration of seizures during status epilepticus. Therefore, liposomal-entrapped curcumin can possess anticonvulsant activity against status epilepticus in mice (Table 1).

To put it briefly, the above data suggest that the administration of liposomal curcumin has numerous beneficial effects which could lead to required clinical applications. These better outcomes take place by means of enhanced solubility, more safety and minimum side effects, more signs of stability in the blood, increased bioavailability and efficacy, owning a potential role as delivery vehicles for the treatment of different cancers, potent anti-inflammatory and antimalaria response, and, finally, anticonvulsant activity.

4. Micelles

A typical micelle is a surfactant molecule aggregate dispersed in a liquid colloid. It is a nanosized vesicular membrane which becomes soluble in water by gathering the hydrophilic heads outside in contact with the solvent and hydrophobic tails inside, which is known as emulsification. Micelles are lipid molecules that arrange themselves in a spherical form in aqueous solutions with a very narrow range from 10 to 100 nm in size, which makes them more stable toward dilution in biological fluids [84]. The shape or morphology of micelles is from amphiphilic block copolymers such as spherical, rodlike, and starlike, as well as vesicles (Table 1). The self-assembly of amphiphilic block copolymer is a reversible process, and the shape varies with the copolymers' composition and length ratio [91]. The functional properties of micelles are based on amphiphilic block copolymers, which come together to form a nanosized core/shell structure in aqueous media. The hydrophobic core area hands out as a pool for hydrophobic drugs, while the hydrophilic shell area stabilizes the hydrophobic core and makes the polymers water soluble. Polymeric micelles can serve as transporters of water-insoluble drugs such as curcumin, which can augment the drug's efficiency by targeting definite cells or organs; therefore, fewer drugs accumulate in healthy tissues and their toxicity reduces, and occasionally higher doses can be administered [92]. In this regard, to overcome the poor water solubility of curcumin, Liu et al. [93] prepared curcumin-loaded biodegradable self-assembled polymeric micelles by solid dispersion method, which was simple and easy to scale up. Release profile showed a significant difference between rapid release of free curcumin and much slower and sustained release of curcumin-loaded micelles. In addition, the preparation of curcumin-loaded micelles based on amphiphilic Pluronic/polycaprolactone block copolymer was investigated by Raveendran et al. [40], which proved to be efficient in enhancing curcumin's aqueous solubility. Some other studies also deliberated on highly surface-active compounds such as poloxamers or Pluronic that can self-assemble into spherical micelle. In vitro results showed that spherical curcumin-loaded mixed micelles might serve as a potential nanocarrier to improve the solubility and biological activity of curcumin [94–96]. In another study, the aqueous solubility of the curcumin was increased by encapsulation within the micelles [97]. Solubilization was directly related to the compatibility between the solubilizate and polycaprolactone as determined by the Flory-Huggins interaction parameter. Molecular modeling study suggested that curcumin tended to interact with polycaprolactone serving as a core embraced by polyethylene glycol as a shell. In addition, Yu et al. [41] showed the structure of modified ε-polylysine micelles and their application in improving cellular antioxidant activity of curcuminoids. Results of their investigation revealed that modified ε-polylysine micelles were able to encapsulate curcuminoids and improve their

water solubility and cellular antioxidative activity compared with free curcuminoids. They suggested that these micelles may be used as new biopolymer micelles for delivering poorly soluble drugs such as curcumin. Another study synthesized curcumin in sodium dodecyl sulfate and cetyltrimethylammonium bromide micelles to overcome the poor water solubility of curcumin and demonstrated antioxidative effects of curcumin analogues against the free-radical-induced peroxidation of linoleic acid in these micelles [98, 99]. Kinetic analysis of the antioxidation processes demonstrated that these compounds exhibited extraordinarily higher antioxidative activity in micelles due to their solubility being higher than free curcumin [98].

Drug release from micelles is governed by different issues including micelle stability, the rate of copolymer biodegradation, and drug diffusion. By the way, Sahu et al. [100] reported the potential of the two most common Pluronic triblock copolymer micelles, Pluronic F127 and F68, for curcumin encapsulation efficiency and stability. Pluronic F127 showed better encapsulation efficiency and good stability for long-term storage than Pluronic F68. Atomic force microscopy (AFM) study revealed that the drug-encapsulated micelles are spherical in shape with diameters below 100 nm. Pluronic-encapsulated curcumin demonstrated slower and sustained release of curcumin from the micelles and considerable anticancer activity in comparison with free curcumin in vitro cytotoxicity study. In addition, Podaralla et al. [42] reported a natural protein core-based polymeric micelle and demonstrated its application for the delivery of hydrophobic anticancer drugs, specifically curcumin. They synthesized novel biodegradable micelles by conjugating methoxy polyethylene glycol and zein, a biodegradable hydrophobic plant protein which can be found in Maize, and then encapsulating with curcumin. Polyethylene glycol zein micelles sustained the curcumin release up to 24 hrs in vitro and significantly enhanced its aqueous solubility and stability with the 3-fold reduction in IC50 value of curcumin. So, since the curcumin is finely protected from possible inactivation by their micellar surroundings, its retention and bioavailability can be enhanced (Table 1).

Aiming to modify the pharmacokinetics of curcumin, Song et al. [43] synthesized a poly(D,L-lactide-co-glycolide)-b-poly(ethylene glycol)-b-poly(D,L-lactide-co-glycolide) (PLGA-PEG-PLGA) with micelles. PLGA-PEG-PLGA micelles provided higher area under the concentration curve (AUC) and enhanced residence time, clearance, and distribution half-life in comparison with curcumin solution. The prolongation of half-life, enhanced residence time, and decreased total clearance indicated that curcumin-loaded micelles could prolong acting time of curcumin in vivo. These results may be related to the curcumin location within the micelles and increased viscosity of copolymer solution at the body temperature. The variation of AUC indicated that the curcumin-loaded micelles provided higher bioavailability than curcumin solution, and the biodistribution study showed that the micelles had decreased drug uptake by liver and spleen and enhanced drug distribution in lung and brain. These results suggested that PLGA-PEG-PLGA micelles would be a potential carrier for curcumin. In addition, Ma et al. [94] demonstrated the pharmacokinetics of both solubilized curcumin and its polymeric micellar formulation in rats by using a simple, rapid, and reliable HPLC method. They concluded that encapsulation of curcumin in the polymeric micellar formulation led to increase in curcumin's half-life and distribution volume.

In addition, curcumin-micelles can be affected by physicochemical characteristics, concentration, and location within the micelles. The polymeric micelles have a prolonged circulation time due to their small size and hydrophilic shell that reduce the drug uptake by the mononuclear phagocyte system [101]. Leung et al. [44] reported that encapsulated curcumin in cationic micelles suppresses alkaline hydrolysis that was studied in three types of micelles composed of the cationic surfactants cetyltrimethylammonium bromide (CTAB) and dodecyltrimethylammonium bromide (DTAB) and the anionic surfactant sodium dodecyl sulfate (SDS). Curcumin underwent rapid degradation in the SDS micellar solution by alkaline hydrolysis at pH of 13, while it was significantly suppressed with a yield of suppression close to 90% in the presence of either CTAB or DTAB micelles. Results from fluorescence spectroscopic studies revealed that curcumin is dissociated from the SDS micelles to the aqueous phase at this pH while curcumin remains encapsulated in CTAB and DTAB micelles at pH 13. The absence of encapsulation and stabilization in the SDS micellar solution resulted in rapid hydrolysis of curcumin. Some other studies showed other curcumin-loaded micelles properties. Wang et al. [102] introduced the sensitive fluorometric method for the determination of curcumin using the enhancement of mixed micelle. This method had the advantages of high sensitivity, selectivity, and stability. The fluorescence of curcumin was greatly enhanced by mixed micelle of sodium dodecylbenzenesulfonate and cetyltrimethylammonium bromide (SDBS-CTAB). This study indicated that fluorescence quantum yield of curcumin in SDBS-CTAB micelle was about 55-fold larger than that of aqueous solution containing 1.0% ethanol, which was in agreement with their fluorescence intensity ratio. As a result, curcumin can be used as a fluorophore in fluorescence polarization anisotropy measurement to determine the critical micellar concentration of surfactant and to study the interaction between them. In addition, Adhikary et al. [45] performed femtosecond fluorescence upconversion experiments on the naturally occurring medicinal pigment, curcumin, in anionic, cationic, and neutral micelles. These micelles were composed of SDS, dodecyltrimethylammonium bromide (DTAB), and Triton X-100. They revealed the curcumin's excited-state kinetics in micelles with fast (3–8 ps) and slow (50–80 ps) components. While deuteration of curcumin had a negligible effect on the fast component, the slow component exhibited a pronounced isotope impact of approximately 1.6, which indicates that micelle-captured curcumin undergoes excited-state intramolecular hydrogen atom transfer. Moreover, Began et al. [46] had attached curcumin to phosphatidylcholine micelles followed by fluorescence measurements. Curcumin in aqueous solution did not inhibit dioxygenation of fatty acids by lipoxygenase 1, but it inhibited the oxidation of fatty acids when bound to phosphatidylcholine micelles.

Results demonstrated that 8.6 μM of curcumin bound to the phosphatidylcholine micelles is required for 50% inhibition of linoleic acid peroxidation. Lineweaver-Burk plot analysis had indicated that curcumin is a competitive inhibitor of lipoxygenase 1 with Ki of 1.7 μM for linoleic acid and 4.3 μM for arachidonic acid, respectively. By using spectroscopic measurement, they revealed that the inhibition of lipoxygenase 1 activity by curcumin can be due to binding to active center iron and curcumin after binding to the phosphatidylcholine micelles acts as an inhibitor of lipoxygenase 1. In a recent investigation, the critical micelle concentration of the amphiphilic polymer was determined by using fluorescent probe. Outcomes indicated that Pluronic/polycaprolactone micelles may be a promising candidate for curcumin delivery to cancer cells of colorectal adenocarcinoma [40]. In another pharmacokinetic study, curcumin micelles demonstrated higher concentration and longer retention time in plasma and tumor sites, so they had stronger inhibitory effects on proliferation, migration, invasion, and tube formation of carcinoma cells than free curcumin; for example, curcumin micelles were shown to be more effective, presumably due to higher concentration in inhibiting tumor growth and prolonged survival in both subcutaneous and pulmonary metastatic tumor models [103].

Investigating the influence of micelles on cytotoxicity of curcumin, specifically in cancer therapy, in vitro study by Raveendran et al. [40] showed that Pluronic/polycaprolactone micelles could be a promising candidate for curcumin delivery to cancer cells regarding the cytotoxicity and cellular uptake of the curcumin-loaded micelles in colorectal adenocarcinoma cells. An investigation by Wang et al. [104] revealed that the encapsulated curcumin maintains its potent antitumor effects; however, curcumin-loaded micelles were more effective in inhibiting tumor growth and spontaneous pulmonary metastasis in subcutaneous 4T1 breast tumor model and prolonged survival of tumor-bearing mice. Immunofluorescent and immunohistochemical studies also showed that tumors of curcumin-loaded micelle-treated mice had more apoptotic cells, fewer microvessels, and fewer proliferation-positive cells [104]. In addition, Yang et al. [19] had conjugated methoxypolyethylene glycol-polylactic acid (mPEG-PLA) micelle to multiple curcumin molecules; the cytotoxicity study results showed that the effect of IC50 of mPEG-PLA-Tris-curcumin on human hepatocellular carcinoma cells was similar to unmodified curcumin. The cellular uptake study demonstrated that these carriers could successfully transport the drug to the cytoplasm of hepatic cells. Micelles containing multiple drug molecules were an efficient means to increase loading and intracellular delivery of low-potency curcumin [19]. Moreover, Mohanty et al. [105] reported that curcumin encapsulated in methoxy poly(ethylene glycol)/poly-epsilon-caprolactone diblock copolymeric (MePEG/PCL) micelle, by varying the copolymer ratio (40 : 60 MePEG/PCL ratio was selected due to its high encapsulation), had increased bioavailability due to intensified uptake, 2.95 times more, with comparative cytotoxic effects by induction of apoptosis in contrast with unmodified curcumin at equimolar concentrations. Overall, these data obviously showed the commitment of a micellar system for efficient solubilization, stabilization, and controlled delivery of the hydrophobic drug, such as curcumin, for cancer therapy.

Concisely, curcumin-loaded micelles can boost the drug's efficiency by targeting definite cells and result in less drug accumulation in healthy tissues and reduction of toxicity. Curcumin's aqueous solubility and much slower and sustained release of drug caused by curcumin-loaded micelles also get in use in several conditions. The retention and bioavailability of curcumin could be elevated since the curcumin is protected from possible inactivation by its micellar surroundings. Locating the curcumin in the micelles can also enhance half-life and residence time and decrease total clearance leading to prolongation of acting time of curcumin. Curcumin micelles can be influenced by physicochemical features including their size and electrical charges, concentration, and location within the micelles. These data obviously showed the commitment of a micellar system for efficient solubilization, stabilization, and controlled delivery of the hydrophobic drug, such as curcumin, for cancer therapy (Table 1).

5. Niosomes

Niosomes are microscopic lamellar constructions of nonionic surfactant of alkyl or dialkyl polyglycerol ether category with cholesterol that were first introduced in the 70s [106, 107]. Niosomes can provide a container for drug molecules with a wide range of solubilities due to presence of hydrophilic, amphiphilic, and lipophilic moieties in the constitution (Table 1). They behave similar to liposomes in vivo and can be used as an effective alternative to liposomal drug carriers, and those properties depend on the composition of the bilayer as well as the method of their production [108]. Surfactant type, encapsulated drug nature, storage temperature, detergents, and use of membrane spanning lipids can affect niosomes stability [107]. Niosomes are also planned for use in a number of potential therapeutic applications, such as anticancer and anti-infective drug targeting agents [84]. They can improve the therapeutic indices of drugs by restricting their action on the target cells. They also improve oral bioavailability of poorly absorbed drugs such as curcumin to design the novel drug delivery system and increase the skin penetration of drugs [47]. In this regard, in an in vitro study which was performed using albino rat skin, proniosomes of curcumin were prepared by encapsulation of the drug in a mixture of Span 80, cholesterol, and diethyl ether to investigate transdermal drug delivery system [109]. The planned systems distinguished between size, drug entrapment, repose angle, hydration rate, and vesicular stability under different storage settings. Results showed that proniosomes are very stable and promising prolonged delivery systems for curcumin [109]. Mandal et al. [48] also designed a comparative study with different microenvironments for photophysical properties of curcumin inside niosomes by means of steady state, time resolved fluorescence spectroscopy, and dynamic light scattering techniques. Outcomes showed that more rigid and confined microenvironments of niosomes improve the steady state fluorescence intensity along with the fluorescence

lifetime of curcumin. The data indicated that niosomes are a good tool for delivery system to suppress the level of degradation of curcumin [48]. In another study, by Rungphanichkul et al., curcuminoid niosomes were developed with a series of nonionic surfactants to enhance skin permeation of curcuminoids. [49]. Results were evaluated based on entrapment efficiency and in vitro penetration of curcuminoids via snake skin. Niosomes drastically enhanced permeation of curcuminoids compared with a vehicle solution of curcuminoids [49]. The fluxes of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin also were consistent with the qualified hydrophobicity of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin, respectively. Data indicated that curcuminoids can be fruitfully prepared as niosomes, and such formulations have superior properties for transdermal drug delivery system [49].

Briefly, niosomes can be a potential delivery system for curcumin in order to suppress the degradation of this agent and increase its life time. It has also been demonstrated that niosomes boost the permeation of curcumin through skin (Table 1).

6. Cyclodextrins

Cyclodextrins (Cds) are a family of complexes prepared from sugar molecules bound together in cyclic oligosaccharides [110]. They are created from starch by using enzymatic switch. Cds are cyclic oligomers of glucose that can form water-soluble inclusion complexes with small molecules and portions of large complexes [111]. They are exceptional molecules with pseudoamphiphilic construction, which are used industrially in pharmaceutical requirements [84]. Cds are also used in agriculture and in environmental engineering in food, drug delivery systems, and chemical industries [110]. They have an interior hydrophobic surface which can provide a place for residence of poorly water-soluble molecules, while the external hydrophilic area makes its solubility possible in the aqueous setting with high stability (Table 1).

To improve the water solubility and the hydrolytic stability of curcumin, Tønnesen et al. [50] prepared cyclodextrin-curcumin complexes by using HPLC and UV/VIS scanning spectrophotometer techniques [50] (Figure 3). Results showed that the hydrolytic stability of curcumin was sturdily improved by the complex, and also the photodecomposition rate was enhanced in organic solvents compared to the free curcumin. As a result, the cavity size and charge of cyclodextrin side-chains influenced the stability and degradation rate of curcumin [50]. In addition, other investigations on the solubility, phase distribution, and hydrolytic and photochemical stability of curcumin showed that curcumin derivatives were more stable towards hydrolytic degradation in cyclodextrin solutions than free curcumin [51]. The photochemical studies illustrated that curcumin is universally more stable than its other derivatives. Solubility and phase-distribution studies showed that curcuminoids with side groups on the phenyl moiety have higher affinity for the hydroxypropyl-γ-cyclodextrin (HP-γ-CD) than the cyclodextrins. The radical scavenging investigations confirmed that curcumin is more active than its curcuminoids derivatives, and the free phenolic hydroxyl group may possibly be necessary for the scavenging properties [51]. In another study, to increase the solubility of curcumin, Darandale and Vavia [52] employed cyclodextrin-based nanosponges; they formulated the complex of curcumin with β-cyclodextrin nanosponge obtained with dimethyl carbonate as a cross-linker. The loaded nanosponges have shown more solubilization efficiency compared to free curcumin and β-cyclodextrin complex. The characterization of curcumin nanosponge complex confirmed the interactions of curcumin with nanosponges. Moreover, in vitro drug release of curcumin was controlled over a prolonged time period, and the complex was nonhemolytic [52]. Therefore, it seems that CDs are permitting vehicles that can be used for oral delivery to develop the bioavailability of insoluble drugs by molecular dispersion and degradation protection and for intravenous delivery to supply as solubilizers for multifaceted hydrophobic drugs without altering their pharmacokinetic properties [84].

Yadav et al. [53] developed a new cyclodextrin complex of curcumin to increase solubility of curcumin and studied its anti-inflammatory and antiproliferative effects. They showed that cyclodextrin-curcumin complex was more active than free curcumin in inhibiting the inflammatory transcription factor, such as nuclear factor kappa-b (NF-κB). In addition, it suppressed cyclin D1 as a cell proliferation marker, matrix metallopeptidase 9 (MMP-9) as an invasion marker in metastasis, and vascular endothelial growth factor (VEGF) as an angiogenesis marker. Cyclodextrin-curcumin complex was also more active in inducing the death receptors and apoptosis of leukemic cells as well as other cancer cell lines. These suggest that cyclodextrin-curcumin complex has superior characteristics compared to free curcumin for cell uptake and antiproliferative and anti-inflammatory effects [53]. Yadav et al. [54] have also planned curcumin complexes by common methods to evaluate the anti-inflammatory effects of cyclodextrin-curcumin complex for the treatment of inflammatory bowel disease (IBD) in an animal rat model. In vivo results showed that curcumin has higher affinity for hydroxypropyl-β-cyclodextrin than other cyclodextrins. In addition, hydroxypropyl-β-cyclodextrin-curcumin complex proved to be a powerful antiangiogenesis complex. In vivo data also confirmed that the scale of colitis was appreciably attenuated by cyclodextrin-curcumin. In summary, cyclodextrin complex was shown to be valuable in the therapeutic approaches for IBD patients being a nontoxic natural dietary yield [54].

Additionally, Cds can augment bioavailability of insoluble drugs such as curcumin by rising drug solubility and dissolution [84]. They also amplify the permeability of hydrophobic agents by making them accessible at the surface of the membrane's biological barrier. A β-cyclodextrin-encapsulated curcumin drug delivery system was developed by Yallapu and colleagues in order to get better curcumin hydrophilic and drug delivery characteristics [55]. Encapsulated-curcumin efficiency was shown to be improved through increasing the ratio of curcumin to cyclodextrin. Then, an optimized cyclodextrin-curcumin complex was assessed for intracellular uptake and anticancer effects. Cell proliferation and clonogenic examinations showed that β-cyclodextrin-curcumin

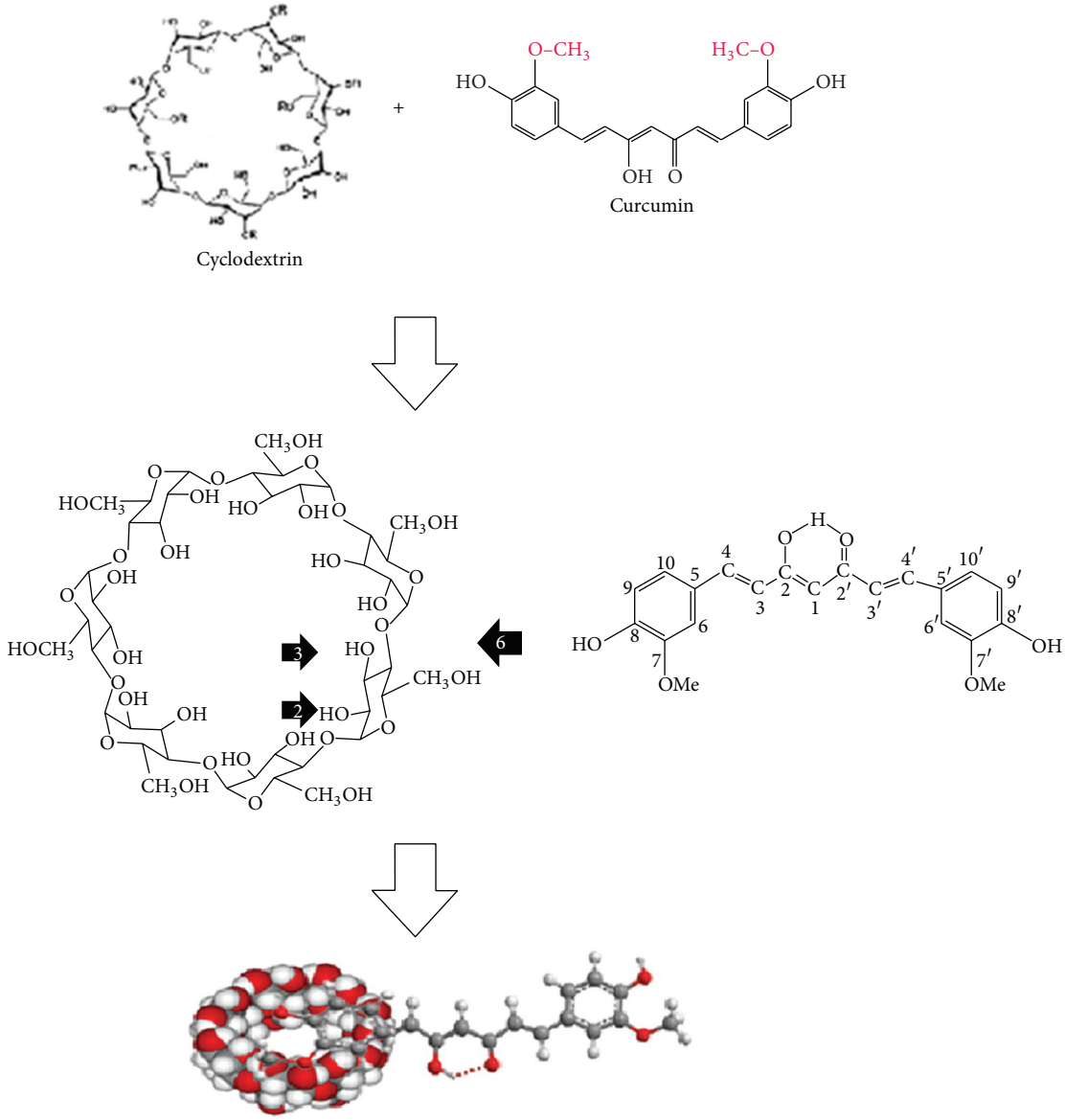


Figure 3: A schematic figure of curcumin connection to the cyclodextrin nanoparticles.

self-assembly augmented curcumin delivery and improved its therapeutic efficacy in prostate cancer cells [55]. Moreover, curcumin-loaded γ-cyclodextrin liposomal nanoparticles as delivery vehicles were also explored by Dhule et al. [34] and evaluated against cancer models. The resulting 2-hydroxypropyl-γ-cyclodextrin/curcumin-liposome complex showed promising anticancer potential both in vitro and in vivo against osteosarcoma and breast cancer. Liposomal curcumin initiated the caspase cascade that led to apoptotic cell death in vitro. In addition, the efficiency of the liposomal curcumin formulation was confirmed in vivo by using a xenograft osteosarcoma model. Data showed that curcumin-loaded γ-cyclodextrin liposomes indicated considerable potential as delivery vehicles for cancer cure [34]. Rahman et al. [30] prepared β-cyclodextrin-curcumin complexes, as a hydrophilic curcumin. They entrapped both native curcumin as a hydrophobic agent and the complexes separately into liposomes and then assessed them for their cytotoxicity in cancerous cell lines. The aqueous solubility of β-cyclodextrin-curcumin complexes enhanced noticeably, and successful entrapment of complexes into prepared liposomes was also achieved. The median effective dose for all curcumin formulations was found to be in a low range for both lung and colon cancer cell lines [30]. Outcomes guaranteed that β-cyclodextrin-curcumin complexes of weakly water-soluble drugs such as curcumin can be tricked within biocompatible vesicles such as liposomes, and this does not prevent their anticancer effects [30]. In another study, a novel curcumin analogue (difluorinated curcumin; CDF) and CDF-β-cyclodextrin-curcumin complex were synthesized to enhance anticancer effects against pancreatic cancer [56]. Results showed that CDF-β-cyclodextrin was found to lower

IC50 value by half when tested against multiple cancer cell lines. Following intravenous administration of CDF-β-cyclodextrin, it was specially accumulated in pancreatic tissue 10 times higher than in serum. As a result, novel curcumin analogue CDF outstanding gathering in pancreas tissue led to its persuasive anticancer effects against pancreatic cancer cells. So, synthesis of such CDF-β-cyclodextrin self-assembly is a successful approach to improve its bioavailability and tissue distribution. Further evaluations on CDF delivery in clinical settings for treatment of human malignancies were suggested by these authors [56]. Moreover, a novel poly(β-cyclodextrin)-curcumin self-assembly was approached to improve curcumin's delivery to prostate cancer cells by Yallapu et al. [112]. Intracellular uptake of the self-assembly was evaluated by means of flow cytometry and immunofluorescence microscopy. The therapeutic values were established by cell proliferation and colony formation tests on prostate cancer cells. Results recommended that the poly(β-cyclodextrin)-curcumin formulation could be a valuable system for developing curcumin delivery and its therapeutic effectiveness in prostate cancer [112]. Additionally, in order to improve solubility and drug delivery of curcumin, Lomedasht et al. [113] exploited a β-cyclodextrin-curcumin inclusion complex and evaluated its cytotoxic effects by MTT assay in vitro. Breast cancer cells were treated with equal concentration of β-cyclodextrin-curcumin and free curcumin. Then, telomerase gene expression was compared by real-time PCR in two groups. In vitro results showed that β-cyclodextrin-curcumin increased curcumin delivery in breast cancer cells [113]. Telomerase gene expression was lower in β-cyclodextrin-curcumin-treated cells than free curcumin-treated cells. As a result, β-cyclodextrin-curcumin complex was more effectual than free curcumin in telomerase expression inhibition. Rocks et al. [114] have used cyclodextrins as an excipient permitting a significant enhancement of curcumin solubility and bioavailability. Then, complex's effects were evaluated in cell cultures as well as in vivo, in an orthotopic lung tumor mouse model. Cell proliferation in the presence of curcumin-cyclodextrin complex was decreased while apoptosis rates were increased in lung epithelial tumor cells in vitro. For in vivo experiments, cells were grafted into lungs of C57Bl/6 mice treated by an oral administration of a nonsoluble form of curcumin, Cds alone, or curcumin-CD complexes, combined with or not combined with gemcitabine [114]. In addition, the size of orthotopically implanted lung tumors was noticeably reduced by curcumin complex administration in comparison with nonsolubilized curcumin. Moreover, curcumin-cyclodextrin complex potentiated the gemcitabine-mediated antitumor effects. Results underlined a prospective preservative effect of curcumin with gemcitabine, thus providing a proficient remedial alternative for anti-lung cancer treatment [114]. Moreover, for noninvasive imaging, encapsulated 4-[3,5-bis(2-chlorobenzylidene-4-oxo-piperidine-1-yl)-4-oxo-2-butenoic-acid] (CLEFMA) was developed by using hydroxypropyl β-cyclodextrin [115]. CLEFMA possessed more persuasive antiproliferative effects in lung adenocarcinoma without any impact on normal lung fibroblasts. It seems that CLEFMA liposomes retained the antiproliferative effectiveness of free CLEFMA while sustaining its nontoxic character in normal lung fibroblasts. In addition, tumor volume extensively reduced after treatment with CLEFMA, to 94% in rat xenograft tumors. Outcomes revealed the usefulness of liposomes to supply as a carrier for CLEFMA, and this study was the first to exhibit the efficacy of novel curcuminoid CLEFMA in a preclinical model [115].

To sum up, these collected data show that Cds help increase the hydrolytic stability of curcumin, photodecomposition rate, protection against decomposition, bioavailability, and molecular dispersion compared to the free curcumin without altering their pharmacokinetic characteristics (Table 1). These data also confirm that cyclodextrin-curcumin complex has a priority against free curcumin in cell uptake, antiproliferative and anti-inflammatory effects by suppression of cyclin D1, MMP-9, and VEGF, and induction of death receptors and apoptosis.

7. Dendrimers

Dendrimers are a group of greatly branched globular polymers which are created with structural control rivaling traditional biomolecules. They were introduced in the mid-1980s and are referred to as synthetic proteins. Dendrimers are a series of polymeric architectures with different chemical and surface-related properties. They have much more accurately controlled structures, with a globular shape and a single molecular weight rather than a distribution of molecular weights in comparison with the traditional linear polymers [116]. A number of properties put together dendrimers' exceptional nanostructures with the interior-surface architecture or generations (Table 1). The dendrimer structure, consisting of a core, branched interiors, and numerous surface functional groups, serves as a platform to which additional substrates can be added to this spherical molecule in a highly controlled manner. This nanospace represents an isolated environment, thus decreasing toxicity associated with the payload. The well-defined organization, dense spherical form, size, monodispersity, and controllable "surface" functionalities of dendrimers make them brilliant applicants for assessment as drug delivery services [117]. In addition, the biocompatibility silhouette of dendrimers donates to their effectiveness in molecular imaging. This biocompatibility can be increased via functionalization with small molecules. Increased biocompatibility is also associated with lower generation branch cells with anionic or neutral groups compared to similar branch cells of higher generations which have cationic surface groups.

To test whether dendrimer curcumin displays both cytotoxicity and water solubility, Debnath et al. [57] generated dendrimer curcumin conjugate, a water-soluble and effective cytotoxic agent against breast cancer cell lines. In vitro results showed that dendrimer curcumin conjugate dissolved in water was significantly more effective in inducing cytotoxicity against SKBr3 and BT549 human breast cancer cells and effectively induced cellular apoptosis measured by caspase-3 activation. In another study, the interaction of curcumin dendrimers with cancer cells, serum proteins, and human red blood cells was studied by Yallapu et al. [58]. They assessed

dendrimers' potential application for in vivo preclinical and clinical studies. Protein interaction studies were conducted using particle size analysis, zeta potential, and western blot techniques. To evaluate its acute toxicity and hemocompatibility, curcumin-dendrimer was incubated with human red blood cells. In addition, the cellular uptake of curcumin-dendrimer was assessed by using curcumin levels in cancer cells using ultraviolet-visible spectrophotometry. Results showed a remarkable capacity of the dendrimer curcumin nanoformulation to bind to plasma protein. However, no significant changes were observed in the zeta potential and the extensive hemolysis of the dendrimer curcumin formulation. Results showed that the positively charged amino surface groups cause destabilize the cell membrane and cell lysis. This type of lytic effect on erythrocytosis is extremely dangerous when administered in vivo. Therefore, polyethylene glycol conjugation of dendrimer formulations may be required to decrease this activity [118, 119].

Cao et al. [59] investigated the interactions between polyamidoamine-C (a dendrimers) and curcumin by using fluorescence spectroscopy and molecular modeling methods. Results showed that the polyamidoamine-C12 25% formation together with curcumin induced the fluorescence quenching of polyamidoamine-C12 25%. Curcumin entered the interface of polyamidoamine-C12 25% with mainly five classes of binding sites by hydrophobic bonds, hydrogen bonds, and van der Waals forces interactions. The larger values of binding constants indicated that polyamidoamine-C12 25% holds the curcumin strongly. Furthermore, in another study, polyamidoamine encapsulated curcumin inhibited telomerase activity in human breast cancer cell line [60]. These researchers also used telomerase repeat amplification protocol (TRAP) assay and determined relative telomerase activity (%RTA). In vitro results demonstrated that dendrimers have no cytotoxicity in human breast cancer cell line. Also, polyamidoamine encapsulating curcumin concentration increased while %RTA decreased. These results suggested that polyamidoamine encapsulating curcumin had a dose-dependent cytotoxicity effect on breast cancer cell line through downregulation and inactivation of telomerase and inducing apoptosis by enhancing curcumin uptake by cells (Table 1). So, polyamidoamine can be considered as a fine carrier especially for hydrophobic agents.

The stability of curcumin and its antitumor properties were improved by using dendrosomal nanoparticles in vitro and in vivo by our team's work [61–63, 120]. The made dendrosomal nanoparticle-curcumin is a neutral, amphipathic, and biodegradable nanomaterial with variable monomers suitable for inert cell drug porters. It is a new type of biocompatible polymeric particle taken from plant fatty acids which keeps curcumin size at 80 nm (Table 1). Acute and chronic toxicity of dendrosomal nanoparticle-curcumin was investigated in mice. Our results shed new light on dendrosomal nanoparticle-curcumin's potential biocompatibility for in vitro and in vivo biological systems. In addition, the protective and the therapeutic effects of dendrosomal nanoparticle-curcumin were assessed on an animal model of breast cancer through apoptosis, proliferation, and angiogenesis pathways. In our study, dendrosomal nanoparticle-curcumin significantly suppressed proliferation of human and mouse carcinoma cells. In vitro results showed not only that dendrosomes have significantly increased the uptake of curcumin but also that dendrosomal nanoparticle-curcumin inhibited the growth of cancer cells rather than normal ones by inducing apoptosis. In toxicity profile, based on hematological, blood chemical, and histological examinations, minimal hepatic and renal toxicity were seen with high dendrosomal nanoparticle-curcumin doses. In addition, in vivo results showed that tumor incidence, weight, and size were significantly declined in dendrosomal nanoparticle-curcumin-treated group. Dendrosomal nanoparticle-curcumin also induced the expression of proapoptotic Bax protein and reduced antiapoptotic Bcl-2 protein expression relative to the control group. Moreover, proliferative and angiogenic markers were lowered in dendrosomal nanoparticle-curcumin-treated animals. These findings point to the features of the polymeric carrier as a promising drug-delivery system for cancer therapy. In another study, we also evaluated the antiproliferative and anticarcinogenic effects of dendrosomal nanoparticle-curcumin in rat colon cancer. Our results demonstrated the potential anticancer effects of dendrosomal nanoparticle-curcumin in a typical animal model of colon cancer. The results provide evidence that nanoparticle-curcumin exerts significant chemoprotective and chemotherapeutic effects on colon cancer through inhibition of cell proliferation and apoptosis induction [61, 63]. These tunable properties make dendrimers more attractive agents for biomedical applications compared to other nanovectors such as micelles, liposomes, or emulsion droplets (Table 1). Therefore, they are being preferred as carriers which are the foundation for new types of anticancer entities. Although the application of dendrimers as drug-delivery instruments has been advertised as a major area of their potential application, this part has really been little studied [121].

So, mentioned studies suggest that dendrimer curcumin conjugate in water was significantly more effective in inducing cytotoxicity through downregulation and inactivation of telomerase activity and in inducing apoptosis by induction of the expression of proapoptotic Bax protein and reduction of antiapoptotic Bcl-2 protein expression since curcumin uptake enhances.

8. Nanogels

Nanogels are self-possessed of cross-linked three-dimensional polymer chain networks which are created through covalent linkages and can be customized to gel networks with biocompatible and degradable properties. The porosity among these cross-linked networks not only provides a perfect reservoir for loading drugs but also keeps them from environmental degradation [58]. The swelling of nanogels in an aqueous setting is controlled by using the polymer chemical structure, cross-linking degree, and the polyelectrolyte gel's charge density and/or by pH value, ionic strength, and chemical nature of low molecular mass (Table 1). Furthermore, nanogels can be chemically modified to incorporate

various ligands for targeted drug delivery, triggered drug release, or preparation of composite materials [122].

Nanogels are developed as carriers for drug delivery and can be planned to spontaneously absorb biologically active molecules via creation of salt bonds, hydrogen bonds, or hydrophobic interactions that can enhance oral and brain bioavailability of low-molecular-weight drugs and biomacromolecules [122]. An important criterion for a nanogel carrier with widespread biomedical abilities is to have good stability in biological fluids, which would prohibit aggregation. In this regard, Gonçalves et al. (2012) applied a self-assembled dextrin nanogel as curcumin delivery system by using dynamic light scattering and fluorescence measurements. They showed that the stability and loading efficiency of curcumin-loaded nanogel depend on the nanogel/curcumin ratio. The in vitro release profile in HeLa cell cultures indicated that dextrin nanogel may act as a suitable carrier for the controlled release of curcumin [123]. Various nanogel properties can be attained by altering the chemical functional groups, cross-linking density, and surface-active and stimuli-responsive elements [58]. Nanogels demonstrate excellent potential for systemic drug delivery that should have a few common features including a smaller particle size (10–200 nm), biodegradability and/or biocompatibility, prolonged half-life, high stability, higher amount of drug loading and/or entrapment, and molecules protection from immune system [58]. Mangalathillam et al. (2011) loaded curcumin into chitin nanogels and analyzed it by dynamic light scattering (DLS), scanning electron microscope (SEM), and Fourier transform infrared spectroscopy (FTIR). Then, the nanogel's cytotoxicity was analyzed on human dermal fibroblast and human melanoma cells. The curcumin-chitin nanogels showed higher release at acidic pH compared to neutral pH. The in vitro results showed that curcumin-chitin nanogels have had a specific toxicity on melanoma cells in a concentration range of 0.1–1.0 mg/mL, but less toxicity towards normal cells [64]. The confocal analysis confirmed the high uptake of curcumin-chitin nanogels by human melanoma cells. In addition, it was indicated that curcumin-chitin nanogels at the higher concentration of the cytotoxic range may show comparable apoptosis in comparison with free curcumin. The curcumin-chitin nanogels also showed a 4-fold increase in steady state transdermal flux of curcumin in comparison with free curcumin. The histopathology studies showed loosening of the horny layer of the epidermis, facilitating penetration with no observed signs of inflammation in the group treated with curcumin-chitin nanogels [64]. These results suggested the formulated curcumin-chitin nanogels' explicit advantage for the treatment of melanoma by effective transdermal penetration.

Drug release from nanogels' networks depends on the interaction of hydrophobic and hydrogen complication and/or coordination of drug molecules with the polymer chain networks. Preclinical studies suggest that nanogels can be used for the efficient delivery of biopharmaceuticals in cells as well as for increasing drug delivery across cellular barriers [124]. Wu et al. [125] designed a class of water-dispersible hybrid nanogels for intracellular delivery of hydrophobic curcumin. They synthesized hybrid nanogels by coating the Ag/Au bimetallic nanoparticles with a hydrophobic polystyrene gel layer as internal shell and a subsequent thin hydrophilic nonlinear poly(ethylene glycol-) based gel layer as external shell. The Ag/Au core nanoparticles not only emitted well-built fluorescence for imaging and monitoring at the cellular level but also exhibited burly absorption in the near-infrared region for photothermal conversion and significantly improved the therapeutic efficacy. Furthermore, while the internal polystyrene gel layer was introduced to provide strong hydrophobic interactions with curcumin for high drug loading yields, the external nontoxic and thermoresponsive poly(ethylene glycol) analog gel layer was designed to trigger the release of the preloaded curcumin by either variation of surrounding temperature or exogenous irradiation with near-infrared light. These results suggest that such designed multifunctional hybrid nanogels are properly suited for in vivo and clinical trials by promising natural medicine of curcumin to the forefront of therapeutic agents for cancers and other diseases. In addition, hyaluronic acid- (HA-) based nanogel-drug conjugates with enhanced anticancer activity were designed by Wei et al. for the targeting of CD44-positive and drug-resistant tumors [65]. These authors synthesized nanogel-drug conjugates based on membranotropic cholesteryl-HA for efficient targeting and suppression of drug-resistant tumors. This class of tumors expresses CD44 receptors, cellular glycoproteins which bind to HA. These nanogel conjugates have significantly increased the bioavailability of poorly soluble drugs such as curcumin. In this study, the small nanogel particles with a hydrophobic core and high drug loads were formed after ultrasonication [65]. These nanogel particles demonstrated a sustained drug release following the hydrolysis of biodegradable ester linkage. Importantly, cholesteryl-HA-drug nanogels demonstrated a 2–7 times higher cytotoxicity in CD44-expressing drug-resistant human breast and pancreatic adenocarcinoma cells [65]. These nanogels were efficiently internalized via CD44 receptor-mediated endocytosis and simultaneous interaction with the cancer cell membrane [65]. Anchoring by cholesterol moieties in cellular membrane caused more efficient drug accumulation in cancer cells. The cholesteryl-HA nanogels were able to penetrate multicellular cancer spheroids and exhibited a higher cytotoxic effect in the system modeling tumor environment than both HA-drug conjugates and free drugs [65].

Overall, the proposed design of nanogel-drug conjugates can allow significantly enhancing drug bioavailability, stability, loading efficiency, effective transdermal penetration, cancer cell targeting, and treatment efficacy against drug-resistant cancer cells and multicellular spheroids (Table 1).

9. Chitosans

Chitosan is a linear polysaccharide composed of randomly disseminated deacetylated and acetylated units. It is made commercially by deacetylation of chitin, which is the structural component of crustaceans' exoskeleton and fungi cell walls. Unlike other biodegradable polymers, chitosan is the only one exhibiting a cationic character due to its primary amino groups that responsible for various effects in drug

delivery systems [126]. It displays particular properties, for example, solubility in various media, polyoxysalt creation, polyelectrolyte behavior, metal chelations, and structural uniqueness (Table 1). One study showed that the fluorescence intensity of curcumin can be greatly improved in the presence of chitosan by bovine and human serum albumin [104]. The method has been profitably used for the determination of human serum albumin in real samples. Data analysis recommended that the highly enhanced fluorescence of curcumin resulted from synergic effects of favorable hydrophobic microenvironment provided by bovine serum albumin and chitosan and efficient intermolecular energy transfer between bovine serum albumin and curcumin. Bovine serum albumin may bind to chitosan through hydrogen bonds, which causes the protein conformation to switch from β-fold to α-helix. Curcumin can combine with bovine serum albumin from β-fold to α-helix and can also combine with the bovine serum albumin-chitosan complex via its center carbonyl carbon. Therefore, chitosan plays a key role in promoting the energy transfer process by shortening the distance between bovine serum albumin and curcumin [104].

Polycaprolactone nanocarriers decorated with a mucoadhesive polysaccharide chitosan containing curcumin were also developed [127]. In order to optimize the preparation conditions, these nanocarriers were prepared by the nanoprecipitation method by using different molar masses and concentrations of chitosan and triblock surfactant poloxamer. Chitosan-coated nanocarriers revealed positive surface charge and a mean particle radius ranging between 114 and 125 nm, confirming the decoration of the nanocarriers with the mucoadhesive polymer, through hydrogen bonds between ether and amino groups, from poloxamer and chitosan, respectively. Dynamic light scattering studies have shown monodisperse nanocarriers. Furthermore, colloidal systems showed mean drug content about 460 lg/mL and encapsulation efficiency higher than 99%. In summary, these nanocarriers showed a vast ability to interact with mucin, also indicating their suitability for mucoadhesive applications when coated with chitosan [127].

On the other hand, curcumin-phytosome-loaded chitosan microspheres were developed by combining polymer- and lipid-based delivery systems to improve the bioavailability and prolong the retention time of curcumin [66]. These complexes were produced by encapsulating curcumin phytosomes in chitosan microspheres using ionotropic gelation. Differential scanning calorimetry and FUTI spectroscopy revealed that the integrity of the phytosomes was protected within the polymeric matrix of the microspheres. In vitro release rate of curcumin from the curcumin-phytosome-loaded chitosan microspheres was slower than curcumin-loaded chitosan microspheres. Pharmacokinetic studies showed an increase in curcumin absorption in curcumin-phytosome-loaded chitosan microspheres compared with curcumin phytosomes and curcumin-loaded chitosan microspheres. Moreover, half-life of curcumin in oral administration of curcumin-phytosome-loaded chitosan microspheres was longer than the two other ones. These results indicated that the novel curcumin-phytosome-loaded chitosan microspheres combined system has the advantages of both the chitosan microspheres and the phytosomes, which had better effects of promoting oral absorption and prolonging retention time of curcumin than single curcumin phytosomes or curcumin-loaded chitosan microspheres. Therefore, the phytosome chitosan microspheres may be used as a sustained delivery system for lipophilic compounds with poor water solubility and low oral bioavailability [66]. A study showed that curcumin bound to chitosan nanoparticles was not rapidly degraded in comparison to free curcumin, and the uptake of curcumin-loaded chitosan NPs by mouse's red blood cells (RBC) was much better than free curcumin [67]. Oral delivery of curcumin-loaded chitosan NPs improved the bioavailability of curcumin both in plasma and in RBC. Like chloroquine, conjugated curcumin inhibited parasite lysate induced heme polymerization in vitro in a dose dependent manner, and it had a lower IC50 value than chloroquine. Additionally, feeding of curcumin-loaded chitosan NPs caused a higher survival in mice infected with a lethal strain of *Plasmodium yoelii*. Therefore, binding of curcumin to chitosan NPs improves its chemical stability and bioavailability. In vitro data also suggest that this complex can inhibit hemozoin synthesis which is lethal for the parasite [67].

In another study, chitosan showed promising features as auxiliary agent in drug delivery (e.g., slimming, wound dressing, and tissue engineering). An in situ injectable nanocomposite hydrogel curcumin was effectively developed for use as a treatment in the dermal wound repair process [68]. In vitro release studies disclosed that the encapsulated nanocurcumin was slowly released from the N,O-carboxymethyl chitosan/oxidized alginate hydrogel with the controllable diffusion behavior. Additionally, in vivo wound healing studies revealed that application of nanocurcumin/N,O-carboxymethyl chitosan/oxidized alginate hydrogel could significantly improve the reepithelialization of epidermis and collagen deposition on rat dorsal wounds. DNA, protein, and hydroxyproline content in wound tissue indicated that making a combination by using nanocurcumin and N,O-carboxymethyl chitosan/oxidized alginate hydrogel could significantly accelerate the process of wound healing. So, results suggested that the developed nanocurcumin/N,O-carboxymethyl chitosan/oxidized alginate hydrogel as a promising wound dressing might have potential application in the wound healing [68].

Water-soluble nanocarriers of curcumin were synthesized, characterized, and applied as a stable detoxifying agent for arsenic poisoning [69]. The therapeutic efficacy of encapsulated curcumin nanocarriers was investigated against arsenic-induced toxicity in an animal model. In this regard, sodium arsenite and encapsulated curcumin were orally administered to male Wistar rats for 4 weeks. Arsenic dramatically declined blood d-aminolevulinic acid dehydratase activity and glutathione and increased blood reactive oxygen species. These alterations were accompanied by increases in hepatic total ROS, oxidized glutathione, and thiobarbituric acid-reactive substance levels. By contrast, hepatic glutathione, superoxide dismutase, and catalase activities were considerably declined after arsenic exposure, indicative of oxidative stress. Brain amines levels such as dopamine, norepinephrine, and 5-hydroxytryptamine also showed

considerable changes after arsenic exposure. Coadministration of encapsulated curcumin nanocarriers provided obvious favorable effects on the adverse changes in oxidative stress parameters induced by arsenic. The results revealed that encapsulated curcumin nanocarriers have better antioxidant and chelating potential compared to free curcumin. Therefore, the significant neurochemical and immunohistochemical protection afforded by encapsulated curcumin nanocarriers shows their neuroprotective effectiveness [69]. Chitosan also explains fungistatic, haemostatic, and antitumor effects [70]. In this regard stable vesicles for efficient curcumin encapsulation, delivery, and controlled release have been obtained by coating of liposomes with thin layer of newly synthesized chitosan derivatives [71]. Some special derivatives of chitosan were studied such as the cationic, hydrophobic, and cationic-hydrophobic derivatives. Zeta potential data proved effectual coating of liposomes with all these derivatives. In this regard, the liposomes coated with cationic-hydrophobic chitosan derivatives were the main promising curcumin carriers. They can easily enter cell membrane and release curcumin in a controlled approach, and the biological investigations showed that such organizations are nontoxic for normal murine fibroblasts while toxic for murine melanoma tumors [71].

In a recent study, Pluronic F127 was used to enhance the solubility of curcumin in the alginate-chitosan NPs [128]. Atomic force and scanning electron microscopic analysis demonstrated that the particles were almost spherical in shape (100 ± 20 nm). Fourier transform infrared analysis showed impending interactions among the components in the composite NPs. Furthermore, encapsulated curcumin efficiency confirmed considerable increase over alginate-chitosan NPs without Pluronic. Cytotoxicity assay explained that composite NPs at a concentration of 500 μg/mL were nontoxic for HeLa cells. Moreover, cellular internalization of curcumin-loaded complex was confirmed by green fluorescence inside the HeLa cells [128]. Curcumin-loaded biodegradable thermoresponsive chitosan-g-poly copolymeric NPs were prepared by using ionic cross-linking method [129]. The results showed that these NPs were nontoxic to different cancerous cell lines, whereas the curcumin loaded with NPs showed a specific toxicity for the abovementioned cell lines. Additionally, these results were further approved by flow cytometry analysis which proved increased apoptosis on these cell lines in a concentration-dependent manner. Furthermore, the blood compatibility assay showed the possibility of an IV injection with this formulation. Preliminary study provided clear evidence for the thermal targeting of curcumin by being loaded with novel thermosensitive chitosan-g-PNIPAAm NPs, and efficacies were achieved in cancer therapy. These results indicated that thermoresponsive chitosan-g-poly copolymeric NPs can be a potential nanocarrier for curcumin drug delivery [129]. Novel cationic poly(butyl) cyanoacrylate (PBCA) NPs coated with chitosan were synthesized with curcumin. The transmission electron microscopy showed the spherical shape of prepared NPs along with the particle size. Curcumin NPs demonstrated more therapeutic efficacy than free curcumin against a panel of human hepatocellular cancer cell lines. Encapsulated curcumin with PBCA NPs caused a profound change in the pharmacokinetics of the drug. The elimination half-life of curcumin was increased 52-fold in loaded form with PBCA NPs, and ultimately its clearance was also decreased 2.5-fold. Additionally, the higher plasma concentration of curcumin for curcumin-PBCA NPs might be a result of the NPs size and chitosan coating to keep drug in the blood circulation for a more extended period. Besides, the mean residence time of curcumin-PBCA NPs was longer than free curcumin. These results might be due to accumulation of NPs in endoplasmic reticulum system of organs and sustained release of the drug from them. Furthermore, the carriers' properties, for instance, shape, size, charge, and hydrophilicity, can prolong the retention of them in the blood circulation. There was also a substantial increase in the distribution volume (51-fold) that was quite unexpected. Obviously, it was possible that the larger micellar carriers were sequestered by the reticuloendothelial system or other tissues and truly led to improved distribution volume [130]. Additionally, treatment with curcumin NPs resulted in reduced tumor size and visible blanching of tumors [131].

So far, curcumin-loaded chitosan NPs improve the bioavailability and prolong the retention time of curcumin due to accumulation of NPs in endoplasmic reticulum system and the carriers' features such as shape, size, charge, and hydrophilicity (Table 1). Gathered data also propose that this complex can be lethal for the parasite because of hemozoin synthesis inhibition. Some in vivo experiments also resulted in better wound healing after application of curcumin-loaded chitosan NP polymers by means of better reepithelialization of epidermis and collagen deposition. This complex could also be administered in order to detoxify arsenic through better antioxidant and chelating potential. These compounds gained some achievements in cancer therapy as well.

10. Gold Nanoparticles

Metal nanoparticles have been known since very old times, and gold nanoparticles (AuNPs) with optical and electrochemical uniqueness have proven to be a potent apparatus in nanomedicinal requests [132]. They have also been largely used in immunochemistry, immunohistochemistry, and immunoblotting for electron microscopy. They are often generated in various shapes [132], and their properties are strongly dependent on the conditions in which they are prepared. Moreover, the stability of AuNPs and their capability to combine with biomolecules are their other outstanding properties. AuNPs are studied broadly as imperative drug delivery vectors due to some of their characteristic aspects, such as low cytotoxicity, tunable surface features, and stability in in vivo conditions, and can be easily synthesized and functionalized (Table 1). They can also act as drug pool for small drug molecules, proteins, DNA, or RNA with improved long life in the blood circulation. Rajesh et al. [133] used polyvinyl pyrrolidone (PVP) as a proven drug carrier to curcumin conjugation with AuNPs to enhance solubility of curcumin. Results showed a superior assurance for such conjugates as therapeutic-curcumin-imaging materials in

biomedical field [134]. Kumar et al. (2012) also prepared the chitosan-curcumin nanocapsules with AuNPs via solvent evaporation method. Scanning electron microscopy and transmission electron microscopy were done to describe the drug entrapped nanocapsules. The average diameter of AuNPs was found to be in the range of 18–20 nm, and the nanocapsules were found to be in the range of 200–250 nm. Furthermore, the Fourier transform infrared analysis revealed no possible interactions among the constituents with the chitosan nanoparticles. The drug release studies revealed that curcumin encapsulated chitosan with AuNPs was controlled and steadied when compared with curcumin encapsulated chitosan nanoparticles. Use of in vitro drug release in various kinetic equations indicated a matrix model with uniform distribution of curcumin in the nanocapsules [135]. Additionally, the tunability of AuNPs allows for complete control of surface properties for targeting and sustained release of the bioactive molecules [136].

In a study by Singh et al. [72] curcumin was bound on the surface of AuNPs in order to increase the bioavailability of it. The AuNPs were synthesized by direct decline of HAuCl4 by curcumin in aqueous part. Curcumin acted as both a reducing and capping agent and a stabilizing gold sol for many months. Furthermore, these curcumin-capped AuNPs showed an excellent antioxidant activity which was established by 2,2-diphenyl-l-picrylhydrazyl radical test. Consequently, the practical surface of AuNPs with curcumin may suggest a new way of use of curcumin towards possible drug delivery and therapeutics [72]. In another study, effect of curcumin-conjugated-AuNPs was investigated on peripheral blood lymphocytes [137]. The treated lymphocytes showed typical characteristics of apoptosis which included chromatin condensation and membrane blebbing and occurrence of apoptotic bodies. Results revealed that these conjugated nanoparticles may be used as drugs in nontoxic range [137]. In order to target cancer at a single cell level, gold-citrate nanoparticles were also synthesized with diameters of 13 nm [73]. AuNPs were coated with sodium citrate. Outcomes revealed that cancerous cells were more prone to absorb nanomaterials coated with citrate than normal somatic cells. Moreover, the damage was reversible with AuNPs and the normal dermal fibroblast cells were able to regenerate stress fibers which were lost during exposure. However, cancer cells were unable to recover from the damage inflicted by Au/citrate nanoparticle exposure [73]. Manju and Sreenivasan [136] also formulated a simple method for the fabrication of water-soluble curcumin conjugated AuNPs to target various cancer cell lines. Curcumin conjugated to hyaluronic acid to get a water-soluble compound. They were made AuNPs by diminishing chloroauric acid using hyaluronic acid-curcumin, which played dual roles of a reducing and a stabilizing agent and subsequently anchored folate conjugated PEG. Their interaction with various cancer cell lines was followed by flow cytometry and confocal microscopy. Blood-materials interactions studies proved that the nanoparticles are extremely hemocompatible. Flow cytometry and confocal microscopy results demonstrated considerable cellular uptake and internalization of the particles by various cancer cells [136].

In conclusion, curcumin conjugated AuNPs exhibited more cytotoxicity compared to free curcumin (Table 1). AuNPs also cause targeting and sustained release of curcumin and an excellent antioxidant activity.

11. Silvers

Silver has usually been utilized as an incredibly efficient material for antimicrobial utility [138]. In small concentrations, it is safe for human cells but lethal for the majority of bacteria and viruses [139]. With development of nanotechnology, it has become the metal of choice in restricting microbial growth and expansion in a variety of nanoparticle-related requests [138]. Silver nanoparticles are identified for their brilliant optoelectronic properties originated from surface plasmon resonance. They can be used in optoelectronics, biological labeling, and biological and chemical sensing (Table 1). They have shown excellent antimicrobial activity compared to other available silver antimicrobial agents.

Sodium carboxylmethyl cellulose silver nanocomposite films were attempted for antibacterial applications, so, to improve their applicability, novel film-silver nanoparticle-curcumin complexes have been developed [74]. These films were described by FTIR, UV-visible, X-ray diffraction (XRD), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and TEM techniques. The structured silver nanoparticles had a typical particle size of 15 nm. Curcumin loading into sodium carboxylmethyl cellulose silver nanocomposite films was achieved by diffusion mechanism. The UV analysis showed superior encapsulation of curcumin in the films with higher sodium carboxylmethyl cellulose content. Additionally, it was surveyed that the presence of silver nanoparticles in the films improved the encapsulation of curcumin demonstrating an interaction between them. Moreover, results showed that the sodium carboxylmethyl cellulose films produced with silver nanoparticles have a synergistic effect in the antimicrobial activity against *E. coli*. Furthermore, curcumin loaded with sodium carboxylmethyl cellulose silver nanocomposite films extended considerable inhibition of *E. coli* growth compared with the silver nanoparticles and curcumin alone film. Therefore, the study obviously supplied novel antimicrobial films which were potentially helpful in preventing/treating infections [74]. In another study, novel hydrogel-silver nanoparticle-curcumin composites have been built up to increase its applicability. These were first synthesized by polymerizing acrylamide in the presence of polyvinyl sulfonic acid sodium salt and a trifunctional cross-linker (2,4,6-triallyloxy 1,3,5-triazine) by using redox initiating system. Silver nanoparticles were then produced throughout the hydrogel networks by using in situ method incorporating the silver ions and following drop with sodium borohydride. Curcumin loading into hydrogel-silver nanoparticles complex was earned by diffusion mechanism. An attractive arrangement of silver nanoparticles (shining sun ball in range 5 nm) with apparent smaller grown nanoparticles (1 nm) was detected. A comparative antimicrobial study was performed for hydrogel-silver nanocomposites and hydrogel-silver nanoparticle-curcumin composites. The results indicated that hydrogel-Ag NPs-curcumin composites

have exhibited greater reduction of *E. coli* growth compared with Ag NPs loaded hydrogels. The current work demonstrated that combining hydrogel, nanotechnology, and curcumin is promising for developing novel antimicrobial agents with potential applications in dressing of various types of skin wounds. The entrapped silver nanoparticles and curcumin molecules showed sustained release which advises enormous prolonged therapeutic values [74]. In addition, silver nanoparticles could protect cells against HIV-1 infection and help with the wound healing process and also have essential function as an anti-inflammation, an antiviral, and an anticancer agent [75]. So, the combination of silver nanoparticles and curcumin, besides prolonged therapeutic outcomes and sustained release, has several other useful effects such as anti-inflammatory, anti-infection, anticancer, and wound healing (Table 1).

12. Solid Lipids

Solid lipid nanoparticles (SLNs) are one of the novel potential colloidal carrier systems as alternative materials to polymers for parenteral nutrition. SLNs have typically spherical and submicron colloidal carriers (50 to 1000 nm) and are composed of physiologically tolerated lipid components with solid shape at room temperature (Table 1). They are one of the most fashionable advances to develop the oral bioavailability of poorly water-soluble drugs [76]. Advantages of SLNs are high and improved drug content, ease of scaling up and sterilizing, better control over release kinetics of encapsulated compounds, enhanced bioavailability of entrapped bioactive compounds, chemical protection of incorporated compounds, much easier manufacturing than biopolymeric nanoparticles, conventional emulsion manufacturing methods, and applicability and very high long-term stability application versatility [76].

Kakkar et al. [77] loaded curcumin into SLNs to improve its oral bioavailability. Curcumin-SLNs with an average particle size of 134.6 nm and a total drug content of <92% were produced by using a microemulsification technique. In vivo pharmacokinetics was performed after oral administration of curcumin-SLNs by using a validated LC-MS/MS method in rat's plasma. Results revealed significant improvement in bioavailability times after administration of curcumin-SLNs with respect to curcumin-solid lipid. Data confirmed that enhanced and reliable bioavailability will help in establishing its therapeutic impacts [77]. Furthermore, Kakkar et al. [78] incorporated curcumin into SLNs to achieve a significant bioavailability of curcumin. Then, the plasma and brain cryosections were observed for fluorescence under fluorescent/confocal microscope. Biodistribution study was also performed using 99m Tc-labeled curcumin-SLNs and curcumin-solid lipid in mice after oral and intravenous administration. Presence of yellow fluorescent particles in plasma and brain indicated effective delivery of curcumin-SLNs across the gut wall and the blood brain barrier. Blood AU coral value for curcumin-SLNs was 8.135 times greater than curcumin-solid lipid, confirming a prolonged circulation of the former. The ratio of blood AUC intravenous curcumin-SLN/curcumin-solid lipid in blood was ≤1 while the ratio in brain promisingly indicates 30 times higher preferential distribution of curcumin-SLNs into brain confirming their direct delivery [78].

Dadhaniya et al. (2011) examined the adverse effects of a new solid lipid curcumin particle in rats. Administration of the conjugated curcumin showed no toxicologically significant treatment-related changes in the clinical parameters including behavioral observations, ophthalmic examinations, body weights and weight gains, food consumption, and organ weights or the paraclinical parameters including hematology, serum chemistry, and urinalysis. In addition, terminal necropsy revealed no treatment-related gross or histopathology findings [140]. Expansion of SLNs is one of the promising fields of lipid nanotechnology with several potential applications in drug delivery system and clinical medicine and research. The experimental paradigm of cerebral ischemia in rats by curcumin-SLNs was prepared; there was an improvement of 90% in cognition and 52% inhibition of acetylcholinesterase versus cerebral ischemic and neurological scoring, which improved by 79% [78]. Levels of superoxide dismutase, catalase, glutathione, and mitochondrial complex enzyme activities were also significantly increased, while lipid peroxidation, nitrite, and acetylcholinesterase levels decreased after curcumin-SLNs administration. Gamma-scintigraphic studies showed 16.4 and 30 times improvement in brain bioavailability upon oral and intravenous administration of curcumin-SLNs versus curcumin-silver. Results indicated the protective role of curcumin-SLNs against cerebral ischemic insult suggesting that it is packaged suitably for improved brain delivery [78]. Moreover, simultaneous curcumin treatment during the induction of neurotoxicity by aluminum was reported by Kakkar and Kaur (2011). They prepared solid lipid nanoparticles of curcumin with enhanced bioavailability and examined its therapeutic effects in alleviating behavioral, biochemical, and histochemical changes in mice. Adverse effects of aluminum were completely reversed by oral administration of curcumin-SLNs. Treatment with free curcumin showed <15% recovery in membrane lipids and 22% recovery in acetylcholinesterase with respect to aluminum treated group. Histopathology of the brain sections of curcumin-SLNs treated groups also indicated significant improvement [141]. This study emphasized the potential of curcumin-SLNs for treatment of Alzheimer's disease; though, the therapeutic potential of curcumin in terms of reversing the neuronal damage, once induced, is limited due to its compromised bioavailability [141].

Yadav et al. (2009) also developed a novel formulation approach for treating experimental colitis in the rat model by a colon-specific delivery approach. Solid lipid microparticles of curcumin were prepared with palmitic acid, stearic acid, and soya lecithin, with an optimized percentage of poloxamer 188. Then, the colonic delivery system of solid lipid microparticles formulations of curcumin was further investigated for their antiangiogenic and anti-inflammatory activities by using chick embryo and rat colitis models. Data showed that solid lipid microparticles of curcumin proved to be a potent angioinhibitory compound in the chorioallantoic membrane assay. Rats treated with curcumin and its solid lipid microparticle complex showed a faster weight gain

compared with dextran sulfate solution control rats. The increase in whole colon length appeared to be significantly greater in solid lipid microparticle-treated rats when compared with free curcumin and control rats. Moreover, decreased mast cell numbers was observed in the colon mucosa of curcumin-solid lipid microparticle treated rats. The degree of colitis caused by administration of dextran sulfate solution was significantly attenuated by colonic delivery of curcumin-solid lipid microparticles [79]. Being a nontoxic natural dietary product, it seems that curcumin can be useful in the therapeutic strategy for inflammatory bowel disease patients. Wang et al. (2012) aimed to formulate curcumin-SLNs to improve its therapeutic efficacy in an ovalbumin-induced allergic rat model of asthma. in vitro tests were performed in order to check Physiochemical properties of curcumin-SLNs and its release experiments. The pharmacokinetics in tissue distribution and the therapeutic effects were studied in mice. X-ray diffraction analysis revealed the amorphous nature of the encapsulated curcumin. The curcumin concentrations in plasma suspension were considerably superior to free curcumin, and all the tissue concentrations of curcumin increased after curcumin-SLNs administration, especially in lung and liver. In addition, curcumin-SLNs efficiently suppressed airway hyperresponsiveness and inflammatory cell infiltration. It also inhibited the expression of T-helper-2-type cytokinesin bronchoalveolar lavage fluid significantly compared to free curcumin. These observations imply that curcumin-SLNs can be a promising candidate for asthma therapy [80]. In another study, transferrin-mediated SLNs were prepared to increase photostability and anticancer activity of curcumin against breast cancer cells in vitro [81]. Microplate analysis and flow cytometry techniques were used for cytotoxicity and apoptosis studies. The physical characterization showed the suitability of preparation method. Transmission electron microscopy and X-ray diffraction studies revealed the spherical nature and entrapment of curcumin in amorphous form, respectively. Annexin V-FITC/PI double staining, DNA analysis, and reduced mitochondrial potential confirmed the occurrence of apoptosis. The flow cytometric studies disclosed that the anticancer activity of curcumin is enhanced with transferrin-mediated SLNs compared to free curcumin, and apoptosis is the mechanism underlying the cytotoxicity (Table 1). Results indicated the potential of transferrin-mediated SLNs in enhancing the anticancer effect of curcumin in breast cancer cells in vitro [81].

13. Conclusion and Future Perspectives

The use of nanotechnology in medicine and more purposely drug delivery is set to spread quickly. Currently, many substances are under investigation for drug delivery and more specifically for cancer therapy. Fascinatingly, pharmaceutical sciences are using nanoparticles to reduce toxicity and side effects of drugs. Moreover nanoparticles augment solubility and stability of some substances like curcumin. It is now clear that further development of traditional natural compounds with chemopreventive and chemotherapeutic potential such as curcumin will be dictated by the advanced drug delivery systems. Nanotechnology is assumed to be a fundamental setting in drug delivery system and human therapeutics. However, considerable challenges remain in driving this field into clinically practical therapies. Curcumin, an excellent representative derived from traditional natural compounds, has been proven to be effectual in long-term application and preclinical trials. There is no doubt that advance of novel delivery systems of curcumin with better therapeutic effects will be vital for future improvement of curcumin as a therapeutic agent. Thus, it is an enormous implication to overcome the current limitations of curcumin. It seems that only by multidisciplinary collaboration we can bring these promising traditional natural compounds to the forefront of therapeutic agents for different diseases. Therefore, the promise of nanotechnology-based medicine may become a reality with sufficient efforts and further researches. Human trials need to be conducted to establish curcumin's effectiveness in clinical applications as an improved therapeutic modality for treatment of different diseases.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content of the paper.

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Review Article

Roles of Renal Proximal Tubule Transport in Acid/Base Balance and Blood Pressure Regulation

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Sodium-coupled bicarbonate absorption from renal proximal tubules (PTs) plays a pivotal role in the maintenance of systemic acid/base balance. Indeed, mutations in the Na^+-HCO_3^- cotransporter NBCe1, which mediates a majority of bicarbonate exit from PTs, cause severe proximal renal tubular acidosis associated with ocular and other extrarenal abnormalities. Sodium transport in PTs also plays an important role in the regulation of blood pressure. For example, PT transport stimulation by insulin may be involved in the pathogenesis of hypertension associated with insulin resistance. Type 1 angiotensin (Ang) II receptors in PT are critical for blood pressure homeostasis. Paradoxically, the effects of Ang II on PT transport are known to be biphasic. Unlike in other species, however, Ang II is recently shown to dose-dependently stimulate human PT transport via nitric oxide/cGMP/ERK pathway, which may represent a novel therapeutic target in human hypertension. In this paper, we will review the physiological and pathophysiological roles of PT transport.

1. Introduction

Renal proximal tubules (PTs) reabsorb approximately 80% of the filtered bicarbonate from glomerulus, thereby playing a pivotal role in the maintenance of systemic acid-base balance [1]. This process is mostly dependent on Na^+, which is composed of the luminal Na^+/H^+ exchanger and the basolateral Na^+-HCO_3^- cotransporter [1]. Although distal nephron segments are also involved in the systemic acid/base regulation, acid-base transporters in these segments often cannot completely compensate for defects in bicarbonate absorption from PTs. Indeed, mutations in the Na^+-HCO_3^- cotransporter NBCe1, which mediates a majority of bicarbonate exit from the basolateral membrane of PTs, are known to cause a severe type of proximal renal tubular acidosis associated with ocular and other extrarenal manifestations [2].

On the other hand, PTs reabsorb approximately 65% of the filtered NaCl, thereby also contributing to the regulation of plasma volume and blood pressure. For example, hypertension is frequently associated with metabolic syndrome, and insulin-mediated stimulation of PT transport may play a role in this association [3, 4]. In addition, angiotensin (Ang) II is pivotal in the regulation of blood pressure, and stimulation of PT transport may play a critical role in Ang II-mediated hypertension [5, 6]. In this review, we will focus on the roles of PT transport in the maintenance of acid-base homeostasis as well as the regulation of blood pressure.

2. Roles of PT Transport in Acid/Base Balance

In PTs, the luminal Na^+/H^+ exchanger type 3 (NHE3) together with the basolateral Na^+-HCO_3^- cotransporter NBCe1 is thought to mediate a majority of sodium-coupled bicarbonate absorption from this segment [1, 7]. Although the basolateral membrane of PTs contains Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchangers [7], these transporters cannot effectively compensate for the loss of NBCe1 function. By contrast, the loss of NHE3 function may be at least partially compensated by the other luminal transporters such as NHE8 [8].

In 1983 Boron and Boulpaep identified the functional existence of electrogenic Na^+-coupled HCO_3^- transport activity in the basolateral membrane of isolated salamander PTs [9]. Subsequently, Kondo and Frömter revealed that this electrogenic Na^+-HCO_3^- cotransport activity is robust in S1 and S2 segments but almost absent in S3 segment of isolated rabbit PTs [10]. Yoshitomi and colleagues initially reported that the Na^+-HCO_3^- cotransporter in rat PTs in vivo functions with $1Na^+$ to $3HCO_3^-$ stoichiometry [11]. On the other hand, Seki and colleagues revealed that the Na^+-HCO_3^- cotransporter in isolated rabbit PTs functions with $1Na^+$ to $2HCO_3^-$ stoichiometry [12]. Later, Müller-Berger and colleagues found that the Na^+-HCO_3^- cotransporter in isolated rabbit PTs can change its transport stoichiometry depending on the incubation conditions [13]. Interestingly, NBCe1 expressed in *Xenopus* oocytes can also change its transport stoichiometry depending on changes in cytosolic Ca^{2+} concentrations [14]. Consistent with these data, Gross and colleagues reported that stoichiometry of NBCe1 is cell-type specific [15].

In 1997 Romero and colleagues succeeded in the first molecular cloning of NBCe1 from salamander kidney [16]. Among the three major variants, NBCe1A is transcribed from the alternative promoter in exon 1 and abundantly expressed in the basolateral membrane of PTs, representing the major bicarbonate exit pathway in this nephron segment [1]. Another variant NBCe1B is transcribed from the dominant promoter in exon 1 and differs from NBCe1A only at the N-terminus [17]. NBCe1B is first cloned from pancreas but is now known to be expressed in a variety of tissues such as intestinal tracts, ocular tissues, and brain [18–20]. On the other hand, NBCe1C is predominantly expressed in brain and differs from NBCe1B only at the C-terminus [21]. Consistent with the indispensable role of NBCe1 in acid/base homeostasis, Igarashi and colleagues found that inactivating mutations in NBCe1 cause a severe type of proximal renal tubular acidosis (pRTA) associated with ocular abnormalities [2]. Until now 12 different homozygous mutations have been found in pRTA patients [22]. These patients invariably presented with ocular abnormalities such as band keratopathy, cataract, and glaucoma, suggesting that NBCe1 function is essential for the maintenance of homeostasis in ocular tissues. Indeed, NBCe1 is found to be abundantly expressed in several human ocular tissues such as corneal endothelium, lens epithelium, and trabecular meshwork cells [20].

NBCe1 in brain may also play several physiological roles [23]. Indeed, Suzuki and colleagues revealed that defective membrane expression of NBCe1B may cause migraine with or without hemiplegia [24]. NBCe1B activity in astrocytes may be indispensable for the regulation of synaptic pH and neuron excitability.

Two types of NBCe1-deficient mice, NBCe1-KO mice [25] and W516X-knockin mice [26], present with very severe acidemia due to pRTA and die within 30 days. Functional analysis using isolated PTs from W516X-knockin mice confirmed that the normal NBCe1 activity is essential for bicarbonate absorption from this nephron segment [26]. Alkali therapy significantly prolonged the survival of W516X-knockin mice. Detailed analysis of ocular tissues in these mice revealed that NBCe1 plays a critical role in the maintenance of corneal transparency also in mice [26].

Unlike NBCe1-deficient mice, NHE3-KO mice present with only mild acidemia [27]. Although NHE8 seems to partially compensate for the loss of NHE3 function, NHE3/NHE3-double KO mice also present with relatively mild acidemia [8]. So far, mutations in NHE3 or NHE8 have not been found in human pRTA patients.

3. Roles of Hyperinsulinemia in Hypertension Associated with Metabolic Syndrome

Certain risk factors such as abdominal adiposity, glucose intolerance, dyslipidemia, and hypertension tend to cluster within individuals. Insulin resistance with obesity is thought to be a key factor for this association, which is now termed as metabolic syndrome [28]. Several different mechanisms such as activation of renin-angiotensin-aldosterone system (RAAS), enhancement of sympathetic nervous system, or hyperinsulinemia may be involved in the occurrence of hypertension associated with insulin resistance [29, 30]. Among these factors, hyperinsulinemia-induced hypertension seems to be an attractive hypothesis in view of the antinatriuretic action of insulin [3, 4]. Indeed, insulin is known to stimulate sodium absorption from several nephron segments. For example, insulin may stimulate sodium absorption from distal convoluted tubules by phosphorylating the Na^+-Cl^- cotransporter NCC through the with-no-lysine kinase 4 (WNK4)/STE20/SPS1-related proline-alanine-rich kinase (SPAK) pathway [31]. In cortical collecting duct (CCD) cells insulin is thought to stimulate sodium absorption by activating the activity of epithelial Na^+ channel ENaC [32–34], though a recent study failed to confirm the stimulatory effect of insulin on the ENaC activity in isolated mammalian CCD [35]. In PTs, insulin enhances sodium absorption by stimulating the luminal NHE3, the basolateral Na^+/K^+-ATPase, and the basolateral NBCe1 [36–39].

Insulin can relax vascular tones through the phosphatidylinositol 3 kinase (PI3K)/Akt-dependent nitric oxide (NO) production, and simple hyperinsulinemia may not necessarily induce hypertension [40]. Notably, however, the vasodilator action of insulin is reported to be attenuated in insulin resistance [41, 42]. Therefore, hyperinsulinemia can be an important factor in hypertension associated with metabolic syndrome, if the stimulatory effects of insulin on renal sodium absorption are preserved even in the systemic insulin resistance.

In support of this hypothesis, recent studies have clarified that defects in insulin signaling at the level of insulin receptor substrate (IRS) proteins are frequently associated with human insulin resistant states, resulting in the occurrence of cell-type specific insulin resistance [43, 44]. The two major substrates IRS1 and IRS2 may mediate distinct pathways in insulin signaling, and they are not functionally interchangeable in many insulin-sensitive tissues [43–45]. Importantly, in adipocytes of human subjects with noninsulin-dependent

diabetes mellitus the expression of IRS1 protein is found to be markedly reduced, accompanied with the severe reduction of insulin-mediated glucose uptake [46]. By contrast, the reduction of IRS2 expression seems to be a key factor in several forms of insulin resistance in liver [47].

To clarify the relative importance of IRS1 and IRS2 in the stimulatory effect of insulin on PT transport, Zheng and colleagues compared the effects of insulin on sodium-coupled bicarbonate absorption in isolated PTs from IRS1-KO and IRS2-KO mice. They found that the PI3 K-dependent stimulatory effect of insulin on PT transport was preserved in IRS1-KO mice but markedly attenuated in IRS2-KO mice. Furthermore, insulin-induced Akt phosphorylation was also preserved in IRS1-KO mice but not in IRS-2 KO mice [48]. These results indicate that IRS2 is the main substrate that mediates the stimulatory effects of insulin on PT transport. Importantly, insulin can induce antinatriuresis even in insulin resistant rats and humans [49, 50]. Moreover, PT sodium transport seems to be enhanced in insulin resistant humans [51, 52], suggesting that the stimulatory effect of insulin on PT transport may be preserved in common forms of insulin resistance. Consistent with this view, a recent study showed that the expression of IRS2 as well as insulin-mediated Akt phosphorylation in renal tubules is preserved in Zucker fatty rats that show marked insulin resistance due to defective leptin signaling [53]. In liver, hyperinsulinemia is known to suppress the expression of IRS2, thereby attenuating the insulin signaling in liver [47, 54]. Future studies are required to determine whether the IRS2-dependent stimulatory insulin signaling in PTs is preserved in common forms of insulin resistance.

Interestingly, the IRS1-dependent insulin signaling in glomeruli seems to be attenuated in insulin resistance [53]. Because insulin signaling may be required not only for the nitric oxide (NO) production by glomerular endothelium but also for the preservation of normal podocyte functions [53, 55], insulin resistance in glomeruli may promote the occurrence and progression of diabetic nephropathy. In fact, the treatment with insulin sensitizers thiazolidinediones (TZDs) can protect podocyte from injury independently of glycemic control [56]. However, TZDs, especially when used with insulin, may induce edema formation as a side effect, probably by stimulating sodium absorption from PT and/or distal tubules [57, 58]. Unfortunately, this side effect may offset the beneficial effects of TZDs on the insulin signaling in glomeruli.

4. Effects of Ang II on PT Transport

There are two major Ang II receptors (AT), AT_1 and AT_2. AT_1 receptors are further subdivided into AT_{1A} and AT_{1B} in rodents [59]. While AT_1 may be the main receptors that mediate the effects of Ang II on blood pressure, AT_2 may be also partially involved in blood pressure regulation [60]. Ang II can regulate blood pressure via AT_1 receptors in both renal and extrarenal tissues. To clarify the relative importance of these receptors in blood pressure homeostasis, Coffman and colleagues performed kidney cross-transplantation between wild-type and AT_{1A}-KO mice [5, 6]. They found that renal and extrarenal AT_{1A} receptors almost equally contribute to the maintenance of baseline blood pressure. However, renal AT_{1A} receptors are indispensable for the occurrence of Ang II-induced hypertension and cardiac hypertrophy. They further showed that specific deletion of AT_{1A} receptors from PTs alone is sufficient to lower blood pressure and provides substantial protection against Ang II-induced hypertension [61]. These results indicate that the stimulatory effect of Ang II on PT sodium transport is quite important in blood pressure regulation.

Paradoxically, however, the effects of Ang II on PT transport are biphasic: transport is stimulated by picomolar to nanomolar concentrations of Ang II, while it is inhibited by nanomolar to micromolar concentrations of Ang II [62, 63]. The effects of Ang II on NHE3, Na^+/K^+ ATPase, and NBCe1 in PTs are all known to be biphasic [64–67]. Notably, intrarenal concentrations of Ang II are much higher than those in plasma [68]. Accordingly, the inhibitory effect of Ang II on PT transport could have some physiological significance.

Controversial data have been reported as to the receptor subtype(s) responsible for the biphasic effects of Ang II on PT transport [69, 70]. However, Horita and colleagues, by analyzing the NBCe1 activity in isolated PTs, found that the biphasic effects of Ang II added to bath perfusate were lost in AT_{1A}-KO mice [71]. Instead, very high concentrations of Ang II added to bath perfusate induced a slight stimulation of NBCe1 activity, which was probably mediated by AT_{1B} [71, 72]. Zheng and colleagues, by analyzing the bicarbonate absorption rates from isolated PTs, also found that the biphasic effects of Ang II added to luminal perfusate were lost in AT_{1A}-KO mice [73]. These results clearly indicate that both luminal and basolateral AT_{1A} receptors mediate the biphasic effects of Ang II on PT transport.

Regarding the signaling pathways, the activation of PKC and/or the decrease in intracellular cAMP concentrations, which may ultimately result in ERK activation, are thought to mediate the stimulatory effect of Ang II [67, 72, 74]. On the other hand, the activation of phospholipase A_2 (PLA_2)/arachidonic acid/5,6-epoxyeicosatrienoic acid (EET) pathway and/or the NO/cGMP pathway is thought to mediate the inhibitory effect of Ang II [67, 72, 75]. Consistent with this view, Li and colleagues found that the biphasic effects of Ang II were lost and all the concentrations of Ang II induced a similar stimulation of NBCe1 activity in isolated PTs from cytosolic PLA_2-KO mice [72].

While the biphasic effects of Ang II on PT transport have been reported in rats, mice, and rabbits [62, 63, 65, 66, 71, 73], little has been known about the effects of Ang II on human PT transport. To clarify this issue, Shirai and colleagues recently examined the effects of Ang II in isolated human PTs obtained from nephrectomy surgery for renal carcinoma [76]. Surprisingly, they found that Ang II, unlike that in the other species, induced a dose-dependent, profound stimulation of human PT transport via AT_1-dependent ERK activation. In wild-type mice, the inhibitory effect of Ang II was dependent on the NO/cGMP/cGMP-dependent kinase II (cGKII) pathway. In cGKII-KO mice, the inhibitory effect of Ang II was lost but

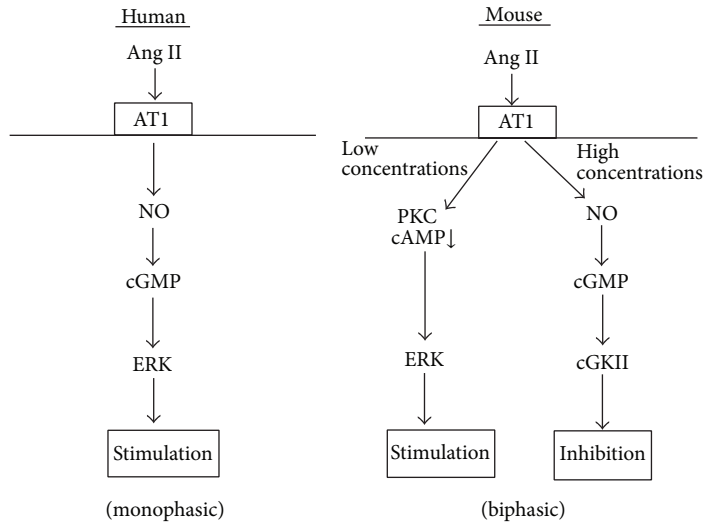

Figure 1: Ang II signaling in mouse and human PTs. In mouse PTs, low concentrations of Ang II induce transport stimulation via either PKC activation or decrease in intracellular cAMP resulting in ERK activation, while high concentrations of Ang II induce transport inhibition via NO/cGMP/cGKII pathway. In human PTs, by contrast, Ang II induces dose-dependent transport stimulation via NO/cGMP/ERK pathway.

the NO/cGMP pathway failed to induce the ERK-dependent NBCe1 activation. By sharp contrast, in human PTs, the NO/cGMP pathway mediated the stimulatory effect of Ang II via cGKII-independent ERK activation. Thus, as shown in Figure 1, the contrasting responses to NO/cGMP pathway seem to be largely responsible for the different modes of PT transport regulation by Ang II in humans and the other species.

At present the molecular mechanisms underlying the species differences in PT response to NO/cGMP pathway remain unknown. However, previous studies suggest that such species differences may indeed exist. For example, NO is generally thought to work as inhibitory on PT transport in rodents [77, 78]. Furthermore, salt loading into rodents is known to enhance renal NO synthesis, which may facilitate sodium excretion and preservation of normal blood pressure [79, 80]. In human subjects, however, salt loading fails to induce an adaptive increase in renal NO synthesis [81, 82]. Thus, the role of NO/cGMP in adaptive natriuretic response to salt loading is clearly established in rodents but not in human subjects. Taken together with these considerations, the study by Shirai and colleagues [76] suggests that the unopposed, marked stimulation of PT transport by high intrarenal concentrations of Ang II may play an important role in the pathogenesis of human hypertension. Furthermore, the human-specific stimulatory effect of NO/cGMP pathway on PT transport may represent a novel therapeutic target in hypertension.

5. Conclusion

In this paper, we reviewed the physiological and pathophysiological roles of PT transport. Sodium-coupled bicarbonate absorption from PTs plays a critical role in the systemic acid/base balance. Indeed, inactivating mutations in NBCe1 cause severe pRTA associated with ocular and other extrarenal abnormalities. Sodium transport in PTs may also play an important role in blood pressure regulation. In particular, the stimulatory effect of insulin on PT transport may be involved in the pathogenesis of hypertension associated with metabolic syndrome. Unlike in other species, Ang II dose-dependently stimulates human PT transport via NO/cGMP/ERK pathway, which may represent a novel therapeutic target in human hypertension.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Distinct Action of Flavonoids, Myricetin and Quercetin, on Epithelial Cl^- Secretion: Useful Tools as Regulators of Cl^- Secretion

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Epithelial Cl^- secretion plays important roles in water secretion preventing bacterial/viral infection and regulation of body fluid. We previously suggested that quercetin would be a useful compound for maintaining epithelial Cl^- secretion at a moderate level irrespective of cAMP-induced stimulation. However, we need a compound that stimulates epithelial Cl^- secretion even under cAMP-stimulated conditions, since in some cases epithelial Cl^- secretion is not large enough even under cAMP-stimulated conditions. We demonstrated that quercetin and myricetin, flavonoids, stimulated epithelial Cl^- secretion under basal conditions in epithelial A6 cells. We used forskolin, which activates adenylyl cyclase increasing cytosolic cAMP concentrations, to study the effects of quercetin and myricetin on cAMP-stimulated epithelial Cl^- secretion. In the presence of forskolin, quercetin diminished epithelial Cl^- secretion to a level similar to that with quercetin alone without forskolin. Conversely, myricetin further stimulated epithelial Cl^- secretion even under forskolin-stimulated conditions. This suggests that the action of myricetin is via a cAMP-independent pathway. Therefore, myricetin may be a potentially useful compound to increase epithelial Cl^- secretion under cAMP-stimulated conditions. In conclusion, myricetin would be a useful compound for prevention from bacterial/viral infection even under conditions that the amount of water secretion driven by cAMP-stimulated epithelial Cl^- secretion is insufficient.

1. Introduction

Water secretion across epithelial tissues contributes to prevention of our body from bacterial/viral infection and regulation of body fluid content. The water secretion across epithelial tissues is driven by epithelial Cl^- secretion [1–3]. The epithelial Cl^- secretion consists of two steps: (1) the Cl^--entry step across the basolateral membrane via Cl^- transporter participating in Cl^- uptake into the intracellular space such as Na^+-K^+-$2Cl^-$ cotransporter (NKCC) and (2) the Cl^--releasing step across the apical membrane via Cl^- channels such as cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel at the apical membrane [4, 5]. The continuous Cl^- secretion requires stimulation of Cl^- uptake transporter such as NKCC [6, 7]. Flavonoids and flavonoid-like compounds have various actions in cell function [8, 9]. Our previous studies have reported that a flavonoid, quercetin, elevates epithelial Cl^- secretion under basal conditions, but decreases it under cAMP-stimulated conditions [10] by modifying activity of NKCC [3]. These reports suggest that quercetin regulates the activity of NKCC leading epithelial Cl^- secretion to a moderate level irrespective of cAMP-induced stimulation and that quercetin would be a useful compound to achieve a moderate level

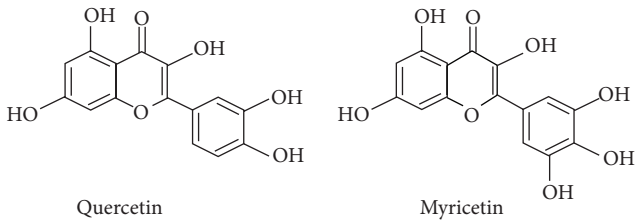

Figure 1: Structures of quercetin and myricetin.

of epithelial Cl^- secretion. However, in some cases, cAMP-induced stimulation is not large enough to maintain an adequate level of epithelial Cl^- secretion. Myricetin has been reported to demonstrate promotive and protective effects on intestinal tight junctional barriers of epithelia and it has antiviral function [11–13]. Therefore, it would be important to identify a useful compound with stimulatory actions on epithelial Cl^- secretion even under cAMP-stimulated conditions. Our study examines the effects of quercetin and myricetin on Cl^- secretion in the absence and presence of cAMP-stimulation of renal A6 cells.

2. Materials and Methods

2.1. Chemicals and Materials. We obtained forskolin, benzamil, NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), quercetin, myricetin, and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA) and epithelial A6 cells from American Type Culture Collection (ATCC). Forskolin, benzamil, NPPB, bumetanide, quercetin, and myricetin were dissolved in DMSO. We applied forskolin of 10 μM, benzamil of 10 μM, NPPB of 100 μM, bumetanide of 100 μM, quercetin of 100 μM, and myricetin of 100 μM to the bath solution as the final concentration. The concentrations of forskolin, quercetin and myricetin used in the present study were determined from the observations obtained in previous reports [7, 10]. The structures of quercetin and myricetin are shown in Figure 1.

2.2. Cell Culture. Renal epithelial A6 cells derived from *Xenopus laevis* were obtained from American Type Culture Collection (Rockville, MD, USA) at *passage 68*. We cultured A6 cells (*passages 73–84*) on plastic flasks at 27°C in a humidified incubator with 1.0% CO_2 in air in a culture medium containing 75% (vol/vol) NCTC-109, 15% (vol/vol) distilled water and 10% (vol/vol) fetal bovine serum. Then, we seeded cells onto permeable tissue culture-treated Transwell filter cups (Costar, Cambridge, MA, USA) for electrophysiological measurements at a density of 5×10^4 cells/well for 11–15 days.

2.3. Measurement of Transepithelial Conductance (Gt). We transferred monolayers of A6 cells subcultured on tissue culture-treated Transwell filter cups to a modified Ussing chamber (Jim's Instrument, Iowa City, IA, USA) designed to hold the filter cup and continuously measured transepithelial potential difference (PD) by a high-impedance millivoltmeter (VCC-600, Physiologic Instrument, San Diego, CA, USA) [7]. We applied a pulse of 1 μA constant current every 10 s for 0.5 s to the A6 monolayer under open-circuit conditions. This enabled us to calculate the transepithelial conductance (Gt) from the change in the PD (ΔPD) caused by the 1 μA constant-current pulse using Ohm's law (Gt = 1 μA/ΔPD mV). We applied 100 μM NPPB (a nonselective Cl^- channel blocker [3, 14, 15]) to the apical solution for detection of NPPB-sensitive conductance. We measured an NPPB-sensitive conductance by calculating the difference between the Gt just before and 30 minutes after application of 100 μM NPPB. This difference of Gt represents an NPPB-sensitive conductance. In the present study, we use the NPPB-sensitive conductance as the apical Cl^- conductance. The NPPB-sensitive conductance indicates the apical Cl^- channel conductance, since the apical Cl^- conductance is much smaller than the basolateral Cl^- conductance [16]. Further, apical application of 100 μM NPPB diminishes the apical Cl^- conductance but not the basolateral Cl^- conductance [7, 16]. Bumetanide has been shown to have no effects on the NPPB-sensitive conductance [7]. These observations [7, 16] indicate that the NPPB-sensitive conductance can be used as the apical Cl^- conductance.

2.4. Measurement of Short-Circuit Current (Isc). As reported previously [7, 16], we measured a short-circuit current (Isc) in A6 cells. The Isc measured directly by clamping the PD to 0 mV was identical to the calculated current as Gt·PD (equivalent current); namely, the monolayer had a linear current-voltage relationship. In the present study, we show an equivalent current (Gt·PD) as Isc. A positive current represents a net flow of anions from the basolateral solution to the apical one [7].

2.5. Solutions. The solution used in the present study contained 120 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES with pH 7.4.

2.6. Temperature. All experiments shown in the present study were performed at 24-25°C.

2.7. Data Presentation. Values of Isc and Gt are shown as the mean, and the error bar indicates SEM. ANOVA was used for statistical analysis, and $P < 0.05$ was considered significant.

3. Results

3.1. Effects of Quercetin and Myricetin on Isc under Basal Conditions. A6 cells can exhibit Cl^- secretion and Na^+ absorption [7, 17–22]. Since the purpose of the present study was to examine transport properties of Cl^- secretion, we added benzamil (10 μM) to the apical solution to block Na^+ absorption via epithelial Na^+ channel (ENaC) in A6 cells (Figure 2). We used 10 μM benzamil, because it has been demonstrated to completely block ENaC activity [18, 23, 24]. DMSO (a solvent control for quercetin and myricetin (Figure 1)) applied to both apical and basolateral solutions had no effect on Isc (closed triangles as in Figure 2(a)), and 100 μM NPPB applied to the apical solution abolished

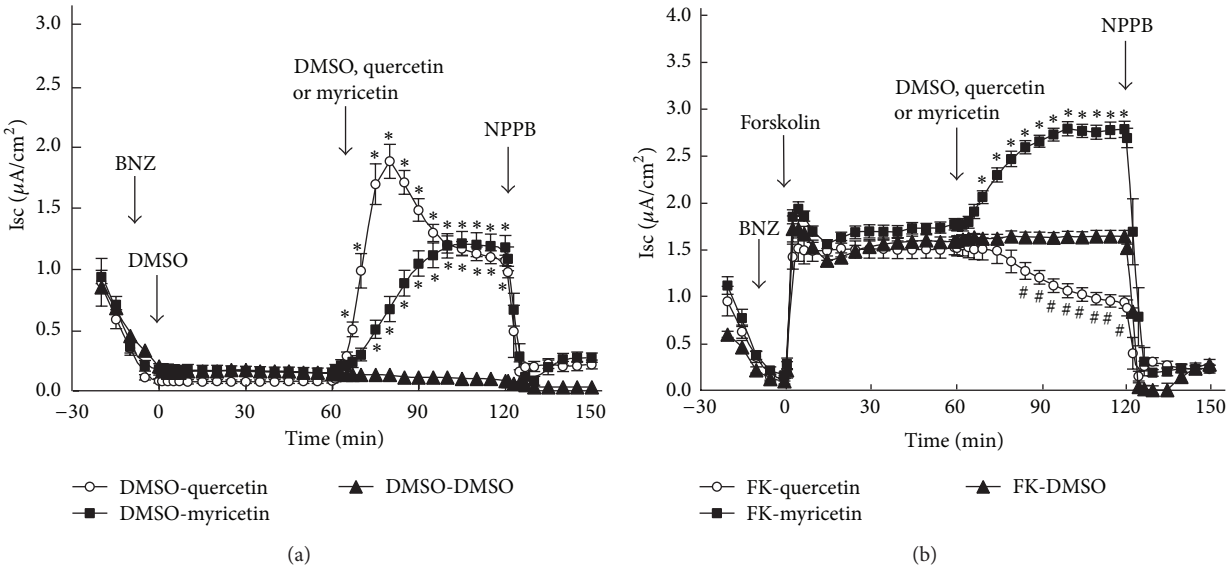

Figure 2: Effects of quercetin and myricetin on Isc under basal (a) and forskolin-stimulated conditions (b). (a) Benzamil (BNZ, a blocker of epithelial Na^+ channel: ENaC; 10 μM) was applied to the apical solution at −10 min. DMSO (dimethyl sulfoxide; a solvent for forskolin; 0.1% as the final concentration in Isc measuring solutions) was added to both apical and basolateral solutions at 0 min (open circles, closed squares, and closed triangles). Quercetin (100 μM; open circles), myricetin (100 μM; closed squares), or DMSO (a solvent for quercetin and myricetin; 0.1%; closed triangles) was applied to both apical and basolateral solutions at 60 min. NPPB (a nonspecific blocker of Cl^- channels; 100 μM) was applied to the apical solution at 120 min (open circles, closed squares, and closed triangles). $n = 5$ for DMSO, $n = 6$ for quercetin, and $n = 7$ for myricetin. (b) Benzamil (BNZ, 10 μM) was applied to the apical solution at −10 min. Forskolin (10 μM) was added to both apical and basolateral solutions at 0 min (open circles, closed squares, and closed triangles). Quercetin (100 μM; open circles), myricetin (100 μM; closed squares), or DMSO (a solvent for quercetin and myricetin; 0.1%; closed triangles) was applied to both apical and basolateral solutions at 60 min. NPPB (100 μM) was applied to the apical solution at 120 min (open circles, closed squares and closed triangles). $n = 6$ for DMSO, $n = 5$ for quercetin, and $n = 8$ for myricetin. The values marked with $*$ (open circles and closed squares) are significantly larger than DMSO (closed triangles; $P < 0.05$). The values marked with # (closed squares) are significantly smaller than DMSO (closed triangles; $P < 0.05$).

the benzamil-insensitive Isc (*i.e.*, the residual Isc after application of benzamil was abolished) (Figure 2(a)). Quercetin significantly stimulated Isc (open circles as in Figure 2(a)), and NPPB abolished the quercetin-stimulated Isc (open circles as in Figure 2(a)) suggesting that quercetin stimulated a Cl^--dependent Isc. Myricetin also elevated Isc (closed squares as in Figure 2(a)), and the myricetin-elevated Isc was sensitive to NPPB (closed squares as in Figure 2(a)). Quercetin or myricetin showed no effects on Isc in A6 cells pretreated with NPPB (data not shown). These observations indicate that quercetin and myricetin stimulate the Cl^- secretion.

3.2. Effects of Quercetin and Myricetin on Isc under Forskolin-Stimulated Conditions. In order to examine the effects of quercetin and myricetin in the presence of elevated cAMP levels, we used forskolin to activate adenylyl cyclase to increase cellular cAMP. As shown in Figure 2(a) (closed triangles), DMSO, a solvent control for forskolin, had no effect on Isc. Though, forskolin stimulated Isc (open circles, closed squares, and closed triangles in Figure 2(b)), but not in the presence of NPPB (data not shown), suggesting that forskolin stimulated an NPPB-sensitive Isc. Under forskolin-stimulated conditions, DMSO (a solvent control for quercetin and myricetin) had no effect on Isc (closed triangles as in Figure 2(b)). However, in the presence of forskolin, quercetin significantly diminished Isc (open circles as in Figure 2(b)) unlike that observed during basal conditions (open circles as in Figure 2(a)). On the other hand, myricetin significantly stimulated Isc (closed squares as in Figure 2(b)) in the presence of forskolin similar to that under basal conditions (closed squares as in Figure 2(a)). Further, we applied bumetanide (a blocker of NKCC) to study the Isc observed in the present study. Application of bumetanide (100 μM) almost completely diminished the Isc irrespective of the presence of forskolin, quercetin, or myricetin without any effects on Gt: the Isc in the presence of bumetanide was reduced to ~0.2 μA/cm^2 irrespective of the presence of forskolin, quercetin, or myricetin. Further, the presence of bumetanide did not significantly influence effects of forskolin, quercetin, or myricetin on Gt (data not shown). Thus, these observations indicate that the Isc observed in the present study is mediated by NKCC irrespective of the presence of forskolin, quercetin, or myricetin.

3.3. NPPB-Sensitive Isc under Basal and Forskolin-Stimulated Conditions. In Figure 3, we show the NPPB-sensitive Isc under various experimental and control conditions in the absence and presence of forskolin-stimulated Isc as shown in Figure 2. Under basal conditions, quercetin increased the NPPB-sensitive Isc ($^*P < 0.001$ compared with DMSO

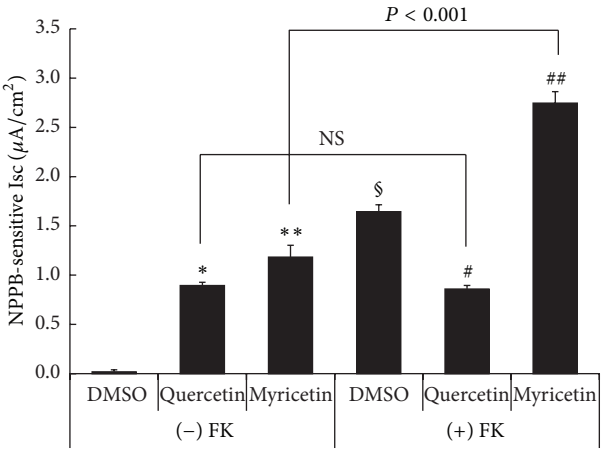

FIGURE 3: NPPB-sensitive Isc. The NPPB-sensitive Isc was measured as the difference of Isc just before and 30 min after addition of 100 μM NPPB to the apical solution. $n = 5$ for DMSO, $n = 6$ for quercetin, and $n = 7$ for myricetin without forskolin ((−) FK). $n = 6$ for DMSO, $n = 5$ for quercetin, and $n = 8$ for myricetin with forskolin ((+) FK). Under basal conditions ((−) FK), the values of quercetin-stimulated Isc (∗) and myricetin-stimulated NPPB-sensitive Isc (∗∗) were significantly larger than the NPPB-sensitive Isc with DMSO alone (solvent control; $P < 0.001$). The value of Isc with DMSO alone under forskolin-stimulated conditions (DMSO marked with § in (+) FK) was significantly larger than that with DMSO alone under basal conditions (DMSO in (−) FK; $P < 0.001$). Under forskolin-stimulated conditions ((+) FK), the value of quercetin-stimulated Isc (Quercetin in (+) FK marked with #) was significantly smaller than that with DMSO alone (DMSO in (+) FK; $P < 0.001$). On the one hand, under forskolin-stimulated conditions ((+) FK), the value of myricetin-stimulated Isc (##) was significantly larger than that with DMSO alone (DMSO in (+) FK; $P < 0.001$). The value of quercetin-stimulated Isc was identical irrespective of forskolin stimulation (Quercetin in (−) FK versus Quercetin in (+) FK; NS, no significant difference), while the value of myricetin-stimulated Isc under forskolin-stimulated conditions (Myricetin in (+) FK) was significantly larger than that under basal condition (Myricetin in (−) FK; $P < 0.001$).

as in Figure 3) and myricetin also increased the NPPB-sensitive Isc ($^{**}P < 0.001$ compared with DMSO as in Figure 3). Quercetin and myricetin had similar effects on the NPPB-sensitive Isc under basal conditions ((−) FK, Figure 3), although the quercetin-stimulated NPPB-sensitive Isc ($0.90 \pm 0.03\ \mu A/cm^2$) was slightly smaller than the myricetin-stimulated one ($1.18 \pm 0.12\ \mu A/cm^2$; $P < 0.05$). The NPPB-sensitive Isc in forskolin-treated cells (DMSO in (+) FK, Figure 3) was significantly larger than that in DMSO in the absence of forskolin ((−) FK; $^{\S}P < 0.001$ compared with DMSO in (−) FK, Figure 3), indicating that forskolin increased the NPPB-sensitive Isc. Under forskolin-stimulated conditions ((+) FK, Figure 3), the NPPB-sensitive Isc in quercetin-treated cells was significantly smaller than that in DMSO-treated cells ($^{\#}P < 0.001$ compared with DMSO in (+) FK, Figure 3), indicating that quercetin decreased the forskolin-stimulated NPPB-sensitive Isc. Contrasting the quercetin results, under forskolin-stimulated conditions ((+) FK, Figure 3), the NPPB-sensitive Isc in myricetin-treated cells was significantly larger than that in DMSO-treated cells ($^{\#\#}P < 0.001$ compared with DMSO in (+) FK, Figure 3), indicating that myricetin increased the forskolin-stimulated NPPB-sensitive Isc. Thus, myricetin induced an NPPB-sensitive Isc at the same level irrespective of prior forskolin stimulation of Isc ($1.10 \pm 0.12\ \mu A/cm^2$ in the presence of forskolin, $1.16 \pm 0.12\ \mu A/cm^2$ in the absence of forskolin; no significant difference). Therefore, myricetin had a synergistic effect on forskolin-activated Cl^- secretion. Under forskolin-stimulated conditions, quercetin did not stimulate, but rather diminished the NPPB-sensitive Isc (Quercetin versus DMSO in (+) FK, Figure 3; $^{\#}P < 0.001$) identical to that under basal conditions (Quercetin in (−) FK, Figure 3; no significant difference (NS) between Quercetin in (−) FK and (+) FK, Figure 3). In other words, forskolin had no effects on the NPPB-sensitive Isc in the presence of quercetin (compare Quercetin in (+) FK with Quercetin in (−) FK, Figure 4; NS); that is, quercetin abolished the action of forskolin on the NPPB-sensitive Isc.

3.4. NPPB-Sensitive Conductance under Basal and Forskolin-Stimulated Conditions. Figure 4 shows the NPPB-sensitive conductance obtained by application of NPPB (100 μM) to the apical solution as described in the method. Under basal conditions, quercetin increased the NPPB-sensitive conductance ($^{*}P < 0.001$ compared with DMSO in (−) FK, Figure 4) and myricetin also increased the NPPB-sensitive Gt ($^{**}P < 0.001$ compared with DMSO in (−) FK, Figure 4). However, the quercetin-stimulated NPPB-sensitive conductance ($37.00 \pm 2.69\ \mu S/cm^2$) was slightly smaller than that stimulated by myricetin ($53.00 \pm 4.73\ \mu S/cm^2$; $P < 0.05$, Figure 4). The NPPB-sensitive conductance in forskolin-treated cells (DMSO in (+) FK, Figure 4) was much larger than that in DMSO in the absence of forskolin ((−) FK; $^{\S}P < 0.005$ compared with DMSO in (−) FK, Figure 4), meaning that forskolin increased the NPPB-sensitive conductance. Under forskolin-stimulated conditions ((+) FK, Figure 4), the NPPB-sensitive conductance in quercetin-treated cells was slightly but significantly larger than that in DMSO-treated cells ($^{\#}P < 0.05$ compared with DMSO in (+) FK, Figure 4), indicating that quercetin increased the forskolin-stimulated NPPB-sensitive conductance. Under forskolin-stimulated conditions ((+) FK, Figure 4), the NPPB-sensitive conductance in myricetin-treated cells was much larger than that in DMSO-treated cells ($^{\#\#}P < 0.001$ compared with DMSO in (+) FK, Figure 4), indicating that myricetin increased the forskolin-stimulated NPPB-sensitive conductance. Thus, unlike the NPPB-sensitive Isc under the forskolin-stimulated conditions, both quercetin and myricetin increased the NPPB-sensitive conductance under the forskolin-stimulated conditions ($^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ compared with DMSO in (+) FK, Figure 4), although myricetin had much larger effects on the NPPB-sensitive conductance than quercetin ($275.83 \pm 15.72\ \mu S/cm^2$ in the presence of myricetin versus $130.50 \pm 10.01\ \mu S/cm^2$ in the presence of quercetin under forskolin-stimulated

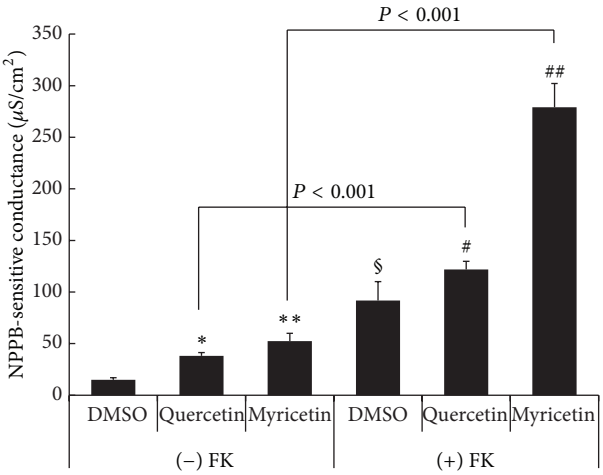

Figure 4: NPPB-sensitive conductance. The NPPB-sensitive conductance was measured as the difference of Isc just before and 30 min after addition of 100 μM NPPB to the apical solution. n = 4 for DMSO, n = 7 for quercetin, and n = 11 for myricetin without forskolin ((−) FK). n = 11 for DMSO, n = 7 for quercetin, and n = 10 for myricetin with forskolin ((+) FK). Under basal conditions ((−) FK), the values of quercetin-stimulated conductance (∗) and myricetin-stimulated NPPB-sensitive conductance (∗∗) were significantly larger than the NPPB-sensitive conductance with DMSO alone (solvent control; $P < 0.001$). The value of Isc with DMSO alone under forskolin-stimulated conditions (DMSO marked with § in (+) FK) was significantly larger than that with DMSO alone under basal conditions (DMSO in (−) FK; $P < 0.005$). Under forskolin-stimulated conditions ((+) FK), the value of quercetin-stimulated Isc (Quercetin in (+) FK marked with #) was slightly but significantly larger than that with DMSO alone (DMSO in (+) FK; $P < 0.05$). Further, under forskolin-stimulated conditions ((+) FK), the value of myricetin-stimulated Isc (##) was significantly larger than that with DMSO alone (DMSO in (+) FK; $P < 0.001$). The quercetin-stimulated NPPB-sensitive conductance under forskolin-stimulated conditions (Quercetin in (+) FK) was significantly larger than that under basal condition (Quercetin in (−) FK; $P < 0.001$). The myricetin-stimulated NPPB-sensitive conductance under forskolin-stimulated conditions (Myricetin in (+) FK) was significantly larger than that under basal condition (Myricetin in (−) FK; $P < 0.001$).

conditions; $P < 0.001$). Further, interestingly the NPPB-sensitive Isc in the presence of quercetin was not affected by application of forskolin (compare Quercetin in (−) FK with Quercetin in (+) FK as in Figure 3; NS), but the NPPB-sensitive conductance in the presence of quercetin was much larger under forskolin-stimulated condition than that under basal conditions (compare Quercetin in (−) FK with Quercetin in (+) FK as in Figure 4; $P < 0.001$) like myricetin (see Myricetin in (−) FK with Myricetin in (+) FK as in Figure 4; $P < 0.001$). In other words, forskolin increased the NPPB-sensitive conductance in the quercetin-treated cells (compare Quercetin in (+) FK with Quercetin in (−) FK as in Figure 4; $P < 0.001$); nevertheless, forskolin had no effects on the NPPB-sensitive Isc in the quercetin-treated cells (compare Quercetin in (+) FK with Quercetin in (−) FK as in Figure 3; NS).

4. Discussion

Epithelial Cl^- secretion plays an important role in prevention from bacterial/viral infection through washout of bacteria and viruses located on the surface of apical membrane using water covering the surface of apical membrane produced by Cl^--secretion-generated osmotic gradient across the epithelial cells and also controls body fluid content by regulating water contents [2, 4, 7, 25–30]. The present study indicates that quercetin is useful for moderate stimulation of epithelial Cl^- secretion irrespective of cAMP stimulation and that myricetin can be applied in cases requiring further stimulation of epithelial Cl^- secretion with insufficient cAMP stimulation. Various types of flavonoids have been reported to modulate epithelial Cl^- secretion [9, 31–35]. For example, genistein stimulates jejunal Cl^- secretion via estrogen receptor-mediated pathways [31, 32]. Chao and Hamilton [9] have reported that genistein stimulates jejunal Cl^- secretion via phosphodiesterase modulation. Further, Fischer and Illek [34] have indicated that trimethoxyflavone, aryl hydrocarbon receptor ligand, activates CFTR Cl^- channel stimulating Cl^- secretion in lung epithelial cells. Quercetin is also reported to have stimulatory action on Cl^- secretion in sinonasal epithelium [35]. Niisato et al. [7] have also reported that flavonoids, genistein, daidzein, and apigenin, stimulate epithelial Cl^- secretion.

Although molecular mechanisms of flavonoids' action on epithelial Cl^- secretion are still unclear, we have a consensus that flavonoids stimulate epithelial Cl^- secretion via activation of the CFTR Cl^- channel playing a role in a Cl^--releasing step across the apical membrane and/or Cl^- transporter playing a role in a Cl^- uptake step across the basolateral membrane [6, 7]. Continuous activation of Cl^- uptake transporter such as NKCC is required to continuously stimulate epithelial Cl^- secretion [6, 7]. This continuous activation of Cl^- uptake transporter such as NKCC is one of the most important targets from a therapeutic viewpoint for continuous stimulation of epithelial Cl^- secretion. The present study and our previous report [3] suggest that quercetin continuously increases activity of NKCC by stimulating translocation of NKCC activating factors to the basolateral membrane from intracellular store sites.

There is little mechanistic information on the effects of flavonoids on the function of NKCC during cAMP-dependent Cl^- secretion, although many researchers have reported the stimulatory action of flavonoids on epithelial Cl^- secretion [9, 31–37] and the inhibitory action of cAMP-activated Cl^- secretion [38]. We were surprised that quercetin reduced the forskolin-stimulated Isc, but myricetin tremendously increased the forskolin-stimulated Isc. As mentioned above, the epithelial Cl^- secretion (the NPPB-sensitive Isc) consists of two steps: (1) the Cl^--entry step across the basolateral membrane via Cl^- transporter participating in Cl^- uptake into the intracellular space such as Na^+-K^+-$2Cl^-$ cotransporter (NKCC) and (2) the Cl^--releasing step across the apical membrane via Cl^- channels such as cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel at the apical membrane [4]. Therefore, the stimulatory action of myricetin on the NPPB-sensitive Isc could be explained

by effects of myricetin on the NPPB-sensitive conductance; that is, we suggest that myricetin would increase the epithelial Cl^- secretion by activating the CFTR Cl^- channel at the apical membrane acting as the Cl^--releasing step. However, the inhibitory action of quercetin on the forskolin-stimulated Isc could not be explained by effects of quercetin on the apical NPPB-sensitive conductance, since quercetin increased the NPPB-sensitive conductance (in other words, quercetin activated the Cl^--releasing step via CFTR Cl^- channel; see Figure 4). Namely, if the inhibitory action of quercetin on the forskolin-stimulated Isc is explained by effects of quercetin on the NPPB-sensitive conductance (the Cl^--releasing step), quercetin should diminish the NPPB-sensitive conductance. Therefore, we should consider other possibilities regarding the inhibitory action of quercetin on the forskolin-stimulated Isc. There are, at least, two possible sites of action that quercetin might have on the cAMP-dependent Cl^- secretory process and that is either by down regulating the Na^+-K^+-$2Cl^-$ cotransporter and/or the Na^+, K^+-ATPase. Similar to our data, Schuier et al. [39] have reported that quercetin, morin, and luteolin, all administered at 100 μM, reduce forskolin-stimulated Isc of T84 colonic epithelial cells. These authors have offered no explanation for the action of these flavonoids. However, Collins et al. [33] have demonstrated that the flavone, naringenin, added prior to forskolin, reduces the forskolin-activated Isc of human and rat colons. They have surmised that since that action of naringenin is upstream of the activation of CFTR, naringenin inhibits NKCC. Alternatively, the action of quercetin on the forskolin-stimulated Isc might be by downregulating the Na^+, K^+-ATPase. Indeed, Mezesova et al. [40] have reported that treatment with quercetin ($20\,mg \cdot kg^{-1} \cdot day^{-1}$) in both normotensive and hypertensive rats produces impairment in the affinity of Na^+ binding site of the Na^+, K^+-ATPase like the inhibitory action on Ca^{2+}-ATPase [41, 42]. On the other hand, myricetin showed no inhibitory action but stimulatory action on Isc, suggesting that myricetin would not inhibit NKCC or produce impairment in the affinity of Na^+ binding site of the Na^+, K^+-ATPase unlike quercetin. Further work is necessary to resolve this complex role of quercetin in cAMP-dependent Cl^- secretion.

Although it has been reported that flavonoids including myricetin and quercetin have various action on cell function [3, 10, 31–33, 43–51], the present study clearly indicates that myricetin has stimulatory action on cAMP-activated Cl^- secretion unlike quercetin. From our knowledge, the present study is the first report showing a flavonoid with stimulatory action on cAMP-activated epithelial Cl^- secretion. Thus, using these compounds it could be possible to treat patients with disorders in water secretion across epithelial tissues by regulating epithelial Cl^- secretion to ideal levels depending on pathophysiological states of patient.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hongxin Sun and Naomi Niisato equally contributed to this work.

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