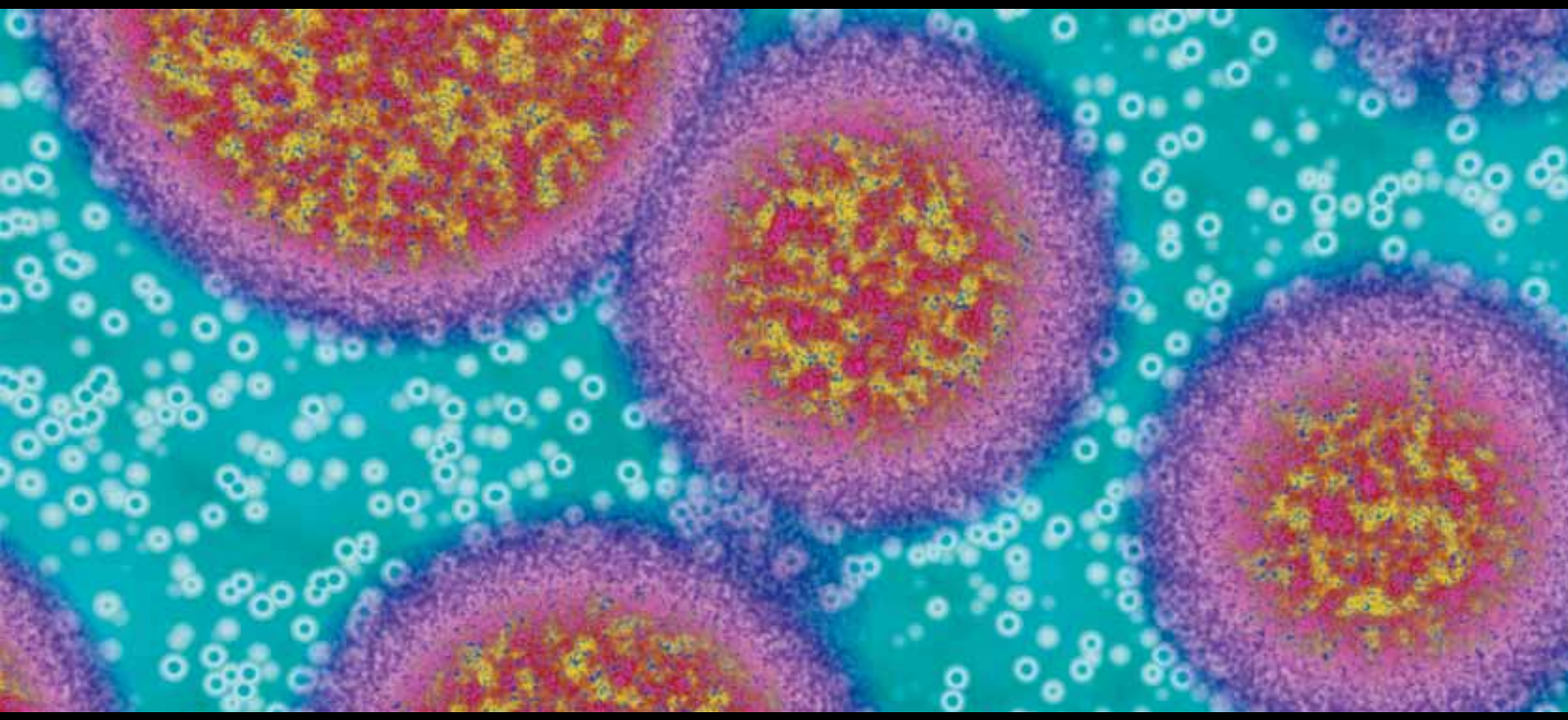
International Journal of Cell Biology

Organelle Cross-Talk in Apoptotic and Survival Pathways

Guest Editors: Lina Ghibelli, Alina Grzanka, Paolo Pinton, and Mariusz R. Wieckowski



Organelle Cross-Talk in Apoptotic and Survival Pathways

International Journal of Cell Biology

Organelle Cross-Talk in Apoptotic and Survival Pathways

Guest Editors: Lina Ghibelli, Alina Grzanka, Paolo Pinton, and Mariusz R. Wieckowski

Editorial Board

Contents

Organelle Cross-Talk in Apoptotic and Survival Pathways, Lina Ghibelli and Alina Grzanka
Volume 2012, Article ID 968586, 2 pages

GSK-3β: A Bifunctional Role in Cell Death Pathways, Keith M. Jacobs, Sandeep R. Bhave, Daniel J. Ferraro, Jerry J. Jaboin, Dennis E. Hallahan, and Dinesh Thotala
Volume 2012, Article ID 930710, 11 pages

ER-Mitochondria Crosstalk during Cerebral Ischemia: Molecular Chaperones and ER-Mitochondrial Calcium Transfer, Yi-Bing Ouyang and Rona G. Giffard
Volume 2012, Article ID 493934, 8 pages

Mitochondrial Dynamics: Functional Link with Apoptosis, Hidenori Otera and Katsuyoshi Mihara
Volume 2012, Article ID 821676, 10 pages

Novel Insights into the Interplay between Apoptosis and Autophagy, Hidemi Rikiishi
Volume 2012, Article ID 317645, 10 pages

Effects of Cisplatin in Neuroblastoma Rat Cells: Damage to Cellular Organelles, Giada Santin, Luigi Scietti, Paola Veneroni, Sergio Barni, Graziella Bernocchi, and Maria Grazia Bottone
Volume 2012, Article ID 424072, 6 pages

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 968586, 2 pages
doi:10.1155/2012/968586

Editorial

Organelle Cross-Talk in Apoptotic and Survival Pathways

Lina Ghibelli[1] and Alina Grzanka[2]

[1] *Dipartimento di Biologia, Universita' di Roma Tor Vergata, via della Ricerca Scientifica, 100133 Roma, Italy*
[2] *Department of Histology and Embryology, Nicolaus Copernicus University, Bydgoszcz, Poland*

Correspondence should be addressed to Lina Ghibelli, ghibelli@uniroma2.it

Received 13 December 2012; Accepted 13 December 2012

Apoptosis and survival pathways are intimately connected signal transduction cascades that determine the fate of cells suffering from insults such as DNA damage, oxidative stress, intracellular Ca^{2+} overload, mild excitotoxicity, accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), and many others, or induced to autoelimination by extracellular signals. The apoptotic pathways, respectively, intrinsic or extrinsic, are executed through molecular signals travelling inside the cells. If the initial studies considered the nucleus, and specifically DNA, as both primary sensor and executioner of apoptosis, active roles of other intracellular organelles have been pointed out in the last two decades, identifying plasma membrane as the initiator of the extrinsic pathway and mitochondria as the pivot of the intrinsic pathway. In apoptosing cells, the outer membrane of mitochondria is permeabilized by the proapoptotic members of the Bcl-2 family, promoting the release of proapoptotic factors such as the primary and secondary activators of caspases (cytochrome c and SMAC/diablo, resp.). Stress signals, typically inducing the intrinsic apoptotic pathway, must be perceived by the damaged cells and translated into signals that promote mitochondria permeabilization, after which caspases are activated. This implies that other organelles participate in apoptotic signaling upstream to mitochondria involvement, acting as sensors of damage or signal transducers. Recently, a key role of the endoplasmic reticulum (ER) is emerging, consisting of the ability of amplifying or dampening the apoptotic signal prior to mitochondria involvement, through the establishment of active Ca^{2+} fluxes that control the physical and functional interaction between mitochondria and ER. Also lysosomes participate to the intrinsic apoptotic signaling, and their dialogs with mitochondria have been described. Other important examples of organelles cross-talk in apoptosis include nucleus-mitochondria exchange of proteins such as p53, which is main controller of the cell response to DNA damage. To date, the role of organelles other than mitochondria in perceiving and signaling cell damage, and the interrelation of the organelles during apoptotic signaling, are still poorly explored matters.

In this special issue dedicated to Organelle Cross-talk in Apoptotic and Survival Pathways, original research papers and review articles are collected with the aim of giving an impulse to this promising field of research on apoptosis and stimulating discussion between interdisciplinary readers.

The paper written by Y.-B. Ouyang and R. G. Giffard describes ER-mitochondria cross-talk during cerebral ischemia. The authors precisely describe the chaperone machineries at both the ER and mitochondrion. Moreover, they contribute the new view that the chaperones organise not only the regulation of Ca^{2+} signaling between these two organelles and control bioenergetics, cell survival, but also cell death decision.

In the paper entitled "*Mitochondrial dynamics: functional link with apoptosis*", written by H. Otera and K. Mihara, the readers are introduced to "intrinsic" apoptotic pathway in the context of regulation and physiologic significance of mitochondrial fusion and fission. The authors of this review underline also that the ER-mitochondria contact is involved in the regulation of mitochondrial energy, lipid metabolism, calcium signaling, and even in mitochondrial fission.

An important issue arising is the cross-talk of apoptosis with autophagy, a stress response pathway controlled by key intracellular regulators such as m-TOR, induced by insults or

conditions ranging from nutrient depletion, protein malfolding, or cell ageing. The paper written by H. Rikiishi focusing on the recent advances regarding autophagy in cancer and the techniques currently available to manipulate autophagy. The author of this paper provides a complete overview of the literature on molecular mechanism of autophagy and its cross-talk with apoptosis. Additionally, the author indicates the technical problems in the study of autophagy and suggest that new methods need to be developed to ensure progress in this area of investigation. Moreover, they highlight that the multifaceted nature of autophagy and its diverse cross-talk with other biological processes must be carefully considered when the autophagic system is targeted for anticancer benefits.

The paper entitled "*Effects of cisplatin in neuroblastoma rat cells: damage to cellular organelles*" written by G. Santin et al. provides the practical analysis of organelle cross-talk during cell death. The authors indicate that B50 neuroblastoma cells treated with cisplatin undergo apoptosis through organelles injury, and not only as a consequent of DNA damage, but also that cisplatin does not induce any autophagic process as alternative response to the treatment.

In conclusion, the studies collected in this issue effectively contribute to the general problem of organelles as target of cell damage versus transducer of the apoptotic signal.

Lina Ghibelli
Alina Grzanka

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 930710, 11 pages
doi:10.1155/2012/930710

Review Article

GSK-3β: A Bifunctional Role in Cell Death Pathways

Keith M. Jacobs,[1] Sandeep R. Bhave,[2] Daniel J. Ferraro,[1] Jerry J. Jaboin,[1,3] Dennis E. Hallahan,[1,3,4] and Dinesh Thotala[1,3]

[1] *Department of Radiation Oncology, Washington University in St. Louis, St. Louis, MO 63108, USA*
[2] *School of Medcine, Washington University in St. Louis, St. Louis, MO 63108, USA*
[3] *Siteman Cancer Center, St. Louis, MO 63110, USA*
[4] *Mallinckrodt Institute of Radiology, St. Louis, MO 63110, USA*

Correspondence should be addressed to Dinesh Thotala, dthotala@radonc.wustl.edu

Received 6 January 2012; Revised 9 March 2012; Accepted 12 March 2012

Academic Editor: Paolo Pinton

Although glycogen synthase kinase-3 beta (GSK-3β) was originally named for its ability to phosphorylate glycogen synthase and regulate glucose metabolism, this multifunctional kinase is presently known to be a key regulator of a wide range of cellular functions. GSK-3β is involved in modulating a variety of functions including cell signaling, growth metabolism, and various transcription factors that determine the survival or death of the organism. Secondary to the role of GSK-3β in various diseases including Alzheimer's disease, inflammation, diabetes, and cancer, small molecule inhibitors of GSK-3β are gaining significant attention. This paper is primarily focused on addressing the bifunctional or conflicting roles of GSK-3β in both the promotion of cell survival and of apoptosis. GSK-3β has emerged as an important molecular target for drug development.

1. Introduction

Glycogen synthase kinase-3 is a ubiquitously expressed protein kinase that exists in two isoforms, α and β. Originally identified based on its role in glycogen biosynthesis based on its inactivating phosphorylation of glycogen synthase, it has since been found to regulate a myriad of functions through Wnt and other signaling pathways [1]. The two isoforms are strongly conserved within their kinase domain but differ greatly at the C-terminus, while the α isoform additionally contains a glycine-rich N-terminus extension [2]. Our paper will focus on the β isoform due to its more established role in cell survival and viability. Glycogen synthase kinase-3 beta (GSK-3β) is involved in the regulation of a wide range of cellular functions including differentiation, growth, proliferation motility, cell cycle progression, embryonic development, apoptosis, and insulin response [1–8]. It has emerged as an important regulator of neuronal, endothelial, hepatocyte, fibroblast, and astrocyte cell death in response to various stimuli [6, 7, 9].

GSK-3β is comprised of 12 exons in humans and 11 exons in mice with the ATG start codon located within exon 1 and the TAG stop codon found in the terminal exon. The gene product is a 46 kDa protein consisting of 433 amino acids in the human and 420 amino acids in the mouse. Figure 1 shows the overall structure of GSK-3β. It is similar to other Ser/Thr kinases [10, 11]. The N-terminal domain is comprised of the first 135 residues and forms a 7-strand β-barrel motif. A small linker region connects the N-terminal domain to the central α-helical domain formed by residues 139 through 342. The ATP-binding site lies at the interface of the N-terminal and α-helical domains. Residues 343 through 433 form the C-terminal domain, which is outside of the classical Ser/Thr kinase core fold. These residues form a helix/loop domain that interacts with the core α-helical domain. The N-terminal amino acids 78 through 92 are necessary for association with p53 (Figure 1). The activity of GSK-3β can be reduced by phosphorylation at Ser-9. Several kinases are able to mediate this modification, including p70S6 kinase, p90RSK, PKC, and Akt [12, 13]. In opposition to the inhibitory phosphorylation of GSK-3β at Ser-9, phosphorylation of GSK-3β at Tyr-216 by ZAK1 or Fyn increases its enzyme activity [14] (Figure 2).

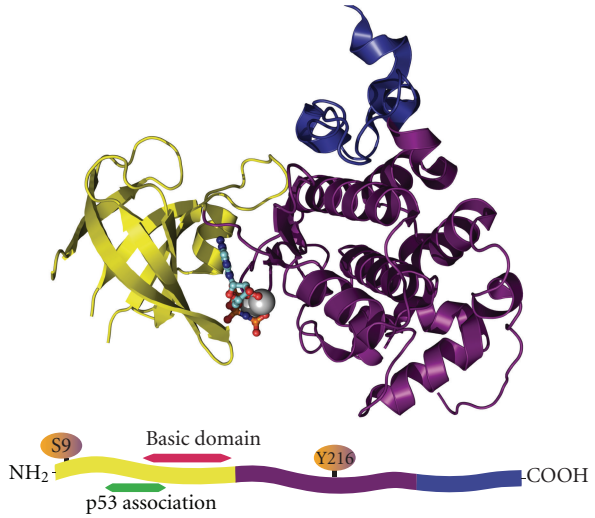


Figure 1: Glycogen synthase kinase-3β (GSK-3β) structure. GSK-3β is a 433 residue protein consisting of 3 distinct structural domains. The N-terminal domain (yellow) consists of the first 134 residues and forms a 7-strand β-barrel. A short linker from the N-terminal domain, residues 135–151 connect the N-terminal domain to the α-helical domain (magenta). The α-helical domain is composed of residues 152–342. Sandwiched between the N-terminal and α-helical domain is the ATP-binding site. The C-terminal domain consists of residues 343–433 (blue). A strand diagram of GSK-3β. Phosphorylation of Ser-9 inactivates the enzyme, while phosphorylation of Tyr-216 activates. The p53 association region and basic domain region are both located in the N-terminal domain. Image was made using PyMol Molecular Graphics Software version 1.3 with the PDB structure 1UV5.

Dysregulation of GSK-3β expression leads to many pathological conditions, including diabetes (or insulin resistance), neuronal dysfunction, Alzheimer's disease [15–18], schizophrenia [19], Dopamine-associated behaviors [20], bipolar disorders [21], Parkinson's disease [22], and cancer. Of special interest is the involvement of GSK-3β in cancer with data supporting a role as a tumor suppressor and tumor promoter, a discrepancy that at least in part depends on both cell type and signaling environment. For example, GSK-3β has been shown to inhibit androgen receptor-stimulated cell growth in prostate cancer, thus acting as a tumor suppressor [23]. In contrast, GSK-3β is highly expressed in colorectal cancer [24, 25] and has been shown to participate in nuclear factor-κB (NF-κB) mediated cell survival in pancreatic cancer [26], thus behaving as a tumor promoter. Moreover, the kinase has dual functions in the regulation of cell survival, where it can either activate or inhibit apoptosis [3, 27], further complicating its involvement in cancer. This paper will focus on how GSK-3β can both activate as well as protect from apoptosis with a focus on oncology.

Regulation of β-catenin levels is a critical step in Wnt signaling. β-Catenin is phosphorylated by GSK-3β and then degraded through the ubiquitin-proteasome system [28–30]. Inhibition of GSK-3β activity leads to stabilization and accumulation of β-catenin in the cytosol, which is shuttled into the nucleus and regulates gene expression (Figure 2). GSK-3β is also involved in cell cycle regulation through the phosphorylation of cyclin D1, which results in the rapid proteolytic turnover of cyclin D1 protein [1, 31] (Figure 2). Direct overexpression of wild-type GSK-3β is known to induce apoptosis in various cell types in culture, and specific inhibitors of GSK-3β are able to stop this apoptotic signaling [6, 7, 9, 32]. The detailed molecular mechanism of GSK-3β's proapoptotic effect is as yet unknown, but it involves regulation of metabolic and signaling proteins, transcription factors, and gene expression [4, 33].

GSK-3β is required for proper development [4] and is ubiquitously expressed in the animal kingdom. GSK-3β protein was originally isolated from skeletal muscle, but though widely expressed, the protein is most abundant in brain tissue, especially neurons. The high level of expression in brain tissue is likely due to its vital role in neuronal signaling. In neuronal cells, GSK-3β is required for dendrite extension and synapse formation in newborns.

2. Regulation of Apoptosis by GSK-3β

GSK-3β has been shown to induce apoptosis in a wide variety of conditions including DNA damage [34], hypoxia [35], endoplasmic reticulum stress [36], and Huntington's disease-associated polyglutamine toxicity [37]. In cell culture studies, apoptosis was either attenuated or fully abrogated by inhibiting GSK-3β in primary neurons [38], HT-22 cells [39], PC12 cells [40], and human SH-SY5Y neuroblastoma cells [36, 41].

GSK-3β promotes apoptosis by inhibiting prosurvival transcription factors, such as CREB and heat shock factor-1 [42], and facilitating proapoptotic transcription factors such as p53 [34]. A list of some alternative conditions where GSK-3β facilitates apoptosis is given in Table 1. A large number of proteins have been shown to interact with the tumor suppressor transcription factor p53 to regulate its actions [43, 44], which has been implicated in the proapoptotic actions of GSK-3β in several studies. Following DNA damage, the normally short-lived p53 protein is stabilized and modified by a complex array of posttranslational modifications, such as phosphorylation, acetylation, methylation, ubiquitination, sumoylation, glycosylation, and neddylation. One of these regulatory proteins is GSK-3β, which forms a complex with nuclear p53 to promote p53-induced apoptosis [34, 45, 46]. GSK-3β binds directly to p53, and the C-terminal region of p53 is necessary for this interaction [45]. GSK-3β was shown to directly phosphorylate p53 at Ser-33 [47] and to mediate p53 phosphorylation at Ser-315 and Ser-376 [48, 49]. GSK-3β also promotes p53-mediated transcription of specific genes and regulates the intracellular localization of p53 [45, 46, 49]. In addition to GSK-3β regulating p53, GSK-3β is also regulated by p53. The activity of GSK-3β is increased by a phosphorylation-independent mechanism of direct binding of p53 to GSK-3β [34]. Nuclear localization of GSK-3β may also be regulated by binding of activated p53 [50].

In addition to direct interaction, GSK-3β can regulate p53 levels through the phosphorylation of the p53-specific E3 ubiquitin ligase MDM2 [69]. Regulation of p53 by MDM2

Table 1: Conditions where GSK-3β facilitates apoptosis.

System or stimulus	Mechanism
C(2) Ceramide-associated damage	Inhibits the phosphorylation of AKT and ERK pathways and through the dephosphorylation of GSK-3β [51]. GSK-3β inhibitors have been shown to inhibit apoptosis through inhibiting dephosphorylation of AKT and GSK-3β [52].
LPS-mediated endotoxic shock	While specific apoptotic studies have not been performed, LPS has been shown to stabilize apoptotic signal-regulating kinase-1 (ASK-1), a serine-threonine kinase associated with stress-induced apoptosis [53].
Immune system	Regulates in apoptosis of activated T-Cells [54].
HIV-mediated neuronal damage	Inhibits NF-κB [55–57].
Neurodegenerative disease-related toxicity and oxidative stress	Neuronal or oligodendrocyte injury or toxicity (including prion peptide) is associated with increased activity of GSK-3β[51, 58–64]. Negative regulators of GSK-3β are associated with increased survival factors [51, 58–64] and neuroprotection [9, 38].
ER stress	ER stress can lead to dephosphorylation of pGSK-3β(S9), leading to stress-induced apoptosis through activated caspase-3 [12–14, 26, 28].
Hypoxia/ischemia	Activates mitochondrial death pathway [35, 65–68].

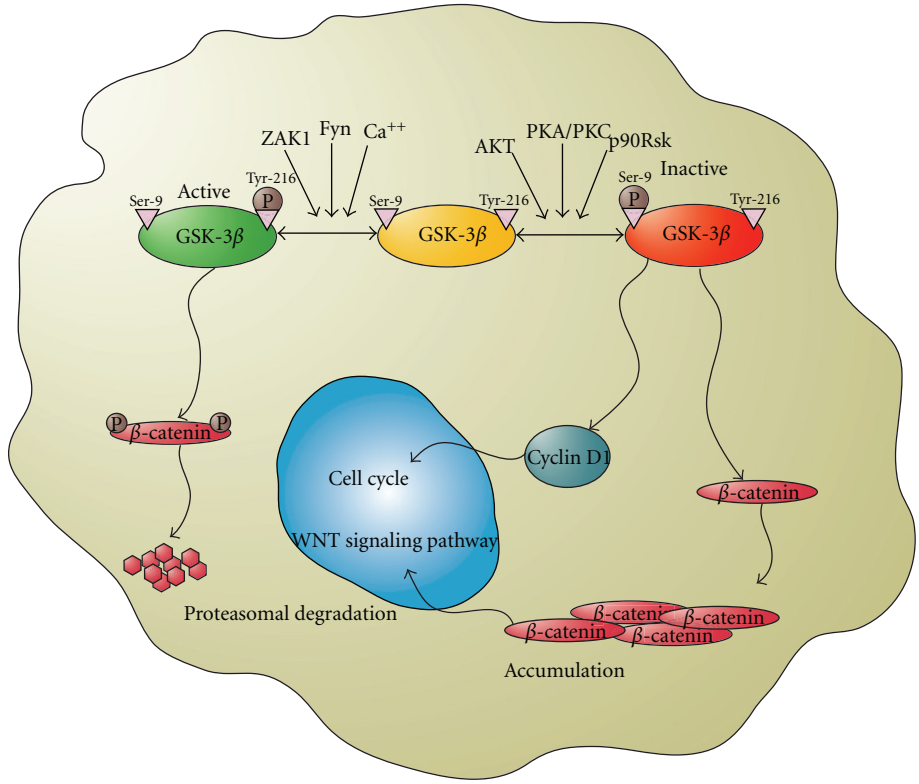


Figure 2: Regulation of GSK-3β. GSK-3β is a multifunctional kinase that has a role in various signaling pathways that regulate cell fate. ZAK1 or Fyn can phosphorylate Tyr-216 which increases the GSK-3β activity. GSK-3β can phosphorylate downstream targets like β-catenin and degrade it through the ubiquitin-proteasome system. Akt and PKC on the other hand can attenuate GSK-3β enzymatic activity by phosphorylating Ser-9. Inhibition of GSK-3β activity therefore leads to stabilization and accumulation of β-catenin in the cytosol, which is shuttled into the nucleus where it functions to regulate gene expression. GSK-3β is also involved in cell cycle regulation through the phosphorylation of cyclin D1, which results in the rapid proteolytic turnover of cyclin D1 protein.

is multifaceted. In the classical model, N-terminal phosphorylation of p53 at Ser-15 (mouse Ser-18) and Ser-20 (mouse Ser-23) inhibits the interaction with MDM2 and thereby prevents MDM2-mediated ubiquitination and the resulting proteasomal degradation of p53 [44] (Figure 3). Stabilized p53 then enters a complex regulatory network to induce DNA binding and transcriptional activation of p53 target genes, in part through the recruitment of coactivators and corepressors. This determines the specific cellular response, which can include survival, growth arrest, DNA repair, or apoptosis [44]. Inhibition of GSK-3β in hippocampal neurons protected it from radiation-induced apoptosis [9, 70].

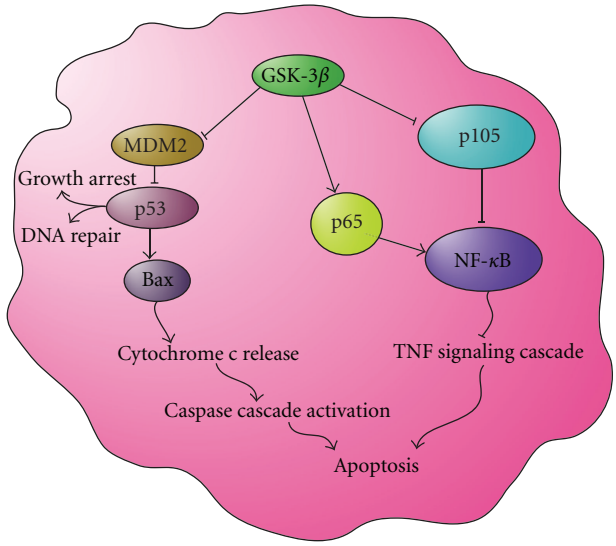


Figure 3: GSK-3β's role in apoptosis signaling. The above schematic shows the role of activated GSK-3β and its role in regulating apoptosis. Active GSK-3β inhibits MDM2 regulation of p53, leading to DNA repair and growth arrest, and in some cases the activation of the caspase cascade through Bax to promote apoptosis. Active GSK-3β also positively regulates NFκB by activating IKK, IκB, and p65, leading to the inhibition of TNF-mediated apoptosis. These actions inhibit the initiation of apoptosis through the TNF signaling cascade.

Similar protection from GSK-3β inhibition has been seen in primary neurons [38]. The mechanism of protection from radiation-induced apoptosis in these cells involves subcellular localization and interaction of GSK-3β, p53, and MDM2. GSK-3β inhibition blocks radiation-induced accumulation of p53 by upregulating levels of MDM2 that subsequently result in decreased radiation-dependent apoptosis [71]. In addition to abrogation of radiation-induced p53 phosphorylation, accumulation, and nuclear translocation, GSK-3β inhibition results in the accumulation of MDM2 and sequestration of GSK-3β, p53, and MDM2 in the cytoplasm where p53 cannot act on its target genes [71]. The role of attenuated p53 function in the prosurvival effects of the GSK-3β inhibitors, has also been previously described [34, 46, 70, 72, 73].

In regulation of the apoptotic response, mammalian cells employ multiple prosurvival proteins from the Bcl-2 family (Bcl-2, Bcl-X_L, Bcl-w, Mcl1, and A1) that antagonize the proapoptotic function of Bax and Bak [34, 74]. Bax and Bak localize to the mitochondrial outer membrane and trigger death signals leading to cytochrome *c* release to the cytosol [74, 75]. Apoptosis requires a group of effector caspases to dismantle the cells. Cytochrome *c* activates caspase-9, which subsequently activates caspase-3 [76]. The activation of caspase-3 is an essential step leading to cleavage of the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP), resulting in genomic DNA fragmentation. Bax protein levels and cleavage (activation) of caspase-3 were increased due to radiation and were abrogated by GSK-3β inhibitors [77] (Figure 3). GSK-3β was also found to be associated with mitochondrial apoptotic signaling. Inhibition of GSK-3β prevented mitochondrial release of cytochrome *c*, which is known to activate caspase-3 and initiate apoptosis [34]. Phosphatidylinositol 3-kinase (PI3-kinase) and its downstream effector, the protein-serine/threonine kinase Akt, a negative regulator of GSK-3β, play an important role in preventing apoptosis by blocking activation of the caspase cascade [78].

3. Survival-Promoting Effects of GSK-3β

GSK-3β is involved in multiple signaling pathways and has many phosphorylation targets. It should therefore not be surprising that GSK-3β has both pro- and antiapoptotic roles. The overall effect of GSK-3β on cell survival varies depending on cell type, transformation status, and the specific signaling pathway being activated. For example, despite evidence for a substantial proapoptotic role of GSK-3β, it is the inhibition of GSK-3β that promotes apoptosis and decreases viability in neuroblastoma cells [79]. Several examples of pro-survival roles of GSK-3β not mentioned here are summarized in Table 2 [80–84].

Additionally, while GSK-3β has been typically identified as an activator of p53-mediated apoptosis [34], conflicting reports suggest an inhibitory effect of GSK-3β signaling on p53 activation. Inhibition of GSK-3β blocks activation of MDM2 by reducing Ser-254 phosphorylation. This prevents p53 degradation and promotes apoptosis despite the induction of p53 ubiquitination. Similarly, ionizing radiation was found to induce an inactivating phosphorylation at Ser-9 of GSK-3β, corresponding to hypophosphorylation of MDM2 and accumulation of p53 [69]. In contrast to its proapoptotic effects, this data suggests that GSK-3β inhibits apoptosis under basal conditions through MDM2-dependent degradation of p53. Overexpression of β-catenin, a downstream signaling factor negatively regulated by GSK-3β, was found to increase basal p53 levels by blocking both MDM2-dependent and independent degradation in neuroblastoma cells [85], providing additional supporting evidence for an inhibitory effect of GSK-3β on p53-mediated apoptosis. Interestingly, a negative feedback loop exists between β-catenin and p53; while β-catenin upregulates p53 levels, the activation of p53 results in degradation of β-catenin through GSK-3β [86]. While the majority of publications suggest a proapoptotic role for GSK-3β in p53 signaling, it is clear that more comprehensive studies are needed in order to fully understand the p53-GSK-3β relationship.

GSK-3β is specifically required for hepatocyte survival in normal embryos, and GSK-3β knockout mice are embryonically lethal between E13.15–14.5. Hepatocyte apoptosis in GSK-3β knockout mice and mouse embryonic fibroblasts results only after exposure to tumor necrosis factor (TNF), while inhibition of GSK-3β in wild-type cells with lithium increases TNF sensitivity. GSK-3β loss in these cells has a detrimental effect on the action of NF-κB, which protects against TNF-induced apoptosis [88]. Other studies have shown that GSK-3β directly promotes NF-κB stability and activation through both the degradation of p105 and

Table 2: Other pro survival roles of GSK-3β.

System	Mechanism
ER stress	Reduces expression of the proapoptotic transcription factor CHOP/GADD153 [87].
Glioblastoma differentiation	Promotes self-renewal through interaction with Bmi1 [81].
Death receptor complex	Inhibits apoptotic signaling and caspase activation [83].
Chemotherapy	Targeted by death-inducing drugs suggesting an inhibitory role [84].
Oncogenic activation	Inhibits apoptotic activation by c-myc [82].
Glucose metabolism	Prevents apoptosis through mitochondrial stabilization [82].

activation of the p65 subunit, suggesting a likely mechanism for lithium-induced TNF hypersensitivity [89, 90] (Figure 3). The role of GSK-3β on NF-κB activation may also be mediated indirectly through inhibition of β-catenin, as cancer cells with high β-catenin levels are especially sensitive to TNF-induced death [91].

Despite the abundance of evidence implicating GSK-3β in protection from TNF-mediated apoptosis, a few conflicting reports further complicate our understanding of the pathway. A more recent study claims that GSK-3 inhibition does indeed reduce NF-κB activity but does not result in TNF-mediated apoptosis, potentially due to the activation of pro-survival genes through Wnt signaling [92]. Similarly, TNF sensitization by lithium in multiple sarcoma cell lines was found to be independent of both GSK-3β and NF-κB [93] while GSK-3β inhibition in prostate cancer and HEK cells actually increased NF-κB activity despite promoting TNF-induced apoptosis [94].

The specifics of apoptosis regulation by GSK-3β remain both ambiguous and complex, requiring further research in order to determine the mechanisms of action responsible for differential control of cell survival. In addition to variations in cell signaling and proliferation status, the effect of GSK-3β on apoptosis may depend on cellular localization. Only cytosolic GSK-3β was found to inhibit TNF-mediated apoptosis [80] while apoptosis enhances nuclear localization [95], suggesting a potential localization-based mechanism for differential apoptotic regulation. Insufficient data is available to explain the contradictory effects proposed for GSK-3β on p53-mediated apoptosis, and a more detailed study is required in order to determine the reasons for these observed differences, but differential localization of p53, MDM2, and GSK-3β may help define the regulatory role of GSK-3β in various systems.

4. Positive Regulators of GSK-3β

Several molecules are known to potentiate the downstream effects of GSK-3β (Table 3). Positive regulators of GSK-3β are often utilized for enhancing the proapoptotic effects of GSK-3β in the context of chemotherapy for cancer treatment (reviewed in [96]). These regulators typically operate through an indirect mechanism, actually serving as inhibitors for upstream negative regulators. For example, GSK-3β activity is increased upon inhibition of PI3-Kinase with wortmannin or LY294002 [97–99]. Many GSK-3β regulators act to inhibit Akt by blocking its activation or kinase activity. The kinase inhibitor staurosporine and the COX-2 inhibitor Celecoxib block the activating phosphorylation of Akt by PDK [100–104]. Additionally, curcumin dephosphorylates Akt to prevent its downstream inactivation of GSK-3β [102], as does the histone deacetylase inhibitor Trichostatin A, in a PP1-dependent manner [105]. Akt/protein kinase B signaling inhibitor-2 (API-2) appears to suppress both Akt activation and kinase activity independent of any upstream inhibitor effects [106].

Alternative GSK-3β regulators have less defined and more indirect mechanisms. The mTOR inhibitor rapamycin has been shown to activate GSK-3β with some studies suggesting a potential influence of the mTOR pathway on GSK-3β regulation through phosphorylation by s6 kinase [107, 108]. Other molecules target the ability of GSK-3β to degrade cyclin D1. Vitamin A derived retinoids and multiple differentiation-inducing factors (DIFs) enhance GSK-3β activation and kinase activity [109–112] as a means for cyclin D inhibition to promote cell cycle arrest and differentiation.

5. Inhibitors of GSK-3β

While a potential therapeutic role of GSK-3β inhibitors has been suggested for some time, they have gained significant interest as a clinical tool over the past decade. GSK-3β inhibitors are currently being utilized for the treatment of various diseases including Alzheimer's disease [113, 114] and other neurodegenerative diseases [18], diabetes, inflammatory disorders [115], radiation damage, and cancer [116]. Various pharmaceutical companies have these inhibitors in clinical trials [116]. A classical example of a nonspecific GSK-3β inhibitor is lithium [21], which has been shown to inhibit GSK-3β with an IC_{50} of approximately 2 mM in an uncompetitive manner with respect to peptide substrate. Lithium was found to inhibit GSK-3β in a competitive manner by binding directly to magnesium-binding sites of the enzyme [117], thus providing evidence for a molecular mechanism for enzyme inactivation by lithium ions. Four distinct regions of GSK-3β have been targeted for inhibition: the Mg^{2+} ATP-binding active site, a separate Mg^{2+}-binding site, the substrate-binding groove, and the scaffold-binding region [33, 118]. Several inhibitors compete with Mg^{2+} and/or ATP to occupy its binding site. However, the specificity of these inhibitors towards GSK-3β relative to other kinases varies significantly (Table 4). Structural studies have further elucidated molecular mechanisms for substrate

Table 3: List of known positive regulators of GSK-3β.

Activator	Activation potency	Mode of activation	Notes
Celecoxib	IC_{50} = 3.5 μM	Inhibits PDK phosphorylation of Akt	COX-2 inhibitor [100].
Staurosporine	IC_{50} = 0.22 μM	Inhibits PDK phosphorylation of Akt	General kinase inhibitor (including PKA/PKC) [101, 103, 104].
Trichostatin A	Unknown	Induces Akt dephosphorylation	HDAC inhibitor acts through PP1 [105].
Curcumin	Unknown	Akt dephosphorylation	Direct target not known [102].
Akt/protein kinase B signaling inhibitor-2 (API-2)	Unknown	Suppresses Akt kinase activity and activation	Does not affect upstream Akt activators [106].
Wortmannin	IC_{50} = 5 nM	Inhibits PI3-Kinase	Indirect effect on GSK-3β [97, 98].
LY294002	IC_{50} = 1.4 μM	Inhibits PI3-Kinase	Likely affects ATP binding to kinase [98, 99].
Rapamycin	Unknown	Potentially inhibits S6K1	mTOR pathway can also inhibit GSK3 [107, 108].
Differentiation-inducing factors (DIFs)	Unknown	Enhances GSK-3β kinase activity and promotes nuclear localization	Reduces inhibitory phosphorylation and enhances activating phosphorylation [111, 112].
Retinoids	Unknown	Reduces inhibitory phosphorylation of GSK-3β	Promotes GSK-3β-dependent cyclin D1 degradation [80, 109].

Table 4: Selected list of known GSK-3β inhibitors.

Inhibitor	Inhibition potency	Mode of inhibition	Notes
Beryllium	IC_{50} = 6 mM	Mg competitor	Also inhibits cdc2
Lithium	K_i =2 mM	Mg competitor	
Anilino maleimides (SB216763, SB415286)	K_i = 10–30 nM	ATP competitor	Does not inhibit a range of other kinases
Arylpyrazolopyridazines (e.g., 6-aryl pyrazole [3,4-b] pyridine 4)	IC_{50} = 0.8–150 nM	ATP competitor	Also inhibits CDK2
Bisindole maleimides (e.g., Ro 31-8220, GF 109203x)	IC_{50} = 5–170 nM	ATP competitor	Also inhibits PKC
Indirubins (6-bromoindirubin-3′-oxime, aka BIO)	IC_{50} = 5–50 nM	ATP competitor	Also inhibits CDKs
Paullones (alsterpaullone)	IC_{50} = 4–80 nM	ATP competitor	Also inhibits CDKs
Pseudosubstrate peptide	K_i = 0.7 mM	Substrate competitor	Specific

selection and GSK3-β inhibition [119–125]. Beryllium was shown to compete with both ATP and Mg^{2+}, while lithium competed only with Mg^{2+} [126].

The small molecule inhibitors of GSK-3 SB-216763 and SB-415286 are structurally distinct maleimides that inhibit GSK-3α/β *in vitro*, with K_is of 9 nM and 31 nM, respectively, in an ATP competitive manner [127]. Hymenialdisine [128] and paullones [129] also inhibit GSK-3β in an ATP competitive manner. Indirubins inhibit GSK-3β in an ATP competitive manner with a IC_{50} of 50–100 nM [130–132]. Small molecule inhibitors like TZDZ8 that are thiadiazolidinones inhibit GSK-3β with a IC_{50} of 2 μM in a noncompetitive manner [133, 134]. The other type of GSK-3β inhibitors is represented by cell-permeable, phosphorylated substrate-competitive peptides which interact with the phospho-recognition motif comprising R96, R180, and K205 to prevent substrate access to the active site. There are also GSK-3β-inhibiting peptides that contain GSK-3β interacting domains, block the interaction between Axin and GSK-3, and prevent β-catenin phosphorylation [135]. In the recent decade small molecule inhibitors of GSK-3β are emerging as a promising drug for treatments against neurodegenerative diseases, radiation damage, Alzheimer's disease, diabetes, and cancer [116].

6. Exploiting the GSK-3β Conundrum

GSK-3β signaling is a complex process influenced not only by cellular type and transformation status, but by environmental and cellular conditions. Survival signals have been mainly determined by studies involving GSK-3β inhibition, through gene silencing or pharmacologic inhibition. The resulting inhibition of apoptosis is complex, and requires further elucidation. However several studies suggest that the effects may at least in part be mediated by the effect of GSK-3β on NF-κB levels. In addition, it is clear that subcellular localization is important, as only cytosolic GSK-3β seems to be able to mediate the survival signals.

Notably, the role in promotion of apoptosis by GSK-3β has been more clearly delineated. It performs this task by both facilitating proapoptotic signals while inhibiting anti-apoptotic molecules. This signal interplay occurs mostly at the level of the mitochondria, and combined with the association with primarily nuclear GSK-3β, suggests a downstream role of GSK-3β in modulation.

So how do we exploit these paradoxical roles of GSK-3β? In healthy cells, the shift to pro-survival modes is important for cell survival under conditions of cellular stress. In these cases, the upstream signals seem to override

the mitochondrial-based apoptotic machinery to allow the cells to escape potentially lethal damage. There have been attempts to exploit these pro-survival roles in neurodegenerative diseases, which are typified by high apoptosis rates. Reduction of disease-associated apoptosis by GSK-3β modulating agents can restore balance to off-kilter apoptotic machinery, resulting in decreased cellular turnover and the resultant protection of the at-risk neuronal population. In addition to diabetes, and neurodegenerative disorders, we believe that GSK-3β inhibition may play a promising role in patients receiving irradiation.

While radiation dose-escalation has been important for the treatment of multiple cranial tumors (e.g., brain metastases, primary gliomas) and benign disorders (e.g., vestibular schwannoma, meningioma), the treatment is limited by the effects of irradiation on healthy surrounding neurons. It has been demonstrated that GSK-3β inhibition can protect hippocampal neurons (in primary culture and murine pups) from irradiation-induced damage [9, 70]. Thotala et al. demonstrated improved survival of intestinal crypt cells and increased latency to murine GI-related death from irradiation [77]. This report suggested that GSK-3β inhibitors could reduce deleterious consequences of intestinal irradiation and possibly improve patient quality of life measures. It would be worthwhile to explore their utility in syngenic murine models of neural cancer, murine tumor xenografts, as well as human clinical trials of patients in the setting of re-irradiation (e.g., recurrent glioma). Reports of radiation protection have also been demonstrated with small molecular inhibitors of GSK-3β in the gastrointestinal system.

In cancer, however, the apoptotic machinery is often defective allowing cells to undergo unregulated proliferation. In this case, negative regulation of GSK-3β can serve to tip the balance in favor of apoptosis. Dickey et al. demonstrated the ability of GSK-3β inhibition to effectively enhance cell death of neuroblastoma cells *in vitro* and in a murine xenograft model [79]. Similar findings have been demonstrated in glioma [81, 82]. The interplay between GSK-3β regulation and other cell death stimuli is being carefully studied across a wide variety of cancer types, and there is promising data suggesting a strong role for this form of therapy in the near future. The bifunctional role of GSK-3β as a facilitator of apoptosis and a mediator of pro-survival signals has important implications in both the generation of novel therapies and the understanding of complex disease states.

The use of both positive and negative regulators of GSK-3β offers exciting treatment possibilities for a multitude of diseases. The complexity of the GSK-3β network requires careful examination, however, when considering modulating its function in a clinical setting. More studies are required to clearly understand the effects of regulating GSK-3β on the multiple signaling pathways involved in growth, development, and metabolism. The effect of GSK-3β on cell survival and apoptosis appears to be context dependent, and the required mode of action will likely depend on the specific pathway, cell type, and disease being targeted. While the vast network of GSK-3β offers a treatment option for multiple diseases, it also requires careful consideration of all the factors involved in order to prepare against potential side effects.

References

[1] S. Frame and P. Cohen, "GSK3 takes centre stage more than 20 years after its discovery," *Biochemical Journal*, vol. 359, no. 1, pp. 1–16, 2001.

[2] B. W. Doble and J. R. Woodgett, "GSK-3: tricks of the trade for a multi-tasking kinase," *Journal of Cell Science*, vol. 116, no. 7, pp. 1175–1186, 2003.

[3] R. S. Jope and G. V. W. Johnson, "The glamour and gloom of glycogen synthase kinase-3," *Trends in Biochemical Sciences*, vol. 29, no. 2, pp. 95–102, 2004.

[4] C. A. Grimes and R. S. Jope, "The multifaceted roles of glycogen synthase kinase 3β in cellular signaling," *Progress in Neurobiology*, vol. 65, no. 4, pp. 391–426, 2001.

[5] J. Luo, "Glycogen synthase kinase 3β (GSK3β) in tumorigenesis and cancer chemotherapy," *Cancer Letters*, vol. 273, no. 2, pp. 194–200, 2009.

[6] M. Pap and G. M. Cooper, "Role of translation initiation factor 2B in control of cell survival by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3β signaling pathway," *Molecular and Cellular Biology*, vol. 22, no. 2, pp. 578–586, 2002.

[7] J. F. Sanchez, L. F. Sniderhan, A. L. Williamson, S. Fan, S. Chakraborty-Sett, and S. B. Maggirwar, "Glycogen synthase kinase 3β-mediated apoptosis of primary cortical astrocytes involves inhibition of nuclear factor κB signaling," *Molecular and Cellular Biology*, vol. 23, no. 13, pp. 4649–4662, 2003.

[8] P. Cohen and S. Frame, "The renaissance of GSK3," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 10, pp. 769–776, 2001.

[9] E. M. Yazlovitskaya, E. Edwards, D. Thotala et al., "Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation," *Cancer Research*, vol. 66, no. 23, pp. 11179–11186, 2006.

[10] E. Ter Haar, J. T. Coll, D. A. Austen, H. M. Hsiao, L. Swenson, and J. Jain, "Structure of GSK3β reveals a primed phosphorylation mechanism," *Nature Structural Biology*, vol. 8, no. 7, pp. 593–596, 2001.

[11] R. Dajani, E. Fraser, S. M. Roe et al., "Crystal structure of glycogen synthase kinase 3β: structural basis for phosphate-primed substrate specificity and autoinhibition," *Cell*, vol. 105, no. 6, pp. 721–732, 2001.

[12] D. A. E. Cross, D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings, "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B," *Nature*, vol. 378, no. 6559, pp. 785–789, 1995.

[13] H. Eldar-Finkelman, R. Seger, J. R. Vandenheede, and E. G. Krebs, "Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway in NIH/3T3 cells," *Journal of Biological Chemistry*, vol. 270, no. 3, pp. 987–990, 1995.

[14] L. Kim, J. Liu, and A. R. Kimmel, "The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification," *Cell*, vol. 99, no. 4, pp. 399–408, 1999.

[15] R. V. Bhat, S. L. Budd Haeberlein, and J. Avila, "Glycogen synthase kinase 3: a drug target for CNS therapies," *Journal of Neurochemistry*, vol. 89, no. 6, pp. 1313–1317, 2004.

[16] F. Hernández, M. Pérez, J. J. Lucas, A. M. Mata, R. Bhat, and J. Avila, "Glycogen synthase kinase-3 plays a crucial role in

tau exon 10 splicing and intranuclear distribution of SC35: implications for Alzheimer's disease," *Journal of Biological Chemistry*, vol. 279, no. 5, pp. 3801–3806, 2004.

[17] T. A. Fulga, I. Elson-Schwab, V. Khurana et al., "Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration in vivo," *Nature Cell Biology*, vol. 9, no. 2, pp. 139–148, 2007.

[18] P. Cohen and M. Goedert, "GSK3 inhibitors: development and therapeutic potential," *Nature Reviews Drug Discovery*, vol. 3, no. 6, pp. 479–487, 2004.

[19] E. S. Emamian, D. Hall, M. J. Birnbaum, M. Karayiorgou, and J. A. Gogos, "Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia," *Nature Genetics*, vol. 36, no. 2, pp. 131–137, 2004.

[20] J. M. Beaulieu, T. D. Sotnikova, W. D. Yao et al., "Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 14, pp. 5099–5104, 2004.

[21] P. S. Klein and D. A. Melton, "A molecular mechanism for the effect of lithium on development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 16, pp. 8455–8459, 1996.

[22] A. P. Kozikowski, I. N. Gaisina, P. A. Petukhov et al., "Highly potent and specific GSK-3β inhibitors that block tau phosphorylation and decrease α-synuclein protein expression in a cellular model of Parkinson's disease," *ChemMedChem*, vol. 1, no. 2, pp. 256–266, 2006.

[23] L. Wang, H. K. Lin, Y. C. Hu, S. Xie, L. Yang, and C. Chang, "Suppression of androgen receptor-mediated transactivation and cell growth by the glycogen synthase kinase 3β in prostate cells," *Journal of Biological Chemistry*, vol. 279, no. 31, pp. 32444–32452, 2004.

[24] A. Shakoori, A. Ougolkov, W. Y. Zhi et al., "Deregulated GSK3β activity in colorectal cancer: its association with tumor cell survival and proliferation," *Biochemical and Biophysical Research Communications*, vol. 334, no. 4, pp. 1365–1373, 2005.

[25] A. Shakoori, W. Mai, K. Miyashita et al., "Inhibition of GSK-3β activity attenuates proliferation of human colon cancer cells in rodents," *Cancer Science*, vol. 98, no. 9, pp. 1388–1393, 2007.

[26] A. V. Ougolkov, M. E. Fernandez-Zapico, D. N. Savoy, R. A. Urrutia, and D. D. Billadeau, "Glycogen synthase kinase-3β participates in nuclear factor κB-mediated gene transcription and cell survival in pancreatic cancer cells," *Cancer Research*, vol. 65, no. 6, pp. 2076–2081, 2005.

[27] E. Beurel and R. S. Jope, "The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways," *Progress in Neurobiology*, vol. 79, no. 4, pp. 173–189, 2006.

[28] J. R. Miller and R. T. Moon, "Signal transduction through β-catenin and specification of cell fate during embryogenesis," *Genes and Development*, vol. 10, no. 20, pp. 2527–2539, 1996.

[29] M. Peifer and P. Polakis, "Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus," *Science*, vol. 287, no. 5458, pp. 1606–1609, 2000.

[30] R. H. Giles, J. H. Van Es, and H. Clevers, "Caught up in a Wnt storm: Wnt signaling in cancer," *Biochimica et Biophysica Acta*, vol. 1653, no. 1, pp. 1–24, 2003.

[31] J. A. Diehl, M. Cheng, M. F. Roussel, and C. J. Sherr, "Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization," *Genes and Development*, vol. 12, no. 22, pp. 3499–3511, 1998.

[32] G. N. Bijur, P. De Sarno, and R. S. Jope, "Glycogen synthase kinase-3β facilitates staurosporine- and heat shock- induced apoptosis. Protection by lithium," *Journal of Biological Chemistry*, vol. 275, no. 11, pp. 7583–7590, 2000.

[33] J. E. Forde and T. C. Dale, "Glycogen synthase kinase 3: a key regulator of cellular fate," *Cellular and Molecular Life Sciences*, vol. 64, no. 15, pp. 1930–1944, 2007.

[34] P. Watcharasit, G. N. Bijur, J. W. Zmijewski et al., "Direct, activating interaction between glycogen synthase kinase-3β and p53 after DNA damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 12, pp. 7951–7955, 2002.

[35] R. D. Loberg, E. Vesely, and F. C. Brosius, "Enhanced glycogen synthase kinase-3β activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism," *Journal of Biological Chemistry*, vol. 277, no. 44, pp. 41667–41673, 2002.

[36] L. Song, P. De Sarno, and R. S. Jope, "Central role of glycogen synthase kinase-3β in endoplasmic reticulum stress-induced caspase-3 activation," *Journal of Biological Chemistry*, vol. 277, no. 47, pp. 44701–44708, 2002.

[37] J. Carmichael, K. L. Sugars, Y. P. Bao, and D. C. Rubinsztein, "Glycogen synthase kinase-3β inhibitors prevent cellular polyglutamine toxicity caused by the Huntington's disease mutation," *Journal of Biological Chemistry*, vol. 277, no. 37, pp. 33791–33798, 2002.

[38] D. A. E. Cross, A. A. Culbert, K. A. Chalmers, L. Facci, S. D. Skaper, and A. D. Reith, "Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death," *Journal of Neurochemistry*, vol. 77, no. 1, pp. 94–102, 2001.

[39] E. M. Yazlovitskaya, E. Edwards, D. Thotala et al., "Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation," *Cancer Research*, vol. 66, no. 23, pp. 11179–11186, 2006.

[40] A. A. Culbert, M. J. Brown, S. Frame et al., "GSK-3 inhibition by adenoviral FRAT1 overexpression is neuroprotective and induces Tau dephosphorylation and β-catenin stabilisation without elevation of glycogen synthase activity," *FEBS Letters*, vol. 507, no. 3, pp. 288–294, 2001.

[41] G. N. Bijur, P. De Sarno, and R. S. Jope, "Glycogen synthase kinase-3β facilitates staurosporine- and heat shock- induced apoptosis. Protection by lithium," *Journal of Biological Chemistry*, vol. 275, no. 11, pp. 7583–7590, 2000.

[42] C. A. Grimes and R. S. Jope, "The multifaceted roles of glycogen synthase kinase 3β in cellular signaling," *Progress in Neurobiology*, vol. 65, no. 4, pp. 391–426, 2001.

[43] M. Oren, "Decision making by p53: life, death and cancer," *Cell Death and Differentiation*, vol. 10, no. 4, pp. 431–442, 2003.

[44] J. P. Kruse and W. Gu, "Modes of p53 Regulation," *Cell*, vol. 137, no. 4, pp. 609–622, 2009.

[45] P. Watcharasit, G. N. Bijur, L. Song, J. Zhu, X. Chen, and R. S. Jope, "Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53," *The Journal of Biological Chemistry*, vol. 278, no. 49, pp. 48872–48879, 2003.

[46] E. Beurel, M. Kornprobst, M. J. Blivet-Van Eggelpoël et al., "GSK-3β inhibition by lithium confers resistance to chemotherapy-induced apoptosis through the repression of CD95 (Fas/APO-1) expression," *Experimental Cell Research*, vol. 300, no. 2, pp. 354–364, 2004.

[47] G. A. Turenne and B. D. Price, "Glycogen synthase kinase3 beta phosphorylates serine 33 of p53 and activates p53's transcriptional activity," *BMC Cell Biology*, vol. 2, 2001.

[48] O. Pluquet, L. K. Qu, D. Baltzis, and A. E. Koromilas, "Endoplasmic reticulum stress accelerates p53 degradation by the cooperative actions of Hdm2 and glycogen synthase kinase 3β," *Molecular and Cellular Biology*, vol. 25, no. 21, pp. 9392–9405, 2005.

[49] L. Qu, S. Huang, D. Baltzis et al., "Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3β," *Genes and Development*, vol. 18, no. 3, pp. 261–277, 2004.

[50] T. Y. Eom, K. A. Roth, and R. S. Jope, "Neural precursor cells are protected from apoptosis induced by trophic factor withdrawal or genotoxic stress by inhibitors of glycogen synthase kinase 3," *Journal of Biological Chemistry*, vol. 282, no. 31, pp. 22856–22864, 2007.

[51] G. Arboleda, Y. Cardenas, Y. Rodriguez, L. C. Morales, L. Matheus, and H. Arboleda, "Differential regulation of AKT, MAPK and GSK3beta during C2-ceramide-induced neuronal death," *Neurotoxicology*, vol. 31, no. 6, pp. 687–693, 2010.

[52] A. Mora, G. Sabio, A. M. Risco et al., "Lithium blocks the PKB and GSK3 dephosphorylation induced by ceramide through protein phosphatase-2A," *Cellular Signalling*, vol. 14, no. 6, pp. 557–562, 2002.

[53] K. T. Noh, Y. M. Park, S. G. Cho, and E. J. Choi, "GSK-3β-induced ASK1 stabilization is crucial in LPS-induced endotoxin shock," *Experimental Cell Research*, vol. 317, no. 12, pp. 1663–1668, 2011.

[54] S. Sengupta, P. Jayaraman, P. M. Chilton, C. R. Casella, and T. C. Mitchell, "Unrestrained glycogen synthase kinase-3β activity leads to activated T cell death and can be inhibited by natural adjuvant," *Journal of Immunology*, vol. 178, no. 10, pp. 6083–6091, 2007.

[55] Y. J. Kang, M. Digicaylioglu, R. Russo et al., "Erythropoietin plus insulin-like growth factor-I protects against neuronal damage in a murine model of human immunodeficiency virus-associated neurocognitive disorders," *Annals of Neurology*, vol. 68, no. 3, pp. 342–352, 2010.

[56] F. Peng, N. K. Dhillon, H. Yao, X. Zhu, R. Williams, and S. Buch, "Mechanisms of platelet-derived growth factor-mediated neuroprotection—implications in HIV dementia," *European Journal of Neuroscience*, vol. 28, no. 7, pp. 1255–1264, 2008.

[57] Z. Sui, L. F. Sniderhan, S. Fan et al., "Human immunodeficiency virus-encoded Tat activates glycogen synthase kinase-3β to antagonize nuclear factor-κB survival pathway in neurons," *European Journal of Neuroscience*, vol. 23, no. 10, pp. 2623–2634, 2006.

[58] O. Goldbaum and C. Richter-Landsberg, "Activation of PP2A-like phosphatase and modulation of tau phosphorylation accompany stress-induced apoptosis in cultured oligodendrocytes," *GLIA*, vol. 40, no. 3, pp. 271–282, 2002.

[59] T. Kihira, A. Suzuki, T. Kondo et al., "Immunohistochemical expression of IGF-I and GSK in the spinal cord of Kii and Guamanian ALS patients: original Article," *Neuropathology*, vol. 29, no. 5, pp. 548–558, 2009.

[60] S. H. Koh, Y. B. Lee, K. S. Kim et al., "Role of GSK-3β activity in motor neuronal cell death induced by G93A or A4V mutant hSOD1 gene," *European Journal of Neuroscience*, vol. 22, no. 2, pp. 301–309, 2005.

[61] M. H. Liang, J. R. Wendland, and D. M. Chuang, "Lithium inhibits Smad3/4 transactivation via increased CREB activity induced by enhanced PKA and AKT signaling," *Molecular and Cellular Neuroscience*, vol. 37, no. 3, pp. 440–453, 2008.

[62] D. F. Martins, A. O. Rosa, V. M. Gadotti et al., "The antinociceptive effects of AR-A014418, a selective inhibitor of glycogen synthase kinase-3 beta, in mice," *Journal of Pain*, vol. 12, no. 3, pp. 315–322, 2011.

[63] M. Pérez, A. I. Rojo, F. Wandosell, J. Díaz-Nido, and J. Avila, "Prion peptide induces neuronal cell death through a pathway involving glycogen synthase kinase 3," *Biochemical Journal*, vol. 372, no. 1, pp. 129–136, 2003.

[64] Z. Tang, P. Arjunan, C. Lee et al., "Survival effect of PDGF-CC rescues neurons from apoptosis in both brain and retina by regulating GSK3β phosphorylation," *Journal of Experimental Medicine*, vol. 207, no. 4, pp. 867–880, 2010.

[65] J. M. Gall, V. Wong, D. R. Pimental et al., "Hexokinase regulates Bax-mediated mitochondrial membrane injury following ischemic stress," *Kidney International*, vol. 79, no. 11, pp. 1207–1216, 2011.

[66] Y. Hotokezaka, K. van Leyen, E. H. Lo et al., "AlphaNAC depletion as an initiator of ER stress-induced apoptosis in hypoxia," *Cell Death & Differentiation*, vol. 16, no. 11, pp. 1505–1514, 2009.

[67] Q. Li, H. Li, K. Roughton et al., "Lithium reduces apoptosis and autophagy after neonatal hypoxia-ischemia," *Cell Death and Disease*, vol. 1, no. 7, article e56, 2010.

[68] A. K. Vasileva, E. Y. Plotnikov, A. V. Kazachenko, V. I. Kirpatovsky, and D. B. Zorov, "Inhibition of GSK-3β decreases the ischemia-induced death of renal cells," *Bulletin of Experimental Biology and Medicine*, vol. 149, no. 3, pp. 303–307, 2010.

[69] R. Kulikov, K. A. Boehme, and C. Blattner, "Glycogen synthase kinase 3-dependent phosphorylation of Mdm2 regulates p53 abundance," *Molecular and Cellular Biology*, vol. 25, no. 16, pp. 7170–7180, 2005.

[70] D. K. Thotala, D. E. Hallahan, and E. M. Yazlovitskaya, "Inhibition of glycogen synthase kinase 3β attenuates neurocognitive dysfunction resulting from cranial irradiation," *Cancer Research*, vol. 68, no. 14, pp. 5859–5868, 2008.

[71] D. K. Thotala, D. E. Hallahan, and E. M. Yazlovitskaya, "Glycogen synthase kinase 3β inhibitors protect hippocampal neurons from radiation-induced apoptosis by regulating MDM2-p53 pathway," *Cell Death and Differentiation*, vol. 19, pp. 387–396, 2011.

[72] R. Lu, L. Song, and R. S. Jope, "Lithium attenuates p53 levels in human neuroblastoma SH-SY5Y cells," *NeuroReport*, vol. 10, no. 5, pp. 1123–1125, 1999.

[73] J. W. Zmijewski and R. S. Jope, "Nuclear accumulation of glycogen synthase kinase-3 during replicative senescence of human fibroblasts," *Aging Cell*, vol. 3, no. 5, pp. 309–317, 2004.

[74] R. J. Youle and A. Strasser, "The BCL-2 protein family: opposing activities that mediate cell death," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 1, pp. 47–59, 2008.

[75] G. Lessene, P. E. Czabotar, and P. M. Colman, "BCL-2 family antagonists for cancer therapy," *Nature Reviews Drug Discovery*, vol. 7, no. 12, pp. 989–1000, 2008.

[76] P. Li, D. Nijhawan, I. Budihardjo et al., "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade," *Cell*, vol. 91, no. 4, pp. 479–489, 1997.

[77] D. K. Thotala, L. Geng, A. K. Dickey, D. E. Hallahan, and E. M. Yazlovitskaya, "A New class of molecular targeted radioprotectors: GSK-3β inhibitors," *International Journal of Radiation Oncology Biology Physics*, vol. 76, no. 2, pp. 557–565, 2010.

[78] S. R. Datta, A. Brunet, and M. E. Greenberg, "Cellular survival: a play in three akts," *Genes and Development*, vol. 13, no. 22, pp. 2905–2927, 1999.

[79] A. Dickey, S. Schleicher, K. Leahy, R. Hu, D. Hallahan, and D. K. Thotala, "GSK-3β inhibition promotes cell death, apoptosis, and in vivo tumor growth delay in neuroblastoma Neuro-2A cell line," *Journal of Neuro-Oncology*, vol. 104, pp. 145–153, 2010.

[80] G. P. Meares and R. S. Jope, "Resolution of the nuclear localization mechanism of glycogen synthase kinase-3: functional effects in apoptosis," *Journal of Biological Chemistry*, vol. 282, no. 23, pp. 16989–17001, 2007.

[81] S. Korur, R. M. Huber, B. Sivasankaran et al., "GSK3beta regulates differentiation and growth arrest in glioblastoma," *PloS One*, vol. 4, no. 10, article e7443, 2009.

[82] S. Kotliarova, S. Pastorino, L. C. Kovell et al., "Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-κB, and glucose regulation," *Cancer Research*, vol. 68, no. 16, pp. 6643–6651, 2008.

[83] M. Sun, L. Song, Y. Li, T. Zhou, and R. S. Jope, "Identification of an antiapoptotic protein complex at death receptors," *Cell Death and Differentiation*, vol. 15, no. 12, pp. 1887–1900, 2008.

[84] R. Venè, P. Larghero, G. Arena, M. B. Sporn, A. Albini, and F. Tosetti, "Glycogen synthase kinase 3β regulates cell death induced by synthetic triterpenoids," *Cancer Research*, vol. 68, no. 17, pp. 6987–6996, 2008.

[85] A. Damalas, A. Ben-Ze'ev, I. Simcha et al., "Excess β-catenin promotes accumulation of transcriptionally active p53," *EMBO Journal*, vol. 18, no. 11, pp. 3054–3063, 1999.

[86] E. Sadot, B. Geiger, M. Oren, and A. Ben-Ze'ev, "Down-regulation of β-catenin by activated p53," *Molecular and Cellular Biology*, vol. 21, no. 20, pp. 6768–6781, 2001.

[87] G. P. Meares, M. A. Mines, E. Beurel et al., "Glycogen synthase kinase-3 regulates endoplasmic reticulum (ER) stress-induced CHOP expression in neuronal cells," *Experimental Cell Research*, vol. 317, no. 11, pp. 1621–1628, 2011.

[88] K. P. Hoeflich, J. Luo, E. A. Rubie, M. S. Tsao, O. Jin, and J. R. Woodgett, "Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation," *Nature*, vol. 406, no. 6791, pp. 86–90, 2000.

[89] F. Demarchi, C. Bertoli, P. Sandy, and C. Schneider, "Glycogen synthase kinase-3β regulates NF-κB1/p105 stability," *Journal of Biological Chemistry*, vol. 278, no. 41, pp. 39583–39590, 2003.

[90] R. F. Schwabe and D. A. Brenner, "Role of glycogen synthase kinase-3 in TNF-α-induced NF-κB activation and apoptosis in hepatocytes," *American Journal of Physiology*, vol. 283, no. 1, pp. G204–G211, 2002.

[91] J. Deng, S. A. Miller, H. Y. Wang et al., "β-catenin interacts with and inhibits NF-κB in human colon and breast cancer," *Cancer Cell*, vol. 2, no. 4, pp. 323–334, 2002.

[92] F. Gotschel, C. Kern, S. Lang et al., "Inhibition of GSK3 differentially modulates NF-kappaB, CREB, AP-1 and beta-catenin signaling in hepatocytes, but fails to promote TNF-alpha-induced apoptosis," *Experimental Cell Research*, vol. 314, no. 6, pp. 1351–1366, 2008.

[93] P. Schotte, G. Van Loo, I. Carpentier, P. Vandenabeele, and R. Beyaert, "Lithium sensitizes tumor cells in an NFκB-independent way to caspase activation and apoptosis induced by tumor necrosis factor (TNF): evidence for a role of the TNF receptor-associated death domain protein," *Journal of Biological Chemistry*, vol. 276, no. 28, pp. 25939–25945, 2001.

[94] X. Liao, L. Zhang, J. B. Thrasher, J. Du, and B. Li, "Glycogen synthase kinase-3beta suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer.," *Molecular Cancer Therapeutics*, vol. 2, no. 11, pp. 1215–1222, 2003.

[95] G. N. Bijur and R. S. Jope, "Proapoptotic stimuli induce nuclear accumulation of glycogen synthase kinase-3β," *Journal of Biological Chemistry*, vol. 276, no. 40, pp. 37436–37442, 2001.

[96] F. Takahashi-Yanaga and T. Sasaguri, "GSK-3β regulates cyclin D1 expression: a new target for chemotherapy," *Cellular Signalling*, vol. 20, no. 4, pp. 581–589, 2008.

[97] A. Arcaro and M. P. Wymann, "Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses," *Biochemical Journal*, vol. 296, no. 2, pp. 297–301, 1993.

[98] M. Pap and G. M. Cooper, "Role of glycogen synthase kinase-3 in the phosphatidylinositol 3- kinase/Akt cell survival pathway," *Journal of Biological Chemistry*, vol. 273, no. 32, pp. 19929–19932, 1998.

[99] C. J. Vlahos, W. F. Matter, K. Y. Hui, and R. F. Brown, "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)- 8-phenyl-4H-1-benzopyran-4-one (LY294002)," *Journal of Biological Chemistry*, vol. 269, no. 7, pp. 5241–5248, 1994.

[100] S. Arico, S. Pattingre, C. Bauvy et al., "Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line," *Journal of Biological Chemistry*, vol. 277, no. 31, pp. 27613–27621, 2002.

[101] M. M. Hill, M. Andjelkovic, D. P. Brazil, S. Ferrari, D. Fabbro, and B. A. Hemmings, "Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase," *Journal of Biological Chemistry*, vol. 276, no. 28, pp. 25643–25646, 2001.

[102] A. R. Hussain, M. Al-Rasheed, P. S. Manogaran et al., "Curcumin induces apoptosis via inhibition of PI3′-kinase/AKT pathway in acute T cell leukemias," *Apoptosis*, vol. 11, no. 2, pp. 245–254, 2006.

[103] F. Meggio, A. Donella Deana, M. Ruzzene et al., "Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2," *European Journal of Biochemistry*, vol. 234, no. 1, pp. 317–322, 1995.

[104] A. Noël, L. Barrier, F. Rinaldi, C. Hubert, B. Fauconneau, and S. Ingrand, "Lithium chloride and staurosporine potentiate the accumulation of phosphorylated glycogen synthase kinase 3β/Tyr216, resulting in glycogen synthase kinase 3β activation in SH-SY5Y human neuroblastoma cell lines," *Journal of Neuroscience Research*, vol. 89, no. 5, pp. 755–763, 2011.

[105] J. P. Alao, A. V. Stavropoulou, E. W. F. Lam, and R. C. Coombes, "Role of glycogen synthase kinase 3 beta (GSK3β) in mediating the cytotoxic effects of the histone deacetylase inhibitor trichostatin A (TSA) in MCF-7 breast cancer cells," *Molecular Cancer*, vol. 5, article 40, 2006.

[106] L. Yang, H. C. Dan, M. Sun et al., "Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt," *Cancer Research*, vol. 64, no. 13, pp. 4394–4399, 2004.

[107] J. Dong, J. Peng, H. Zhang et al., "Role of glycogen synthase kinase 3β in rapamycin-mediated cell cycle regulation and

chemosensitivity," *Cancer Research*, vol. 65, no. 5, pp. 1961–1972, 2005.

[108] H. H. Zhang, A. I. Lipovsky, C. C. Dibble, M. Sahin, and B. D. Manning, "S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt," *Molecular Cell*, vol. 24, no. 2, pp. 185–197, 2006.

[109] M. Benkoussa, C. Brand, M. H. Delmotte, P. Formstecher, and P. Lefebvre, "Retinoic acid receptors inhibit AP1 activation by regulating extracellular signal-regulated kinase and CBP recruitment to an AP1-responsive promoter," *Molecular and Cellular Biology*, vol. 22, no. 13, pp. 4522–4534, 2002.

[110] Y. Ma, Q. Feng, D. Sekula, J. A. Diehl, S. J. Freemantle, and E. Dmitrovsky, "Retinoid targeting of different D-type cyclins through distinct chemopreventive mechanisms," *Cancer Research*, vol. 65, no. 14, pp. 6476–6483, 2005.

[111] J. Mori, F. Takahashi-Yanaga, Y. Miwa et al., "Differentiation-inducing factor-1 induces cyclin D1 degradation through the phosphorylation of Thr286 in squamous cell carcinoma," *Experimental Cell Research*, vol. 310, no. 2, pp. 426–433, 2005.

[112] F. Takahashi-Yanaga, Y. Taba, Y. Miwa et al., "Dictyostelium differentiation-inducing factor-3 activates glycogen synthase kinase-3β and degrades cyclin D1 in mammalian cells," *Journal of Biological Chemistry*, vol. 278, no. 11, pp. 9663–9670, 2003.

[113] M. Medina and J. Avila, "Glycogen synthase kinase-3 (GSK-3) inhibitors for the treatment of Alzheimer's disease," *Current Pharmaceutical Design*, vol. 16, no. 25, pp. 2790–2798, 2010.

[114] J. Avila and F. Hernández, "GSK-3 inhibitors for Alzheimer's disease," *Expert Review of Neurotherapeutics*, vol. 7, no. 11, pp. 1527–1533, 2007.

[115] G. Klamer, E. Song, K. H. Ko, T. A. O'Brien, and A. Dolnikov, "Using small molecule GSK3β inhibitors to treat inflammation," *Current Medicinal Chemistry*, vol. 17, no. 26, pp. 2873–2881, 2010.

[116] S. Phukan, V. S. Babu, A. Kannoji, R. Hariharan, and V. N. Balaji, "GSK3β: role in therapeutic landscape and development of modulators," *British Journal of Pharmacology*, vol. 160, no. 1, pp. 1–19, 2010.

[117] W. J. Ryves, R. Dajani, L. Pearl, and A. J. Harwood, "Glycogen synthase kinase-3 inhibition by lithium and beryllium suggests the presence of two magnesium binding sites," *Biochemical and Biophysical Research Communications*, vol. 290, no. 3, pp. 967–972, 2002.

[118] A. Martinez, A. Castro, I. Dorronsoro, and M. Alonso, "Glycogen synthase kinase 3 (GSK-3) inhibitors as new promising drugs for diabetes, neurodegeneration, cancer, and inflammation," *Medicinal Research Reviews*, vol. 22, no. 4, pp. 373–384, 2002.

[119] M. Aoki, T. Yokota, I. Sugiura et al., "Structural insight into nucleotide recognition in tau-protein kinase I/glycogen synthase kinase 3β," *Acta Crystallographica D*, vol. 60, no. 3, pp. 439–446, 2004.

[120] B. Bax, P. S. Carter, C. Lewis et al., "The structure of phosphorylated GSK-3β complexed with a peptide, FRATtide, that inhibits β-catenin phosphorylation," *Structure*, vol. 9, no. 12, pp. 1143–1152, 2001.

[121] J. A. Bertrand, S. Thieffine, A. Vulpetti et al., "Structural characterization of the GSK-3β active site using selective and non-selective ATP-mimetic inhibitors," *Journal of Molecular Biology*, vol. 333, no. 2, pp. 393–407, 2003.

[122] R. Bhat, Y. Xue, S. Berg et al., "Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418," *The Journal of Biological Chemistry*, vol. 278, no. 46, pp. 45937–45945, 2003.

[123] R. Dajani, E. Fraser, S. M. Roe et al., "Structural basis for recruitment of glycogen synthase kinase 3β to the axin-APC scaffold complex," *EMBO Journal*, vol. 22, no. 3, pp. 494–501, 2003.

[124] D. Shin, S. C. Lee, Y. S. Heo et al., "Design and synthesis of 7-hydroxy-1H-benzoimidazole derivatives as novel inhibitors of glycogen synthase kinase-3β," *Bioorganic and Medicinal Chemistry Letters*, vol. 17, no. 20, pp. 5686–5689, 2007.

[125] H. C. Zhang, L. V. Bonaga, H. Ye, C. K. Derian, B. P. Damiano, and B. E. Maryanoff, "Novel bis(indolyl)maleimide pyridinophanes that are potent, selective inhibitors of glycogen synthase kinase-3," *Bioorganic & Medicinal Chemistry Letters* , vol. 17, no. 10, pp. 2863–2868, 2007.

[126] R. S. Jope, "Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes," *Trends in Pharmacological Sciences*, vol. 24, no. 9, pp. 441–443, 2003.

[127] M. P. Coghlan, A. A. Culbert, D. A. E. Cross et al., "Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription," *Chemistry and Biology*, vol. 7, no. 10, pp. 793–803, 2000.

[128] L. Meijer, A. M. W. H. Thunnissen, A. W. White et al., "Inhibition of cyclin-dependent kinases, GSK-3β and CK1 by hymenialdisine, a marine sponge constituent," *Chemistry and Biology*, vol. 7, no. 1, pp. 51–63, 2000.

[129] D. W. Zaharevitz, R. Gussio, M. Leost et al., "Discovery and initial characterization of the paullones, a novel class of smallmolecule inhibitors of cyclin-dependent kinases," *Cancer Research*, vol. 59, no. 11, pp. 2566–2569, 1999.

[130] R. Hoessel, S. Leclerc, J. A. Endicott et al., "Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases," *Nature Cell Biology*, vol. 1, no. 1, pp. 60–67, 1999.

[131] S. Leclerc, M. Garnier, R. Hoessel et al., "Indirubins inhibit glycogen synthase kinase-3β and CDK5/P25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors?" *Journal of Biological Chemistry*, vol. 276, no. 1, pp. 251–260, 2001.

[132] L. Meijer, A. L. Skaltsounis, P. Magiatis et al., "GSK-3-selective inhibitors derived from tyrian purple indirubins," *Chemistry and Biology*, vol. 10, no. 12, pp. 1255–1266, 2003.

[133] A. Martinez, M. Alonso, A. Castro, C. Pérez, and F. J. Moreno, "First non-ATP competitive glycogen synthase kinase 3 β (GSK-3β) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease," *Journal of Medicinal Chemistry*, vol. 45, no. 6, pp. 1292–1299, 2002.

[134] M. P. Coghlan, A. A. Culbert, D. A. E. Cross et al., "Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription," *Chemistry and Biology*, vol. 7, no. 10, pp. 793–803, 2000.

[135] G. M. Thomas, S. Frame, M. Goedert, I. Nathke, P. Polakis, and P. Cohen, "A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of Axin and β-catenin," *FEBS Letters*, vol. 458, no. 2, pp. 247–251, 1999.

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 493934, 8 pages
doi:10.1155/2012/493934

Review Article

ER-Mitochondria Crosstalk during Cerebral Ischemia: Molecular Chaperones and ER-Mitochondrial Calcium Transfer

Yi-Bing Ouyang and Rona G. Giffard

Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305-5117, USA

Correspondence should be addressed to Yi-Bing Ouyang, ybouyang@stanford.edu

Received 1 December 2011; Accepted 12 January 2012

Academic Editor: Mariusz R. Wieckowski

It is commonly believed that sustained elevations in the mitochondrial matrix Ca^{2+} concentration are a major feature of the intracellular cascade of lethal events during cerebral ischemia. The physical association between the endoplasmic reticulum (ER) and mitochondria, known as the mitochondria-associated ER membrane (MAM), enables highly efficient transmission of Ca^{2+} from the ER to mitochondria under both physiological and pathological conditions. Molecular chaperones are well known for their protective effects during cerebral ischemia. It has been demonstrated recently that many molecular chaperones coexist with MAM and regulate the MAM and thus Ca^{2+} concentration inside mitochondria. Here, we review recent research on cerebral ischemia and MAM, with a focus on molecular chaperones and ER-mitochondrial calcium transfer.

1. Introduction

Stroke is one of the leading causes of death worldwide and a major cause of long-term disability [1]. Although many clinical trials have been completed in stroke patients, none of these have demonstrated protective efficacy except for thrombolysis [2, 3]. Suggested reasons for this failure include the complex interplay among multiple pathways (for review see [4–6]) including excitotoxicity, mitochondrial dysfunction, acidotoxicity, ionic imbalance, oxidative stress, and inflammation, which can all lead to cell death and irreversible tissue injury.

A generally accepted cell death pathway after cerebral ischemia is mitochondrial permeability transition (MPT) pore opening (Figure 1(a)). Ischemia leads to energy deprivation and loss of ion homeostasis. As the cells are unable to maintain a negative membrane potential, they depolarize, leading to the opening of voltage-gated calcium channels and release of excitatory amino acids into the extracellular space [7]. This cascade of events leads to a massive entry of calcium and this increase in free cytosolic calcium is transmitted to the matrix of mitochondria by Ca^{2+} channels and exchangers located on the inner mitochondrial membrane. Recently ER stress was found to be one of the effects of excitotoxicity, that is, exposure to toxic levels of excitatory neurotransmitters, with release of Ca^{2+} from the ER via both ryanodine receptors and IP3R, with release from inositol trisphosphate receptors (IP3Rs) leading to mitochondrial Ca^{2+} overload and activation of apoptosis [8]. Excessive increases in matrix Ca^{2+} alter the permeability of mitochondria and finally open the MPT pore [9], causing the release of cytochrome c [10] and other proapoptotic factors into the cytoplasm. The released cytochrome c activates caspase-3, one of the executioner caspases to initiate cell death. Excessive accumulation of calcium in mitochondria is a key factor in the final outcome of the cascade leading to neural cell death (Figure 1(a)) [11].

Mitochondria can accumulate large amounts of calcium through a Ca^{2+}-selective channel known as the mitochondrial Ca^{2+} uniporter (MCU) [12, 13]. However, MCU has a relatively low Ca^{2+} affinity [14]. It is interesting that in response to cytosolic Ca^{2+} transients not exceeding concentrations of 1–3 μM, mitochondrial Ca^{2+} concentrations rise almost simultaneously to values above 10 μM [15]. The existence of close contact points between the ER and mitochondria (the mitochondria-associated ER membrane,

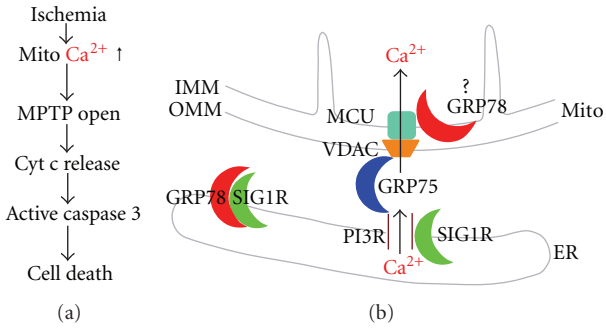


Figure 1: (a) Diagram of cerebral ischemia-induced cell death signaling cascade. (b) Chaperone machinery controls ER-mitochondria Ca^{2+} crosstalk at the MAM. Under normal, resting conditions, SIG1R chaperone forms a complex with GRP78 at the ER. Under stress such as ischemia, SIG1R dissociates from GRP78 and associates with IP3R3 at the MAM, and GRP78 translocates from ER to IMM. ER-mitochondria Ca^{2+} transfer controls cell survival or death decision. Cyt c: cytochrome c; ER: endoplasmic reticulum; GRP75: glucose-regulated protein 75; GRP78: glucose-regulated protein 78 kDa; IMM: inner mitochondrial membrane; IP3R: inositol trisphosphate receptor; MCU: mitochondrial Ca^{2+} uniporter; Mito: mitochondria; MPTP: mitochondrial permeability transition pore; OMM: outer mitochondrial membrane; SIG1R: sigma-1 receptor; VDAC: voltage-dependent anion channel.

MAM) is thought to provide a selective direct pathway for calcium from the ER to mitochondria. Upon cell stimulation, the release of high concentrations of Ca^{2+} at MAM leads to the formation of microdomains of high Ca^{2+} concentration that is crucial for efficient Ca^{2+} uptake by mitochondria [16, 17].

Molecular chaperones are a functionally related group of proteins that assist protein folding in cells and protect cells from injury after cerebral ischemia or other stress. It has been demonstrated recently that MAM coexists with many molecular chaperones [18]. The relationship between molecular chaperones and ER-mitochondrial calcium transfer after cerebral ischemia is an emerging research area and is the focus of this mini review.

2. Cerebral Ischemic Models

Animal models of ischemic stroke are used to study the basic pathophysiological processes and potential therapeutic interventions in this disease; the extension of knowledge gained from these animal models will lead to improvement of medical treatment for human ischemic stroke in the future. Focal cerebral ischemia by middle cerebral artery occlusion (MCAO) in rats or mice is the rodent model most immediately relevant to human stroke. Using this method, transient ischemia is achieved by inserting a suture into the left middle cerebral artery, temporarily blocking blood flow to the middle cerebral artery territory, and removing the suture to allow reperfusion after a duration of minutes to hours depending on the specific study [9, 19, 20].

Glucose deprivation (GD) and combined oxygen-glucose deprivation (OGD) are common *in vitro* models of brain ischemia. Either cell cultures or slice cultures are subjected to medium lacking glucose, and in the case of OGD, also placed in a chamber with very low oxygen levels for a fixed period of time [19, 21–26], followed by restoration of oxygen and glucose to the medium to imitate reperfusion.

3. Molecular Chaperones

Molecular chaperones were originally defined as a functionally related group of proteins that assist protein folding in bacterial, plant, and animal cells. The heat shock proteins of the 70 kDa molecular weight family (HSP70), including HSP72 (cytosol), GRP75/mortalin (mitochondria), and GRP78/BIP (endoplasmic reticulum; ER), are highly evolutionarily conserved and have been extensively studied. Studies, including those from our laboratory, show that all three of these HSP70 family members are protective in both *in vivo* and *in vitro* models of stroke [19, 27–32]. It has been a long-standing observation, as documented for HSP72 [33–35] and GRP75 [36], that cells destined to die fail to produce heat shock proteins, while cells that survive make new heat shock proteins. We recently identified translational arrest of GRP78 due to microRNA181 in focal cerebral ischemia in the mouse [19]. Although *Grp78* mRNA was induced following MCAO both in the core and outside the infarcted area, GRP78 protein was only induced in the penumbra, not within the area of infarction.

Recently a more complex, integrating role of these proteins has been recognized, that of stabilizing intracellular morphological and functional networks through protein-protein interactions with numerous client proteins [37–39]. This chaperoning network concept is increasingly accepted as a basic regulatory mechanism in diverse cellular functions [39, 40]. These networks allow the cell to change phenotype by releasing client proteins from chaperones allowing them to be activated, or in some cases released and degraded. These functional adjustments are rapid, do not require protein synthesis, and facilitate calibrated and integrated adaptation to changing conditions.

In addition to the new concept of the chaperone network, each individual chaperone has been found to have additional functions beyond that of functioning as a molecular chaperone. For example, GRP78 is traditionally considered to be a major endoplasmic reticulum chaperone as well as a master regulator of the unfolded protein response. Due to recent findings that significant amounts of GRP78 are present on the surface of cancer cells, it has emerged as an important regulator of tumor cell viability signaling, and cell surface GRP78 is now being used for therapeutic targeting [41]. In addition to GRP78, the ER calcium-binding protein calreticulin has also been shown to traffic to the plasma membrane and be involved in regulation of cell death [42, 43]. GRP78 plays a critical role in physiologic and pathologic stress [44], including developmental and neurological disorders [45]. As a multifunctional receptor on the cell surface [46], GRP78 may be associated with the AKT and ERK signaling pathways [47]. Because of its multiple

locations and functions, GRP78 may play a central role in the chaperone network. HSP72 also protects brain by regulating distinct pathways of apoptosis and inflammation which both contribute to outcome after cerebral ischemia (for review see [48]). Other ER proteins also participate in cell death regulation, and function outside the ER.

4. The Mitochondria-Associated ER Membrane (MAM)

Although the association of endoplasmic reticulum (ER) with mitochondria was first observed in the 1960s by several independent groups [49, 50], morphological evidence for the physical association or interaction between the ER and mitochondria first emerged in the early 1990s. Such contact has since been observed in mitochondria in many types of cell [51, 52]. Structural and functional interactions of mitochondria with the ER have been demonstrated for rat brain [53]. The close contacts through which ER communicates with mitochondria are referred to as MAM [54]. The distance between the ER and the outer mitochondrial membrane (OMM) was originally estimated to be approximately 100 nm [51, 52]. However, a more recent study using electron tomography showed that the minimum distance is even less, 10 nm at the smooth ER and 25 nm at the rough ER [55]. Actually the spacing between the ER and mitochondria changes with different cell physiological and pathological conditions [56, 57] and artificial modification of this contact can lead to ER stress [55].

Numerous proteins have recently been proposed to participate in the interaction and communication between the mitochondria and the ER, highlighting the emerging role of this region in bioenergetics, cell survival, and cell death [58, 59]. One important structure is the IP3R on the ER and the voltage-dependent anion channel (VDAC) on the OMM which are now thought to be physically coupled through the chaperone Grp75/mortalin (Figure 1(b)) [60]. The sigma-1 receptor (SIG1R) chaperone is enriched in the MAM fraction [61–64] and recruits GRP78. In addition, other Ca^{2+}-binding ER resident chaperones have been found in the MAM fraction, for example, calnexin (CNX), calreticulin, and ERp44 [65–67]. The multifunctional cytosolic sorting protein PACS-2 is another protein that has been found in the MAM fraction [68]. This fraction can also contain adenine nucleotide translocase (ANT) and cyclophylin D, the components of mitochondrial contact sites with similar composition to the mitochondrial permeability transition pore (MPTP). Such close apposition of the MPTP to the ER can sensitize mitochondria to Ca^{2+} signals [69]. Recently, the mitochondrial GTPase mitofusin 2 has been shown to be enriched in MAM as well as localized on the ER, where it interacts with mitofusins on mitochondria to form interorganellar bridges [70].

MAM can be isolated from tissues and cells to investigate the mechanisms and functions involved [60, 71]. Wieckowski et al. provided detailed protocols in 2009 in Nature Protocols [71]. Briefly the procedure consists of two steps: a crude mitochondrial fraction is isolated from tissue or cells by differential centrifugation, and the crude mitochondria are fractionated to the pure mitochondria and MAM fraction by Percoll density gradient.

5. Ca^{2+} Signaling at the MAM during Apoptosis

It is commonly accepted that the main structure responsible for ER-mitochondrial calcium transfer at the MAM is composed of the IP3R on the ER, VDAC on the OMM and MCU on the IMM (Figure 1(b)). Ca^{2+} released upon activation of the IP3R at the ER is taken up into mitochondria via VDAC and then MCU [72, 73].

A major function of MAM is the control of Ca^{2+} signaling between ER and mitochondria, a central topic of major interest both in normal physiology and pathophysiology. This second messenger has been proposed to have multiple roles in modulating intracellular events including bioenergetics and autophagy. Constitutive calcium release via the IP3R was found to be essential for maintaining normal bioenergetics and suppressing autophagy in conditions of ready nutrient availability [74]. In contrast during ER stress, Ca^{2+} increase seems to be required for triggering autophagy [75], though calcium-independent routes to induce autophagy involving interaction of IP3R with Beclin have also been reported [76], and lack of Ca^{2+} release via the IP3R can also induce autophagy [74]. Thus the role of calcium is complex, and induction of autophagy reflects combined input from Ca^{2+} dependent and independent pathways (see recent review [77]). Under prolonged ER stress conditions, as happens in the ischemic core after cerebral ischemia, a slow but sustained increase in mitochondrial matrix free [Ca^{2+}] can occur, which can reach a critical threshold to trigger the opening of MPTP and initiate the apoptotic cascade (Figure 1(a)). Some studies indicate that the induction of apoptosis by ER stress has a mandatory mitochondrial component, further highlighting the intimate connection between these two organelles [78].

The ER can play an important role in regulating apoptosis by adjusting the load of Ca^{2+} imposed upon the mitochondrion. Previous studies have shown that the reduction in the Ca^{2+} amount that can be released from ER to mitochondria decreases the probability of Ca^{2+}-dependent apoptosis. On the other hand, conditions that increase ER Ca^{2+} storage have the opposite effect on Ca^{2+}-dependent apoptosis [79–82]. It has been demonstrated that overexpression of the antiapoptotic protein BCL2 can influence the distribution of Ca^{2+} within the ER/mitochondrial complex. Knockout of the proapoptotic proteins BAX and BAK reduced the resting concentration of ER Ca^{2+} decreasing the uptake of Ca^{2+} by mitochondria after Ca^{2+} release from the ER [81]. The active form of the antiapoptotic protein AKT results in reduced ER Ca^{2+} release, and diminished cellular sensitivity to Ca^{2+}-mediated apoptotic stimuli [79, 82]. Antiapoptotic proteins BCL2 and AKT affect ER calcium homeostasis by differential mechanisms: BCL2 overexpression increases the Ca^{2+} leak from the ER, while AKT hyperactivation induces a decrease in ER Ca^{2+} release, probably through phosphorylation of the IP3R [58, 80].

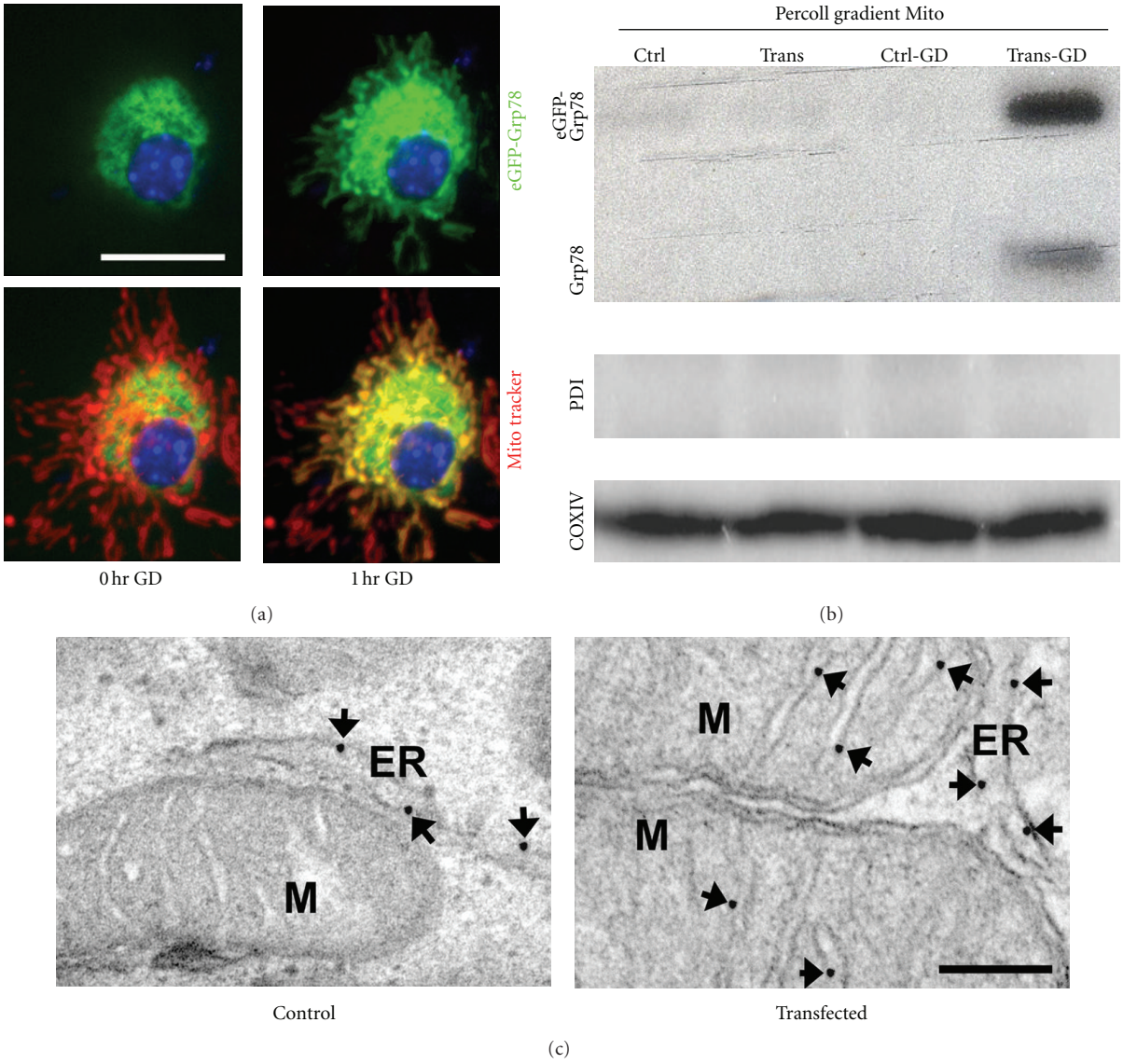


Figure 2: GRP78 retargets to mitochondria with glucose deprivation (GD). (a) Fluorescence photomicrographs were taken before and after 1 hr GD. Under normal conditions, the green fluorescence in eGFP-Grp78 transfected cells shows the normal perinuclear ER localization. After 1 hr GD this changes to a diffuse cytoplasmic pattern overlapping with the mitochondrial distribution visualized by partial overlap with Mito-tracker (red) fluorescence. Overlap is yellow. (b) After 3 hr GD, mitochondria were purified and analyzed by Western blotting using antibodies against GRP78, PDI (an ER-specific marker), or COXIV (a mitochondrial marker). The purified mitochondria do not show contamination with ER marker PDI but do have GRP78. Ctrl: control. Trans: transfected. (c) Submitochondrial localization of GRP78 in control and GD-stressed cells by immunoelectron microscopy. Arrows point to the localization of GRP78. The gold particles were associated exclusively with ER membrane in control cells (left panel). In contrast, immune-EM staining shows GRP78 within mitochondria from 3 hr GD-stressed cells, demonstrating significant mitochondrial labeling, with grains mainly decorating the inner mitochondrial membrane (right panel). M, mitochondrion. Scale bars, 100 nm.

6. Molecular Chaperones Regulate MAM

Some important chaperones are enriched in MAM and may play a key role in regulating Ca^{2+} signaling between ER and mitochondria. It was found that the mitochondrial chaperone GRP75 regulates IP3R-mediated mitochondrial Ca^{2+} signaling [60]. It was demonstrated that isoform 1 of VDAC is physically linked to the ER Ca^{2+}-release channel IP3R through GRP75, highlighting chaperone-mediated conformational coupling between the IP3R and the mitochondrial Ca^{2+} uptake machinery (Figure 1(b)). We have found that overexpression of GRP75 improved mitochondrial function after *in vivo* and *in vitro* cerebral ischemia [31, 83].

ER protein SIG1R, implicated in neuroprotection, carcinogenesis, and neuroplasticity, is a Ca^{2+}-sensitive and ligand-operated receptor chaperone at the MAM [62]. Normally, SIG1R forms a complex at the MAM with another ER chaperone GRP78/BiP (Figure 1(b)). Upon ER Ca^{2+} depletion or after ligand stimulation, SIG1R can dissociate from GRP78 and begin to chaperone conformationally unstable

IP3R (Figure 1(b)) to enhance Ca^{2+} signaling from the ER into mitochondria to increase the production of ATP in the cell through the tricarboxylic acid cycle in the mitochondria [74]. If stimulated by high concentrations of agonists or impacted by extreme ER stress, SIG1Rs translocate from the MAM to the plasma membrane to bind various ion channels, receptors, or kinases [63, 84–86]. An increase of SIG1R in cells counteracts ER stress, whereas decreased levels enhance apoptosis.

Recent evidence [41] indicates that GRP78, like SIG1R, may emerge as a novel interorganelle signaling modulator. As a multifunctional receptor on the cell surface after stress [46], GRP78 may be associated with many signaling pathways [47]. However, until now there has been no detailed research on the importance of GRP78 in MAM except as a binding partner of SIG1R (Figure 1(b)). GRP78 has been found to be one of the VDAC interactors (Table 1 in [60]) together with GRP75, although the authors of the paper never discuss it in the text [60]. We recently found that overexpressing GRP78 preserves respiratory activity and mitochondrial membrane potential, reduces free radical production, reduces mitochondria Ca^{2+} overload, and increases Ca^{2+} uptake capacity in isolated mitochondria after stress [22]. In order to follow GRP78 directly in response to ischemia-like stress, we created a fusion protein consisting of green fluorescent protein (eGFP) fused between the GRP78 N-terminal 18 amino acid ER signal peptide and the remainder of GRP78. We found that eGFP-GRP78 retargets to mitochondria within a short period of GD by fluorescence and immunoelectron microscopy (IEM) as well as Western blotting (Figure 2). The mitochondrial location of GRP78 is mainly on the inner membrane of mitochondria by IEM (Figure 2(c)). A prior report in 9L tumor cells has demonstrated relocalization of GRP78 to mitochondria after induction of ER stress by thapsigargin [87]. As in the case of translocation to the cell surface, cytoplasm, and nucleus after stress [41], the molecular mechanism underlying GRP78 translocation to mitochondria has not yet been elucidated.

The mitochondrial Ca^{2+} uniporter is the primary influx pathway for Ca^{2+} into respiring mitochondria, and hence is a key regulator of mitochondrial Ca^{2+}. Although the uniporter's biophysical properties have been studied extensively, its molecular composition remained elusive for more than 50 years. A very recent report has identified a 40-kDa protein which fulfills the requirements for being the long sought mitochondrial calcium uniporter [72]. Overexpression of MCU alone in one report did not give rise to a marked gain of Ca^{2+} uptake in HeLa cells indicating that additional components or chaperones may be limiting in some settings [88], though other investigators did observe increased Ca^{2+} with overexpression [72]. The MCU is thought to function as part of a complex including at least MICU1 [89]. Considering the fact that overexpressing GRP78 not only reduces mitochondria Ca^{2+} overload in intact cells, but also increases Ca^{2+} uptake capacity in isolated mitochondria [22], it is possible that translocated GRP78 interacts with the uniporter in some way on the IMM and regulates the mitochondrial Ca^{2+}. Future validation of the hypothesis depends on further development of molecular approaches to confirm this property of MCU and its relationship with GRP78.

In summary these findings together support a new emerging picture: chaperone machineries at both the ER and mitochondrion orchestrate the regulation of Ca^{2+} signaling between these two organelles and control bioenergetics, cell survival, and cell death decisions. In the brain, ER calcium release has been found to directly contribute to excitotoxicity, a neuronal death mechanism important both in acute and chronic neurodegenerative diseases. Better understanding the roles of chaperones and Ca^{2+} handling *in vivo* should in the future provide new therapeutic strategies to protect brain cells during ischemia.

Acknowledgments

This work was supported in part by NIH grants NS053898 and GM49831 to RGG. The authors would like to thank William Magruder for help preparing the paper. The authors have no conflicting financial interests.

References

[1] D. Lloyd-Jones, R. J. Adams, T. M. Brown et al., "Executive summary: heart disease and stroke statistics-2010 update: a report from the american heart association," *Circulation*, vol. 121, no. 7, pp. e46–e215, 2010.

[2] J. O. Blakeley and R. H. Llinas, "Thrombolytic therapy for acute ischemic stroke," *Journal of the Neurological Sciences*, vol. 261, no. 1-2, pp. 55–62, 2007.

[3] V. J. Marder, R. Jahan, T. Gruber, A. Goyal, and V. Arora, "Thrombolysis with plasmin: implications for stroke treatment," *Stroke*, vol. 41, no. 10, pp. S45–S49, 2010.

[4] B. K. Siesjo, E. Elmer, S. Janelidze et al., "Role and mechanisms of secondary mitochondrial failure," *Acta Neurochirurgica, Supplement*, vol. 1999, supplement 73, pp. 7–13, 1999.

[5] B. K. Siesjo, Y. B. Ouyang, T. Kristian et al., "Role of mitochondria in immediate and delayed reperfusion damage," in *Maturation Phenomenon in Cerebral Ischemia III*, U. Ito, Ed., Springer Verlag, Berlin, Germany, 1999.

[6] Y. B. Ouyang, T. Kristian, P. A. Li, and B. K. Siesjo, "Mitochondria, free radicals, and ischemic brain damage," in *Pharmacology of Cerebral Ischemia*, J. Krieglstein, Ed., Wissenschaftliche Verlagsgesellschaft, Stuttgart, Germany, 1998.

[7] T. Kristián, Y. Ouyang, and B. K. Siesjö, "Calcium-induced neuronal cell death in vivo and in vitro: are the pathophysiologic mechanisms different?" *Advances in Neurology*, vol. 71, pp. 107–118, 1996.

[8] A. Ruiz, C. Matute, and E. Alberdi, "Endoplasmic reticulum Ca^{2+} release through ryanodine and IP3 receptors contributes to neuronal excitotoxicity," *Cell Calcium*, vol. 46, no. 4, pp. 273–281, 2009.

[9] Y. B. Ouyang, S. Kuroda, T. Kristián, and B. K. Siesjö, "Release of mitochondrial aspartate aminotransferase (mAST) following transient focal cerebral ischemia suggests the opening of a mitochondrial permeability transition pore," *Neuroscience Research Communications*, vol. 20, no. 3, pp. 167–173, 1997.

[10] Y.-B. Ouyang, Y. Tan, M. Comb et al., "Survival- and death-promoting events after transient cerebral ischemia(colon): phosphorylation of Akt, release of cytochrome C, and activation of caspase- like proteases," *Journal of Cerebral Blood Flow and Metabolism*, vol. 19, no. 10, pp. 1126–1135, 1999.

[11] Y. Gouriou, N. Demaurex, P. Bijlenga, and U. de Marchi, "Mitochondrial calcium handling during ischemia-induced cell death in neurons," *Biochimie*, vol. 93, no. 12, pp. 2060–2067, 2011.

[12] Y. Kirichok, G. Krapivinsky, and D. E. Clapham, "The mitochondrial calcium uniporter is a highly selective ion channel," *Nature*, vol. 427, no. 6972, pp. 360–364, 2004.

[13] D. G. Nicholls and S. L. Budd, "Mitochondria and neuronal survival," *Physiological Reviews*, vol. 80, no. 1, pp. 315–360, 2000.

[14] P. Bernardi, "Mitochondrial transport of cations: channels, exchangers, and permeability transition," *Physiological Reviews*, vol. 79, no. 4, pp. 1127–1155, 1999.

[15] R. Rizzuto and T. Pozzan, "Microdomains of intracellular Ca^{2+}: molecular determinants and functional consequences," *Physiological Reviews*, vol. 86, no. 1, pp. 369–408, 2006.

[16] R. Rizzuto, M. Brini, M. Murgia, and T. Pozzan, "Microdomains with high Ca^{2+} close to IP3-sensitive channels that are sensed by neighboring mitochondria," *Science*, vol. 262, no. 5134, pp. 744–747, 1993.

[17] R. Rizzuto, P. Pinton, W. Carrington et al., "Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses," *Science*, vol. 280, no. 5370, pp. 1763–1766, 1998.

[18] T. Hayashi, R. Rizzuto, G. Hajnoczky, and T. P. Su, "MAM: more than just a housekeeper," *Trends in Cell Biology*, vol. 19, no. 2, pp. 81–88, 2009.

[19] Y.-B. Ouyang, Y. Lu, S. Yue et al., "MiR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo," *Neurobiology of Disease*, vol. 45, no. 1, pp. 555–563, 2012.

[20] G. X. Wang, G. R. Li, Y. D. Wang, T. S. Yang, and Y. B. Ouyang, "Characterization of neuronal cell death in normal and diabetic rats following exprimental focal cerebral ischemia," *Life Sciences*, vol. 69, no. 23, pp. 2801–2810, 2001.

[21] Y. B. Ouyang, L. A. Volobоueva, L. J. Xu, and R. G. Giffard, "Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia," *Journal of Neuroscience*, vol. 27, no. 16, pp. 4253–4260, 2007.

[22] Y. B. Ouyang, L. J. Xu, J. F. Emery, A. S. Lee, and R. G. Giffard, "Overexpressing GRP78 influences Ca^{2+} handling and function of mitochondria in astrocytes after ischemia-like stress," *Mitochondrion*, 2010.

[23] Y. B. Ouyang, L. J. Xu, Y. J. Sun, and R. G. Giffard, "Overexpression of inducible heat shock protein 70 and its mutants in astrocytes is associated with maintenance of mitochondrial physiology during glucose deprivation stress," *Cell Stress and Chaperones*, vol. 11, no. 2, Article ID csac. 2006.CSC-182R, pp. 180–186, 2006.

[24] Y. B. Ouyang, S. G. Carriedo, and R. G. Giffard, "Effect of Bcl-xL overexpression on reactive oxygen species, intracellular calcium, and mitochondrial membrane potential following injury in astrocytes," *Free Radical Biology and Medicine*, vol. 33, no. 4, pp. 544–551, 2002.

[25] Y. B. Ouyang and R. G. Giffard, "Bcl-xL maintains mitochondrial function in murine astrocytes deprived of glucose," *Journal of Cerebral Blood Flow and Metabolism*, vol. 23, no. 3, pp. 275–279, 2003.

[26] Y. B. Ouyang, L. Xu, and R. G. Giffard, "Geldanamycin treatment reduces delayed CA1 damage in mouse hippocampal organotypic cultures subjected to oxygen glucose deprivation," *Neuroscience Letters*, vol. 380, no. 3, pp. 229–233, 2005.

[27] B. Hoehn, T. M. Ringer, L. Xu et al., "Overexpression of HSP72 after induction of experimental stroke protects neurons from ischemic damage," *Journal of Cerebral Blood Flow and Metabolism*, vol. 21, no. 11, pp. 1303–1309, 2001.

[28] T. Kudo, S. Kanemoto, H. Hara et al., "A molecular chaperone inducer protects neurons from ER stress," *Cell Death and Differentiation*, vol. 15, no. 2, pp. 364–375, 2008.

[29] Y. Oida, H. Izuta, A. Oyagi et al., "Induction of BiP, an ER-resident protein, prevents the neuronal death induced by transient forebrain ischemia in gerbil," *Brain Research C*, vol. 1208, pp. 217–224, 2008.

[30] S. Rajdev, K. Hara, Y. Kokubo et al., "Mice overexpressing rat heat shock protein 70 are protected against cerebral infarction," *Annals of Neurology*, vol. 47, no. 6, pp. 782–791, 2000.

[31] L. Xu, L. A. Volobоueva, Y. Ouyang, J. F. Emery, and R. G. Giffard, "Overexpression of mitochondrial Hsp70/Hsp75 in rat brain protects mitochondria, reduces oxidative stress, and protects from focal ischemia," *Journal of Cerebral Blood Flow and Metabolism*, vol. 29, no. 2, pp. 365–374, 2009.

[32] L. Xu, X. Xiong, Y. Ouyang, G. Barreto, and R. Giffard, "Heat shock protein 72 (Hsp72) improves long term recovery after focal cerebral ischemia in mice," *Neuroscience Letters*, vol. 488, no. 3, pp. 279–282, 2011.

[33] H. Kinouchi, F. R. Sharp, M. P. Hill, J. Koistinaho, S. M. Sagar, and P. H. Chan, "Induction of 70-kDa heat shock protein and hsp70 mRNA following transient focal cerebral ischemia in the rat," *Journal of Cerebral Blood Flow and Metabolism*, vol. 13, no. 1, pp. 105–115, 1993.

[34] H. Kinouchi, F. R. Sharp, J. Koistinaho, K. Hicks, H. Kamii, and P. H. Chan, "Induction of heat shock hsp70 mRNA and HSP70 kDa protein in neurons in the "penumbra" following focal cerebral ischemia in the rat," *Brain Research*, vol. 619, no. 1-2, pp. 334–338, 1993.

[35] K. Vass, W. J. Welch, and T. S. Nowak, "Localization of 70-kDa stress protein induction in gerbil brain after ischemia," *Acta Neuropathologica*, vol. 77, no. 2, pp. 128–135, 1988.

[36] S. M. Massa, F. M. Longo, J. Zuo, S. Wang, J. Chen, and F. R. Sharp, "Cloning of rat grp75, an hsp70-family member, and its expression in normal and ischemic brain," *Journal of Neuroscience Research*, vol. 40, no. 6, pp. 807–819, 1995.

[37] A. L. Barabási and Z. N. Oltvai, "Network biology: understanding the cell's functional organization," *Nature Reviews Genetics*, vol. 5, no. 2, pp. 101–113, 2004.

[38] P. Csermely, "Strong links are important, but weak links stabilize them," *Trends in Biochemical Sciences*, vol. 29, no. 7, pp. 331–334, 2004.

[39] C. Soti, C. Pál, B. Papp, and P. Csermely, "Molecular chaperones as regulatory elements of cellular networks," *Current Opinion in Cell Biology*, vol. 17, no. 2, pp. 210–215, 2005.

[40] P. Csermely and L. Vigh, Eds., *Molecular Aspects of the Stress Reponse: Chaperones, Membranes and Networks*, Springer Science, New York, NY, USA, 2007.

[41] M. Ni, Y. Zhang, and A. S. Lee, "Beyond the endoplasmic reticulum: a typical GRP78 in cell viability, signalling and therapeutic targeting," *Biochemical Journal*, vol. 434, no. 2, pp. 181–188, 2011.

[42] T. Panaretakis, O. Kepp, U. Brockmeier et al., "Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death," *EMBO Journal*, vol. 28, no. 5, pp. 578–590, 2009.

[43] R. Tufi, T. Panaretakis, K. Bianchi et al., "Reduction of endoplasmic reticulum Ca^{2+} levels favors plasma membrane surface exposure of calreticulin," *Cell Death and Differentiation*, vol. 15, no. 2, pp. 274–282, 2008.

[44] K. T. Pfaffenbach and A. S. Lee, "The critical role of GRP78 in physiologic and pathologic stress," *Current Opinion in Cell Biology*, 2010.

[45] M. Wang, S. Wey, Y. Zhang, R. Ye, and A. S. Lee, "Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders," *Antioxidants and Redox Signaling*, vol. 11, no. 9, pp. 2307–2316, 2009.

[46] M. Gonzalez-Gronow, M. A. Selim, J. Papalas, and S. V. Pizzo, "GRP78: a multifunctional receptor on the cell surface," *Antioxidants and Redox Signaling*, vol. 11, no. 9, pp. 2299–2306, 2009.

[47] L. H. Zhang, X. L. Yang, X. Zhang, J. X. Cheng, and W. Zhang, "Association of elevated GRP78 expression with increased astrocytoma malignancy via Akt and ERK pathways," *Brain Research*, 2010.

[48] R. G. Giffard, R. Q. Han, J. F. Emery, M. Duan, and J. F. Pittet, "Regulation of apoptotic and inflammatory cell signaling in cerebral ischemia: the complex roles of heat shock protein 70," *Anesthesiology*, vol. 109, no. 2, pp. 339–348, 2008.

[49] D. E. Copeland and A. J. Dalton, "An association between mitochondria and the endoplasmic reticulum in cells of the pseudobranch gland of a teleost," *The Journal of biophysical and biochemical cytology*, vol. 5, no. 3, pp. 393–396, 1959.

[50] J. R. Ruby, R. F. Dyer, and R. G. Skalko, "Continuities between mitochondria and endoplasmic reticulum in the mammalian ovary," *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, vol. 97, no. 1, pp. 30–37, 1969.

[51] C. A. Mannella, M. Marko, P. Penczek, D. Barnard, and J. Frank, "The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope," *Microscopy Research and Technique*, vol. 27, no. 4, pp. 278–283, 1994.

[52] B. J. Soltys and R. S. Gupta, "Interrelationships of endoplasmic reticulum, mitochondria, intermediate filaments, and microtubules—a quadruple fluorescence labeling study," *Biochemistry and Cell Biology*, vol. 70, no. 10-11, pp. 1174–1186, 1992.

[53] O. Camici and L. Corazzi, "Phosphatidylserine translocation into brain mitochondria: involvement of a fusogenic protein associated with mitochondrial membranes," *Molecular and Cellular Biochemistry*, vol. 175, no. 1-2, pp. 71–80, 1997.

[54] J. E. Vance, "Phospholipid synthesis in a membrane fraction associated with mitochondria," *Journal of Biological Chemistry*, vol. 265, no. 13, pp. 7248–7256, 1990.

[55] G. Csordás, C. Renken, P. Várnai et al., "Structural and functional features and significance of the physical linkage between ER and mitochondria," *Journal of Cell Biology*, vol. 174, no. 7, pp. 915–921, 2006.

[56] R. Bravo, J. M. Vicencio, V. Parra et al., "Increased ER-mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress," *Journal of Cell Science*, vol. 124, no. 13, pp. 2143–2152, 2011.

[57] M. Chami, B. Oulès, G. Szabadkai, R. Tacine, R. Rizzuto, and P. Paterlini-Bréchot, "Role of SERCA1 Truncated Isoform in the Proapoptotic Calcium Transfer from ER to Mitochondria during ER Stress," *Molecular Cell*, vol. 32, no. 5, pp. 641–651, 2008.

[58] C. Giorgi, D. de Stefani, A. Bononi, R. Rizzuto, and P. Pinton, "Structural and functional link between the mitochondrial network and the endoplasmic reticulum," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 10, pp. 1817–1827, 2009.

[59] M. Lebiedzinska, G. Szabadkai, A. W. E. Jones, J. Duszynski, and M. R. Wieckowski, "Interactions between the endoplasmic reticulum, mitochondria, plasma membrane and other subcellular organelles," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 10, pp. 1805–1816, 2009.

[60] G. Szabadkai, K. Bianchi, P. Várnai et al., "Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca^{2+} channels," *Journal of Cell Biology*, vol. 175, no. 6, pp. 901–911, 2006.

[61] T. Hayashi and T. P. Su, "Regulating ankyrin dynamics: roles of sigma-1 receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 2, pp. 491–496, 2001.

[62] T. Hayashi and T. P. Su, "Sigma-1 receptor chaperones at the ER- mitochondrion interface regulate Ca^{2+} signaling and cell survival," *Cell*, vol. 131, no. 3, pp. 596–610, 2007.

[63] T. P. Su, T. Hayashi, T. Maurice, S. Buch, and A. E. Ruoho, "The sigma-1 receptor chaperone as an inter-organelle signaling modulator," *Trends in Pharmacological Sciences*, vol. 31, no. 12, pp. 557–566, 2010.

[64] Z. Wu and W. D. Bowen, "Role of sigma-1 receptor C-terminal segment in inositol 1,4,5-trisphosphate receptor activation: constitutive enhancement of calcium signaling in MCF-7 tumor cells," *Journal of Biological Chemistry*, vol. 283, no. 42, pp. 28198–28215, 2008.

[65] T. Higo, M. Hattori, T. Nakamura, T. Natsume, T. Michikawa, and K. Mikoshiba, "Subtype-specific and ER lumenal environmentdependent regulation of inositol 1,4,5-trisphosphate receptor type 1 by ERp44," *Cell*, vol. 120, no. 1, pp. 85–98, 2005.

[66] L. M. John, J. D. Lechleiter, and P. Camacho, "Differential modulation of SERCA2 isoforms by calreticulin," *Journal of Cell Biology*, vol. 142, no. 4, pp. 963–973, 1998.

[67] H. L. Roderick, J. D. Lechleiter, and P. Camacho, "Cytosolic phosphorylation of calnexin controls intracellular Ca^{2+} oscillations via an interaction with SERCA2b," *Journal of Cell Biology*, vol. 149, no. 6, pp. 1235–1247, 2000.

[68] T. Simmen, J. E. Aslan, A. D. Blagoveshchenskaya et al., "PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis," *EMBO Journal*, vol. 24, no. 4, pp. 717–729, 2005.

[69] M. R. Wieckowski, G. Szabadkai, M. Wasilewski, P. Pinton, J. Duszyński, and R. Rizzuto, "Overexpression of adenine nucleotide translocase reduces Ca^{2+} signal transmission between the ER and mitochondria," *Biochemical and Biophysical Research Communications*, vol. 348, no. 2, pp. 393–399, 2006.

[70] O. M. de Brito and L. Scorrano, "Mitofusin 2 tethers endoplasmic reticulum to mitochondria," *Nature*, vol. 456, no. 7222, pp. 605–610, 2008.

[71] M. R. M. R. Wieckowski, C. Giorgi, M. Lebiedzinska, J. Duszynski, and P. Pinton, "Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells," *Nature Protocols*, vol. 4, no. 11, pp. 1582–1590, 2009.

[72] D. de Stefani, A. Raffaello, E. Teardo, I. Szabó, and R. Rizzuto, "A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter," *Nature*, vol. 476, no. 7360, pp. 336–340, 2011.

[73] E. Rapizzi, P. Pinton, G. Szabadkai et al., "Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca^{2+} microdomains to mitochondria," *Journal of Cell Biology*, vol. 159, no. 4, pp. 613–624, 2002.

[74] C. Cárdenas, R. A. Miller, I. Smith et al., "Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca^{2+}

transfer to mitochondria," *Cell*, vol. 142, no. 2, pp. 270–283, 2010.

[75] M. Høyer-Hansen, L. Bastholm, P. Szyniarowski et al., "Control of Macroautophagy by Calcium, Calmodulin-Dependent Kinase Kinase-β, and Bcl-2," *Molecular Cell*, vol. 25, no. 2, pp. 193–205, 2007.

[76] J. M. Vicencio, C. Ortiz, A. Criollo et al., "The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1," *Cell Death and Differentiation*, vol. 16, no. 7, pp. 1006–1017, 2009.

[77] J. M. Vicencio, S. Lavandero, and G. Szabadkai, "Ca^{2+}, autophagy and protein degradation: thrown off balance in neurodegenerative disease," *Cell Calcium*, vol. 47, no. 2, pp. 112–121, 2010.

[78] A. Deniaud, O. Sharaf El Dein, E. Maillier et al., "Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis," *Oncogene*, vol. 27, no. 3, pp. 285–299, 2008.

[79] S. Marchi, A. Rimessi, C. Giorgi et al., "Akt kinase reducing endoplasmic reticulum Ca^{2+} release protects cells from Ca^{2+}-dependent apoptotic stimuli," *Biochemical and Biophysical Research Communications*, vol. 375, no. 4, pp. 501–505, 2008.

[80] R. Rizzuto, S. Marchi, M. Bonora et al., "Ca^{2+} transfer from the ER to mitochondria: when, how and why," *Biochimica et Biophysica Acta*, vol. 1787, no. 11, pp. 1342–1351, 2009.

[81] L. Scorrano, S. A. Oakes, J. T. Opferman et al., "BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis," *Science*, vol. 300, no. 5616, pp. 135–139, 2003.

[82] T. Szado, V. Vanderheyden, J. B. Parys et al., "Phosphorylation of inositol 1,4,5-trisphosphate receptors by protein kinase B/Akt inhibits Ca^{2+} release and apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 7, pp. 2427–2432, 2008.

[83] L. A. Volobоueva, M. Duan, Y. Ouyang, J. F. Emery, C. Stoy, and R. G. Giffard, "Overexpression of mitochondrial Hsp70/Hsp75 protects astrocytes against ischemic injury in vitro," *Journal of Cerebral Blood Flow and Metabolism*, vol. 28, no. 5, pp. 1009–1016, 2008.

[84] J. A. Fishback, M. J. Robson, Y. T. Xu, and R. R. Matsumoto, "Sigma receptors: potential targets for a new class of antidepressant drug," *Pharmacology and Therapeutics*, vol. 127, no. 3, pp. 271–282, 2010.

[85] D. Fontanilla, M. Johannessen, A. R. Hajipour, N. V. Cozzi, M. B. Jackson, and A. E. Ruoho, "The hallucinogen N,N-dimethyltryptamine (DMT) is an endogenous sigma-1 receptor regulator," *Science*, vol. 323, no. 5916, pp. 934–937, 2009.

[86] T. Maurice and T. P. Su, "The pharmacology of sigma-1 receptors," *Pharmacology and Therapeutics*, vol. 124, no. 2, pp. 195–206, 2009.

[87] F. C. Sun, S. Wei, C. W. Li, Y. S. Chang, C. C. Chao, and Y. K. Lai, "Localization of GRP78 to mitochondria under the unfolded protein response," *Biochemical Journal*, vol. 396, no. 1, pp. 31–39, 2006.

[88] J. M. Baughman, F. Perocchi, H. S. Girgis et al., "Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter," *Nature*, vol. 476, no. 7360, pp. 341–345, 2011.

[89] F. Perocchi, V. M. Gohil, H. S. Girgis et al., "MICU1 encodes a mitochondrial EF hand protein required for Ca^{2+} uptake," *Nature*, vol. 467, no. 7313, pp. 291–296, 2010.

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 821676, 10 pages
doi:10.1155/2012/821676

Review Article

Mitochondrial Dynamics: Functional Link with Apoptosis

Hidenori Otera and Katsuyoshi Mihara

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan

Correspondence should be addressed to Katsuyoshi Mihara, mihara@cell.med.kyushu-u.ac.jp

Received 30 November 2011; Accepted 17 January 2012

Academic Editor: Lina Ghibelli

Mitochondria participate in a variety of physiologic processes, such as ATP production, lipid metabolism, iron-sulfur cluster biogenesis, and calcium buffering. The morphology of mitochondria changes dynamically due to their frequent fusion and division in response to cellular conditions, and these dynamics are an important constituent of apoptosis. The discovery of large GTPase family proteins that regulate mitochondrial dynamics, together with novel insights into the role of mitochondrial fusion and fission in apoptosis, has provided important clues to understanding the molecular mechanisms of cellular apoptosis. In this paper, we briefly summarize current knowledge of the role of mitochondrial dynamics in apoptosis and cell pathophysiology in mammalian cells.

1. Introduction

Apoptosis, also called programmed cell death, is a crucial physiologic process in the development and homeostasis of multicellular organisms [1]. Perturbation of this vital process leads to a range of diseases, such as ischemia, cancer, neurodegeneration, and autoimmunity [2]. The mitochondrial outer membrane (MOM) serves to coordinate mitochondrial function with extra mitochondrial signaling and participates in the regulation of mitochondrial homeostasis. Mitochondria have a central role in the initiation of apoptosis triggered by intrinsic death signals such as DNA damage (the mitochondrial pathway) by releasing cytochrome *c* and other apoptogenic factors stored in the intermembrane space (IMS) into the cytoplasm [3, 4]. Cytochrome *c* complexed with Apaf-1 activates caspase 9, which leads to the activation of downstream caspases [5].

Mitochondrial morphology changes dynamically by continuous fission and fusion to form small units or interconnected mitochondrial networks, and the balance of these dynamic changes is essential for counteracting deleterious mitochondrial processes. Mitochondrial fusion allows for complementation of damaged mitochondrial DNA and other contents (e.g., lipids, proteins, or metabolites) with the components of healthy mitochondria, thus maintaining normal mitochondrial activity. Mitochondrial fission, on the other hand, plays an important role in the quality control of mitochondria, facilitating the removal of damaged mitochondria to maintain cellular homeostasis [6–10]. Compromise of this quality control system induces cell death, which results in various degenerative disorders [9]. Mitochondrial fission is also essential for the distribution of mitochondria in response to the local demand for ATP or calcium buffering [10]. In addition to these fundamental roles, the dynamic morphologic changes of mitochondria are closely associated with the initial process of apoptosis. The rate of fission increases markedly when cells become committed to apoptosis; apoptotic stimuli such as DNA injury, UV radiation, endoplasmic reticulum (ER) stress, oxygen radicals, or cytokine withdrawal trigger extensive mitochondrial fission accompanied by cristae disorganization and permeabilization of the mitochondrial outer membrane (MOMP), which in turn induces the release of IMS-stored proapoptotic factors, such as cytochrome *c*, to trigger the apoptosis program [11–15]. Although modulation of mitochondrial fusion and fission machineries is considered to influence the apoptotic response of the cells, it remains controversial whether fission is absolutely required for the progression of apoptosis. Nonetheless, perturbations of the mitochondrial dynamics cause cellular dysfunction, particularly of highly polarized cells such as

neurons, and neuronal synaptic loss and cell death in neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, and Huntington's disease), although the functional relation between the morphologic alterations and apoptosis is still insufficiently understood [16].

2. Regulation and Physiologic Significance of Mitochondrial Fusion and Fission

Three types of high-molecular-weight GTPase proteins regulate mitochondrial fusion and fission in mammals [10]. Outer membrane fusion involves two mitofusin proteins (Mfn1 and Mfn2; Fzo1 in yeast) located on the mitochondrial outer membrane (MOM) [17, 18]. An IMS-localized GTPase, OPA1 (Mgm1 in yeast), functions as a hetero-oligomeric complex of the larger size Opa1 (L-Opa1) and the smaller size Opa1 (S-Opa1) in fusion and cristae organization of the inner membrane (MIM) [19, 20]. The cytoplasmic dynamin-related GTPase protein Drp1 (Dnm1 in yeast) translocates to the foci of future mitochondrial fission sites and mediates mitochondrial fission [21–23]. Mitochondrial fission factor (Mff), and mitochondrial dynamics (Mid) 51/mitochondrial elongation factor 1 (MIEF1), and the variant Mid49 were recently reported to function as Drp1 receptors on the MOM [10, 24–27], although detailed mechanisms of Mff and MiD/MIEF1 proteins and their relation in Drp1-dependent mitochondrial fission remain to be clarified. The function of the mammalian homolog of yeast Fis1, which is thought to regulate mitochondrial fission as in yeast remains controversial [10, 24, 25].

Mutations in the mitochondrial fusion factors Mfn2 and OPA1 result in neurodegenerative disorders, such as Charcot-Marie-Tooth Neuropathy 2a and Dominant Optic Atrophy I, respectively [16, 19, 20]. Mitochondrial fusion factor knockout (KO) mice are lethal before embryonic day 12.5 (E12.5 for Mfn1 KO) or embryonic day 11.5 (E11.5 for Mfn2 KO), suggesting that both Mfn isoforms are essential for embryonic development in mammals [17]. Cells lacking both Mfn1 and Mfn2 exhibit severe cellular defects, including poor cell growth, heterogeneity of inner membrane potential, and decreased respiration, indicating that mitochondrial fusion has an essential role in maintaining functional mitochondria. Depletion of Mfn2 in neurons in mice leads to highly specific degeneration of Purkinje neurons [17]. The mitochondria in these mutant cells are fragmented and fail to distribute to the long and branched neurites, indicating that fusion also plays an important role in mitochondrial distribution in polarized cells. Depletion of both Mfn isoforms in skeletal muscle results in muscle atrophy [28]. Homozygous mutation of OPA1 in mice leads to embryonic lethality by E13.5 in mice, while heterozygous mutation causes a slow onset of degeneration in the optic nerves [29]. Pancreatic beta-cell-specific OPA1 KO mice have compromised glucose-stimulated insulin secretion and ATP production due to a defect in respiratory complex IV, suggesting that the function of OPA1 in the maintenance of the respiratory chain is physiologically relevant to beta cells [30].

The dynamin-related GTPase Drp1 localizes mainly in the cytoplasm and plays a central role in mitochondrial fission in mammals. It is composed of an N-terminal GTPase domain thought to provide mechanical force, a dynamin-like middle domain, a connecting domain ("B" in Figure 1), and a C-terminal GTPase effector domain (GED) (Figure 1). Compared with mitochondrial fusion, however, the *in vivo* function of Drp1-dependent mitochondrial fission is poorly understood. During mitochondrial fission, Drp1 existing as small oligomers in the cytoplasm assembles into larger oligomeric structures at the mitochondrial fission sites depending on GTP binding and then severs the mitochondrial membrane by GTP hydrolysis. A heterozygous, dominant-negative mutation of the Drp1 gene (A395D in the middle domain) was identified in a newborn girl with severe pleiotrophic defects, including abnormal brain development and optic atrophy, who died at 37 days of age (Figure 1) [31]. To elucidate the detailed physiologic roles of mitochondrial fission *in vivo*, we and another group generated tissue-specific Drp1 KO mice [32, 33]. Drp1 KO mice die at around E12.5 with developmental abnormalities, particularly in the forebrain. Neuron-specific Drp1 KO mice are born, but die within a day of birth due to neurodegeneration, although Drp1 is dispensable for the viability of mouse embryonic fibroblast (MEF) cells. In primary cultured neural Drp1 KO cells, enlarged mitochondrial clumps are sparsely distributed in the neurites and the synaptic structures are lost. These findings suggest that the Drp1-deficiency causes the abnormal distribution of fused and aggregated mitochondria in polarized cells and these spatiotemporal defects might inhibit the ATP supply and Ca^{2+} signaling, eventually preventing synapse formation. Similarly, Drp1-dependent mitochondrial fission is essential for immune synapse formation in T-cell receptor signaling [34]. A missense mutation in mouse Drp1 in the middle domain, which is essential for oligomerization (Python mice; C452F mutation), leads to cardiomyopathy [35]. The physiologic relevance of Drp1 in other tissues that might underlie various human diseases remains to be elucidated.

Drp1 activity is regulated by various posttranslational modifications and changes in these modifications are related to several disorders (Figure 1). In the early mitotic phase, Ser616 in human Drp1 is specifically phosphorylated by the Cdk1/cyclinB complex, which promotes mitochondrial fission to facilitate stochastic distribution of the mitochondria to daughter cells [36]. Under oxidative stress conditions, protein kinase Cδ mediates phosphorylation of Ser579 in human Drp1 isoform 3 (Ser616 in the human Drp1 isoform 1), leading to mitochondrial fission and impaired mitochondrial function, which contributes to hypertension-induced brain injury [37]. Phosphorylation at Ser637 in the GED domain of human Drp1 by cAMP-dependent protein kinase (PKA) stimulates Drp1 GTPase activity and releases Drp1 from the mitochondria by inhibiting oligomeric assembly on the membrane to promote mitochondrial network extension and cell viability [38]. This reaction is reversed by calcineurin-mediated dephosphorylation [39, 40]. Polyglutamine expansions in huntingtin protein, the cause of Huntington's disease, superactivate calcineurin through enhanced calcium levels, and increase mitochondrial recruitment of Drp1, leading to apoptosis due to mitochondrial fission, cristae

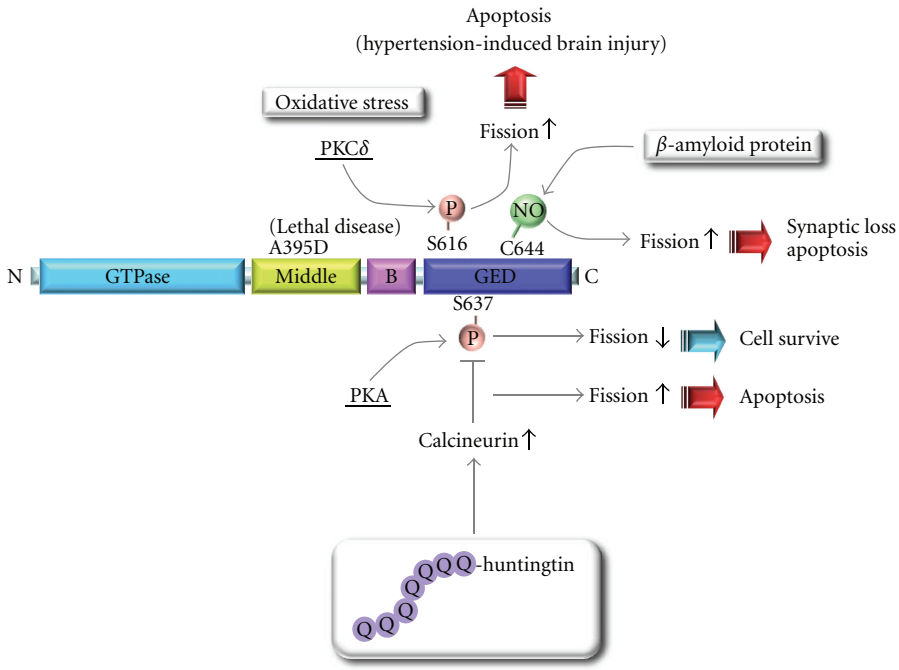


Figure 1: Domain structure of Drp1 and schematic view of the regulation of Drp1 by posttranslational modifications. Drp1 activity is regulated by various posttranslational modifications and changes in these modifications are related to several disorders. Under oxidative stress, protein kinase Cδ (PKCδ) phosphorylates Drp1 at Ser616 in the GED domain. Drp1 is recruited to mitochondria and stimulates mitochondrial fission, leading to apoptosis in hypertension-induced brain. Cyclic-AMP-dependent protein kinase (PKA) phosphorylates Drp1 at Ser637 in the GED domain. This reaction releases Drp1 from mitochondria to the cytosol, leading to mitochondrial elongation and suppression of apoptosis vulnerability of the cells. Calcineurin dephosphorylates Drp1 at Ser637 and promotes mitochondrial fragmentation and cell vulnerability to apoptosis. Polyglutamine expansion in huntingtin protein activates calcineurin and increases mitochondrial fragmentation and cell vulnerability to apoptosis. β-Amyloid protein increases S-nitrosylation of Drp1 at Cys644 in the GED domain to trigger mitochondrial fission by activating GTPase, thereby causing synaptic loss. All amino acid numbering is based on the human Drp1 splice variant 1 sequence.

disintegration, and cytochrome *c* release (Figures 1 and 3) [41]. Further, mutant huntingtin protein directly binds Drp1 and increases its GTPase activity, leading to mitochondrial fragmentation and defects in anterograde and retrograde mitochondrial transport and neuronal cell death [42, 43]. β-Amyloid protein, a key mediator of Alzheimer's disease, is reported to induce S-nitrosylation of Drp1 at Cys644 in the GED domain to trigger mitochondrial fission by activating GTPase and thereby causing synaptic loss (Figure 1) [44], although this model has been challenged [45]. Thus, mitochondrial morphologic balance shift toward fission makes cells susceptible to apoptosis and vice versa (Figure 1).

3. Regulation of Mitochondrial Apoptosis by Bcl-2 Family Proteins

Mitochondria play a central role in apoptotic initiation by providing proapoptotic factors that are involved in caspase activation, and chromosome condensation and fragmentation [15]. Multiple cellular pathways trigger apoptosis [46]: an extrinsic pathway that is initiated by the binding of a death ligands to the plasma-membrane-localized receptor, resulting in the rapid activation of caspases in the cytoplasm and an intrinsic (mitochondrial) pathway where mitochondria play a central role governed by pro- and antiapoptotic Bcl-2 family proteins. The extrinsic pathway does not directly involve the mitochondria, and activation of the initiator caspase (caspase-8) is mediated by the death-inducing signaling complex [47]. Conversely, the intrinsic pathway is initiated by the release of cytochrome *c* from the IMS accompanied by MOMP and cristae disorganization, which activates procaspase-9 through Apaf-1 [3, 4, 15]. Although the extrinsic and intrinsic pathways have long been considered independent from each other, evidence that caspase-8 also activates the intrinsic pathway has led to a more complex view of apoptosis in which crosstalk exists between the two pathways [48, 49].

The Bcl-2 family proteins regulate the MOM integrity and contribute to the release of proapoptotic factors from the IMS to the cytoplasm by MOMP [50, 51]. Irrespective of the precise mechanism, the antiapoptotic members of the Bcl-2 family tend to stabilize the barrier function of the MOM, whereas proapoptotic Bcl-2 family proteins such as Bax or Bak tend to antagonize such function and permeabilize the MOM. Upon apoptotic stimuli, caspase-8 activated by the death-inducing signaling complex cleaves the proapoptotic BH3-only protein Bid to the active truncated form (tBid).

The activated tBid is then recruited to the cardiolipin-rich region on the MOM by MTCH2, a half-type carrier superfamily protein [52]. tBid either interacts with antiapoptotic Bcl-2 family proteins such as Bcl-2 or Bcl-XL to inhibit their antiapoptotic functions [48, 49, 53], or triggers targeting and oligomerization of cytoplasmic Bax into the MOM and Opa1-dependent cristae disorganization, leading to the release of IMS-stored proapoptotic factors such as cytochrome *c* [54]. Bak, in contrast to Bax, is constitutively localized in the MOM associating with voltage-dependent anion channel 2 (VDAC2) as an inactive form in the ~400-kDa complex, and tBid activates Bak by releasing it from the complex, leading to MOMP [55–58]. Bcl-XL interacts with Bax on the mitochondrial surface and retrotranslocates it to the cytosol, thereby preventing Bax-induced MOMP in healthy cells [59]. As discussed below, mitochondrial membrane dynamics have an important role in the regulation of MOMP and apoptosis.

4. The Role of Mitochondrial Fission in Apoptosis

It is generally accepted that the mitochondrial network collapses into small spherical structures in response to apoptotic stimuli, and that proapoptotic and antiapoptotic Bcl-2 family member proteins play important roles in regulating mitochondrial morphology [15]. During apoptosis, cytosolic Bax is recruited to the MOM and colocalizes with Drp1 and Mfn2 at mitochondrial sites where fission subsequently occurs [60]. Bak, which initially localizes uniformly on the MOM, also coalesces into discrete foci at mitochondrial fission sites during apoptosis. tBid-triggered Bax/Bak activation correlates with a reduction in mitochondrial fusion, possibly through the inhibition of Mfn2 and eventually leads to mitochondrial fragmentation [61, 62]. Upon Bax activation, Drp1 stably associates with the MOM through Bax/Bak-dependent SUMO modifications of Drp1 [63]. This mitochondrial fragmentation is caspase independent and occurs concomitantly with MOMP, cristae disorganization, and subsequent cytochrome *c* release (Figure 2) [64, 65]. Increased mitochondrial fission in apoptotic cells apparently parallels the release of cytochrome *c*, and inhibition of fission by Drp1-RNA interference (RNAi) delays the release of cytochrome *c*, suggesting that the release of cytochrome *c* from the IMS is intimately involved in mitochondrial fission [65]. Consistent with these data, Mff depletion by RNAi results in extensive mitochondrial elongation, delayed cytochrome *c* release, and retardation of apoptosis [24, 66]. Similarly, MEFs from Drp1-KO mice exhibit a delay in cytochrome *c* release, caspase activation and nuclear DNA fragmentation [32, 33]. Notably, mitochondria with network structures that are subtly different from the structures observed prior to cytochrome *c* release are frequently detected in Drp1 KO cells after the release of cytochrome *c* and seem to undergo fragmentation in the advanced stage of apoptosis, suggesting that Drp1-independent mitochondrial fragmentation likely occurs late after the release of cytochrome *c* [32]. This suggests that Drp1-independent fission might participate in

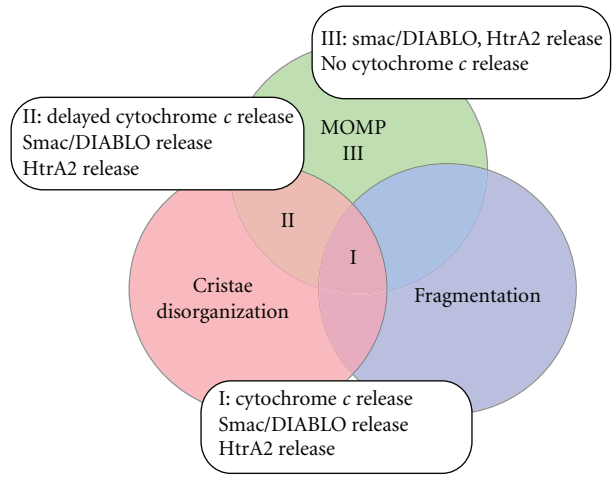


Figure 2: Relation between mitochondrial structural changes and the release of IMS-stored apoptogenic factors. During apoptosis in wild-type cells, mitochondrial fragmentation normally occurs concomitantly with MOMP, cristae disorganization, and subsequent release of the IMS-stored apoptogenic factors (e.g., cytochrome *c*, smac/DIABLO, HtrA2/omi). Mitochondrial fission facilitates these reactions (State I). In contrast, elongated but cristae-disrupted mitochondria (i.e., Drp1- and OPA1-double RNAi cells; Drp1-KO and OPA1 RNAi cells) exhibit a significant delay only in the cytochrome *c* release in response to apoptotic stimuli, because of the absence of mitochondrial fragmentation. Of note, however, MOM targeting and oligomeric assembly of Bax and the release of the IMS-soluble Smac/DIABLO normally proceed (State II). State III: presumed MOM permeabilized state in the absence of both cristae disorganization and mitochondrial fragmentation. MOMP: mitochondrial outer membrane permeabilization.

mitochondrial fission during apoptosis; for example, *Drosophila* PMI and its human homolog TMEM11 of MIM, both of which regulate mitochondrial morphology in a manner independent of Drp1 and Mfn [67]. Taken together, these findings indicate that the delay in cytochrome *c* release in these cells is relatively modest, suggesting that the Drp1-Mff system is dispensable, but facilitates the normal progression of apoptosis [32, 33]. Conversely, the inhibition of mitochondrial fragmentation by the activation of fusion-related proteins, such as Mfn1, Mfn2, or Opa1 antagonizes apoptosis progression.

A pharmacologic inhibitor of Drp1-GTPase, mdivi-1, inhibits tBid-dependent cytochrome *c* release from isolated mitochondria that are incapable of undergoing fission *in vitro*. These findings suggest either that mdivi-1 inhibits other Drp1 functions than mediating mitochondrial fission or that it inhibits molecules other than Drp1 that regulate cytochrome *c* release [68]. Martinou and coworkers recently demonstrated that Drp1 promotes the formation of a non-bilayer hemifission intermediate in which the activated and oligomerized Bax forms a hole, leading to MOMP [69].

Therefore, although mitochondrial fragmentation is indeed associated with apoptosis, excessive mitochondrial fragmentation can occur in a variety of conditions independently of apoptosis processes, such as that occurring upon exposure

to carbonyl cyanide m-chlorophenyl hydrazone (CCCP), uncoupling agents that disrupt the electrochemical potential of the MIM [70]. Thus, how Drp1 contributes to apoptosis is an important issue for future studies.

5. Cristae Remodeling and Apoptosis

Opa1, localizing in the inner membrane as a hetero-oligomeric complex of large and small size forms, regulates MIM fusion and is necessary for maintenance of the cristae junctions independently of mitochondrial fusion. The majority of cytochrome *c* is confined within the cristae folds and the complete release and mobilization of cytochrome *c* in the IMS require cristae remodeling or cristae-junction opening [71]. Opa1 depletion by RNAi leads to fragmented mitochondria with disrupted cristae structures and an increase in the sensitivity to the apoptotic stimuli [65, 72, 73]. Further, during early apoptosis, the Opa1 hetero-oligomer is disrupted, the cristae widen, and cytochrome *c* is released into the IMS. Of note, we demonstrated that Opa1 RNAi HeLa cells have disrupted cristae and efficient sensitivity to apoptosis, based on the cytochrome *c* release. In contrast, Opa1/Drp1 or Opa1/Mff double-RNAi cells have elongated but cristae-disrupted mitochondria, and exhibit a significant delay in the cytochrome *c* release in response to apoptotic stimuli (State II in Figure 2) [24]. Importantly, however, mitochondrial targeting of Bax and the release of the IMS-soluble Smac/DIABLO proceeded with the same kinetics as in the control cells. Similarly, in Drp1 KO MEFs or Drp1 RNAi HeLa cells, Bax/Bak-mediated MOMP occurs independently of Drp1 and is separable from cytochrome *c* release. These results suggest that cristae disorganization and mitochondrial fission as well as MOMP (State I in Figure 2) are essentially required for efficient cytochrome *c* release and each can limit the initial apoptosis progression. In contrast to these observations, detailed analysis with transmission electron microscopy and three-dimensional electron microscope tomography revealed that neither cristae reorganization nor cristae-junction opening is required for the complete release of cytochrome *c* [74]. Thus, the requirement of Opa1-dependent cristae remodeling for cytochrome *c* release remains to be reconciled.

6. Mitochondrial Morphologic Responses in Cell Survival

Many lines of evidence indicate that the efficiency of oxidative phosphorylation by the mitochondrial electron transport chain is affected by the degree of mitochondrial connectivity; a highly connected mitochondrial network correlates with increased ATP production efficiency. Mitochondria hyperfuse and form a highly interconnected network when cells are exposed to modest levels of stress (e.g., UV irradiation, actinomycin D treatment), named stress-induced mitochondrial hyperfusion (SIMH) [75]. SIMH depends on Mfn1, Opa1, and the MIM protein SLP-2, and delays the activation of Bax and MOMP similar to the beneficial effect of mitofusin overexpression. This seems to be a counterstress action of the cells that is necessary for survival by increased mitochondrial ATP production. Under nutrient starvation, mitochondrial fission is repressed in response to PKA-dependent Drp1 phosphorylation of Drp1 Ser637 due to increased cAMP levels (Figure 1), resulting in elongation of the mitochondria with a higher density of cristae and a capacity for efficient ATP production. This response protects mitochondria from autophagosomal degradation and sustains cell viability [76, 77]. Taken together, enhanced fusion in the mitochondrial fusion/fission balance promotes cell survival. Alternatively, dysfunctional or damaged mitochondria are selectively eliminated by autophagic degradation (termed mitophagy): the process essential for maintaining mitochondrial quality and cell function. For example, accumulation of a causal gene product of Parkinson's disease, PTEN-induced mitochondrial protein kinase 1 (PINK1) on depolarized mitochondria facilitates recruitment of cytoplasmic Parkin, an E3 ubiquitin ligase, to mitochondria to initiate mitophagy [78–80]. The Parkin-PINK1 system thus monitors damaged mitochondria, and dysfunction of this mechanism is a possible cause of inflammation or Parkinson's disease [81–86]. As it is thought that mitochondrial fission is related to the progression of mitophagy, inhibition of mitochondrial fission by the dominant negative mutant of Drp1 or specific inhibitor of Drp1-GTPase mdivi-1 compromises Parkin-PINK1-dependent mitophagy [83]. Together, mitochondrial fusion and fission are more likely to be involved in mitochondrial quality control in healthy cells. A recent report demonstrated that the A-kinase anchoring protein 1 (AKAP1) localized on the MOM is involved in this reaction. Thus, the PKA/AKAP1 complex-calcineurin system regulates mitochondrial morphology and cell viability by controlling the translocation of Drp1 to the mitochondria (Figure 1) [87].

7. Communication between the Mitochondria and ER in Apoptosis

In yeast, the ER mitochondria encounter structure (ERMES comprising cytosolic Mdm12, mitochondrial Mdm10, Mdm34, and Gem1, and ER membrane protein Mmm1), identified by synthetic biology and biochemical approaches [88–90], are involved in phospholipid transport. A similar structure is expected to exist in mammalian cells; a mammalian homolog of yeast Gem1, MIRO, is detected in the proximity of the ER-mitochondria [90]. Ca^{2+} is a key regulator of not only cell survival but also cell death in response to various apoptotic stimuli (Figure 3). The mitochondria and ER have close contacts that are physiologically important for the transfer of Ca^{2+}, lipids, and metabolites, and therefore, for the regulation of mitochondrial metabolism and other complex cellular processes including apoptosis. Mfn2 and its regulator Trichoplein/mitostatin localizing on the mitochondria-associated ER membranes (MAM) is involved in tethering mitochondria and ER through hetero- or homotypic interactions with mitochondrial Mfn1 and Mfn2, thereby regulating Ca^{2+} transfer from the ER to the mitochondria [91, 92]. Interactions between the mitochondria and ER are also supported by the finding that the ER

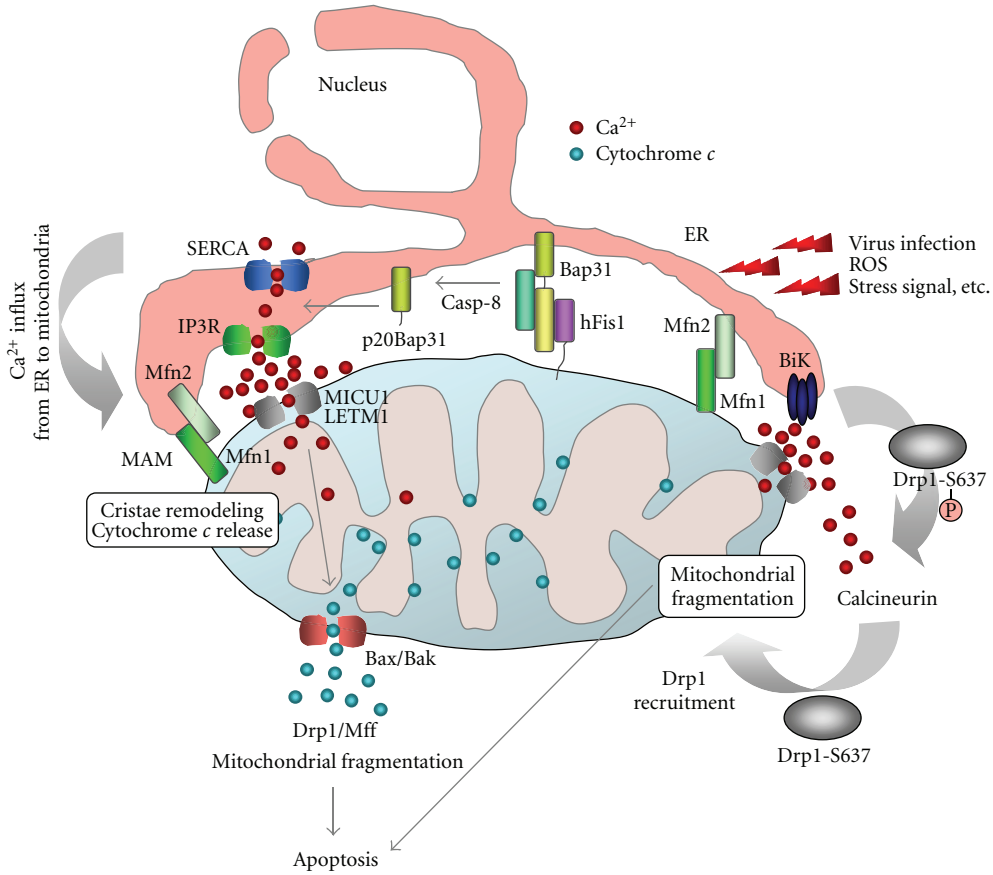


Figure 3: Hypothetical models of the role of contacts between mitochondria and ER in apoptosis. The hFis1/Bap31 platform transmits the mitochondrial stress signal to the ER via the activation of procaspase-8. The cytosolic region of the ER integral membrane protein Bap31 is cleaved by activated caspase-8 to generate proapoptotic p20Bap31, which causes rapid transmission of ER calcium signals to the mitochondria via the IP3 receptor. At close ER-mitochondria contact sites, mitochondria takes up calcium into the matrix via the mitochondrial calcium channels MICU1 or LETM1. The massive influx of calcium leads to mitochondrial fission, cristae remodeling, and cytochrome *c* release. Mfn2 is enriched in the mitochondria-associated membranes (MAM) of the endoplasmic reticulum (ER), where it interacts with Mfn1 and Mfn2 on the mitochondria to form interorganellar bridges. Upon apoptosis signal, a BH3-only member of the Bcl-2 family, Bik, induces Ca^{2+} release from the ER and, in turn, induces Drp1 recruitment to the mitochondria and their fragmentation and cristae remodeling. SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase. MICU1, mitochondrial calcium uptake 1. LETM1, leucine zipper/EF hand-containing transmembrane 1.

can elicit mitochondrial apoptosis. ER targeting of Bik, a BH3-only member of the Bcl-2 family, induces Ca^{2+} release from the ER and its concomitant uptake by the mitochondria, which in turn induces Drp1 recruitment to the mitochondria and their fragmentation and cristae remodeling (Figure 3) [93]. Mammalian mitochondrial Fis1 is an ortholog of yeast Fis1 thought to be involved in recruitment of Drp1 to the mitochondria as in yeast [94]. Although recent experiments revealed that Fis1 is not necessary for Drp1-dependent mitochondrial fission in mammals [10, 24, 25], it might have another important role. In this context, overexpression of hFis1 (for human Fis1) induces mitochondrial fragmentation concomitant with Bax/Bak-dependent release of cytochrome *c* into the cytosol [95]. Of note, hFis1 does not directly activate Bax and Bak, but induces ER Ca^{2+}-dependent mitochondrial dysfunction, leading to mitochondrial apoptosis [96]. Interestingly, Iwasawa et al. recently demonstrated that hFis1 localized to the MAM transmits an apoptosis signal from the ER to mitochondria by interacting with Bap31 at the ER to form a platform for the recruitment of the initiator procaspase-8. Apoptotic signals induce cleavage of Bap31 into p20Bap31, which causes the rapid transmission of ER calcium to the mitochondria through inositol triphosphate receptors at the ER-mitochondria junction [97]. Ca^{2+} influx into the mitochondria stimulates Drp1-dependent mitochondrial fission and cytochrome *c* release. Thus, the hFis1-Bap31 complex, bridging the mitochondria and ER, functions as a platform to activate the initiator procaspase in apoptosis signaling (Figure 3). Recently Green and collaborators provided evidence that contacts between mitochondria and other organelles such as ER are involved in regulation of the levels of sphingolipid metabolites that are required for Bax/Bak activation [98]. Distinct from the substrate or ion transfer function of the ER-mitochondria

contact, Friedman et al. [99] recently demonstrated that mitochondrial fission occurs at contact regions between the mitochondria and the ER. At the contacts, the ER wraps around the mitochondria to form constrictions, where Drp1 and Mff accumulate and facilitate mitochondrial fission. Interestingly, ER-localized Mfn2 which was shown to be involved in tethering mitochondria and ER [91] is not involved in this reaction [99].

8. Conclusions

Although key proteins regulating mammalian mitochondrial dynamics have been identified during the past decade, molecular mechanisms, their coordination, and physiologic functions in distinct tissues are poorly understood, especially in fission reaction: the mechanism of recruitment of Drp1 to fission foci, functional relation between Mff and Mid proteins in the Drp1 recruitment, and regulation of the foci assembly and disassembly. Furthermore, involvement of Fis1 in the regulation of mitochondrial dynamics and its physiologic function remain to be investigated. It is generally agreed, but not completely admitted, that mitochondrial fission/fusion dynamics are related to apoptosis and a balance sift toward fission enhances apoptotic susceptibility of essentially all types of cells. Accumulating evidence, however, suggest that Bax/Bak-dependent MOMP and cytochrome *c* mobilization within IMS after cristae disintegration are essential for cytochrome *c* release and Drp1 facilitates these processes. The mechanisms coordinating these reactions and the effect of Bcl-2 family proteins on mitochondrial fission and fusion machineries remain to be analyzed at the molecular level. Recent studies have revealed that the ER-mitochondria contacts (MAM structures) are involved in the regulation of mitochondrial energy, lipid metabolism, calcium signaling, and even in mitochondrial fission. Identification of additional structural components, regulation of assembly of these structures, and relation between various complexes will reveal novel aspects of cell physiology regulation through communication between mitochondria and ER.

In conclusion, mitochondrial fusion and fission machineries have crucial function in regulating cell physiology, and investigation of the relation between mitochondrial dynamics and their physiologic function will provide exciting breakthrough in cell biology and various disorders.

Acknowledgment

This work has been supported by and Culture of Japan to Katsuyoshi Mihara and grants from the Ministry of Education, Science, and Culture of Japan to Katsuyoshi Mihara and Hidenori Otera, and from Naito foundation and Sumitomo foundation to Hidenori Otera.

References

[1] E. H. Baehrecke, "How death shapes life during development," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 10, pp. 779–787, 2002.

[2] P. Saikumar, Z. Dong, V. Mikhailov, M. Denton, J. M. Weinberg, and M. A. Venkatachalam, "Apoptosis: definition, mechanisms, and relevance to disease," *American Journal of Medicine*, vol. 107, no. 5, pp. 489–506, 1999.

[3] J. C. Martinou and D. R. Green, "Breaking the mitochondrial barrier," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 1, pp. 63–67, 2001.

[4] N. Zamzami and G. Kroemer, "The mitochondrion in apoptosis: how Pandora's box opens," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 1, pp. 67–71, 2001.

[5] P. A. Parone, D. James, and J. C. Martinou, "Mitochondria: regulating the inevitable," *Biochimie*, vol. 84, no. 2-3, pp. 105–111, 2002.

[6] G. Twig, A. Elorza, A. J. A. Molina et al., "Fission and selective fusion govern mitochondrial segregation and elimination by autophagy," *The EMBO Journal*, vol. 27, no. 2, pp. 433–446, 2008.

[7] G. Twig, B. Hyde, and O. S. Shirihai, "Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view," *Biochimica et Biophysica Acta*, vol. 1777, no. 9, pp. 1092–1097, 2008.

[8] R. J. Youle and D. P. Narendra, "Mechanisms of mitophagy," *Nature Reviews Molecular Cell Biology*, vol. 12, no. 1, pp. 9–14, 2011.

[9] K. Okamoto and N. Kondo-Okamoto, "Mitochondria and autophagy: crinical interplay between the two homeostats," *Biochimica et Biophysica Acta*. In press.

[10] H. Otera and K. Mihara, "Molecular mechanisms and physiologic functions of mitochondrial dynamics," *Journal of Biochemistry*, vol. 149, no. 3, pp. 241–251, 2011.

[11] R. J. Youle and M. Karbowski, "Mitochondrial fission in apoptosis," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 8, pp. 657–663, 2005.

[12] P. A. Parone and J. C. Martinou, "Mitochondrial fssion and apoptosis: an ongoing trial," *Biochimica et Biophysica Acta*, vol. 1763, no. 5-6, pp. 522–530, 2006.

[13] D. Arnoult, "Mitochondrial fragmentation in apoptosis," *Trends in Cell Biology*, vol. 17, no. 1, pp. 6–12, 2007.

[14] D. F. Suen, K. L. Norris, and R. J. Youle, "Mitochondrial dynamics and apoptosis," *Genes and Development*, vol. 22, no. 12, pp. 1577–1590, 2008.

[15] J. C. Martinou and R. Youle, "Mitochondria in apoptosis: bcl-2 family members and mitochondrial dynamics," *Developmental Cell*, vol. 21, no. 1, pp. 92–101, 2011.

[16] H. Chen and D. C. Chan, "Mitochondrial dynamics—fusion, fission, movement, and mitophagy—in neurodegenerative diseases," *Human Molecular Genetics*, vol. 18, no. 2, pp. R169–R176, 2009.

[17] H. Chen, S. A. Detmer, A. J. Ewald, E. E. Griffin, S. E. Fraser, and D. C. Chan, "Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development," *Journal of Cell Biology*, vol. 160, no. 2, pp. 189–200, 2003.

[18] A. Santel and M. T. Fuller, "Control of mitochondrial morphology by a human mitofusin," *Journal of Cell Science*, vol. 114, no. 5, pp. 867–874, 2001.

[19] C. Alexander, M. Votruba, U. E. Pesch et al., "OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28," *Nature Genetics*, vol. 26, no. 2, pp. 211–215, 2000.

[20] C. Delettre, G. Lenaers, J. M. Griffoin et al., "Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy," *Nature Genetics*, vol. 26, no. 2, pp. 207–210, 2000.

[21] E. Smirnova, L. Griparic, D. L. Shurland, and A. M. Van der Bliek, "Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells," *Molecular Biology of the Cell*, vol. 12, no. 8, pp. 2245–2256, 2001.

[22] Y. Yoon, K. R. Pitts, and M. A. McNiven, "Mammalian dynamin-like protein DLP1 tubulates membranes," *Molecular Biology of the Cell*, vol. 12, no. 9, pp. 2894–2905, 2001.

[23] E. Ingerman, E. M. Perkins, M. Marino et al., "Dnm1 forms spirals that are structurally tailored to fit mitochondria," *Journal of Cell Biology*, vol. 170, no. 7, pp. 1021–1027, 2005.

[24] H. Otera, C. Wang, M. M. Cleland et al., "Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells," *Journal of Cell Biology*, vol. 191, no. 6, pp. 1141–1158, 2010.

[25] H. Otera and K. Mihara, "Discovery of membrane receptor for mitochondrial fission GTPase Drp1," *Small GTPases*, vol. 3, pp. 167–172, 2011.

[26] C. S. Palmer, L. D. Osellame, D. Laine, O. S. Koutsopoulos, A. E. Frazier, and M. T. Ryan, "MiD49 and MiD51, new components of the mitochondrial fission machinery," *EMBO Reports*, vol. 12, no. 6, pp. 565–573, 2011.

[27] J. Zhao, T. Liu, S. Jin et al., "Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission," *The EMBO Journal*, vol. 30, no. 14, pp. 2762–2778, 2011.

[28] H. Chen, M. Vermulst, Y. E. Wang et al., "Mitochondrial fusion is required for mtdna stability in skeletal muscle and tolerance of mtDNA mutations," *Cell*, vol. 141, no. 2, pp. 280–289, 2010.

[29] V. J. Davies, A. J. Hollins, M. J. Piechota et al., "Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function," *Human Molecular Genetics*, vol. 16, no. 11, pp. 1307–1318, 2007.

[30] Z. Zhang, N. Wakabayashi, J. Wakabayashi et al., "The dynamin-related GTPase Opa1 is required for glucose-stimulated ATP production in pancreatic beta cells," *Molecular Biology of the Cell*, vol. 22, no. 13, pp. 2235–2245, 2011.

[31] H. R. Waterham, J. Koster, C. W. Van Roermund, P. A. Mooyer, R. J. Wanders, and J. V. Leonard, "A lethal defect of mitochondrial and peroxisomal fission," *The New England Journal of Medicine*, vol. 356, no. 17, pp. 1736–1741, 2007.

[32] N. Ishihara, M. Nomura, A. Jofuku et al., "Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice," *Nature Cell Biology*, vol. 11, no. 8, pp. 958–966, 2009.

[33] J. Wakabayashi, Z. Zhang, N. Wakabayashi et al., "The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice," *Journal of Cell Biology*, vol. 186, no. 6, pp. 805–816, 2009.

[34] F. Baixauli, N. B. Martín-Cófreces, G. Morlino et al., "The mitochondrial fission factor dynamin-related protein 1 modulates T-cell receptor signalling at the immune synapse," *The EMBO Journal*, vol. 30, no. 7, pp. 1238–1250, 2011.

[35] H. Ashrafian, L. Docherty, V. Leo et al., "A mutation in the mitochondrial fission gene Dnm1l leads to cardiomyopathy," *Plos genetics*, vol. 6, no. 6, p. e1001000, 2010.

[36] N. Taguchi, N. Ishihara, A. Jofuku, T. Oka, and K. Mihara, "Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission," *Journal of Biological Chemistry*, vol. 282, no. 15, pp. 11521–11529, 2007.

[37] X. Qi, M. H. Disatnik, N. Shen, R. A. Sobel, and D. Mochly-Rosen, "Aberrant mitochondrial fission in neurons induced by protein kinase Cδ under oxidative stress conditions *in vivo*," *Molecular Biology of the Cell*, vol. 22, no. 2, pp. 256–265, 2011.

[38] C. R. Chang and C. Blackstone, "Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology," *Journal of Biological Chemistry*, vol. 282, no. 30, pp. 21583–21587, 2007.

[39] J. T. Cribbs and S. Strack, "Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death," *EMBO Reports*, vol. 8, no. 10, pp. 939–944, 2007.

[40] G. M. Cereghetti, A. Stangherlin, M. de Brito et al., "Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 41, pp. 15803–15808, 2008.

[41] V. Costa, M. Giacomello, R. Hudec et al., "Mitochondrial fission and cristae disruption increase the response of cell models of Huntington's disease to apoptotic stimuli," *EMBO Molecular Medicine*, vol. 2, no. 12, pp. 490–503, 2010.

[42] W. Song, J. Chen, A. Petrilli et al., "Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity," *Nature Medicine*, vol. 17, no. 3, pp. 377–383, 2011.

[43] U. Shirendeb, M. J. Calkins, M. Manczak et al., "Mutant Huntingtin's interaction with mitochondrial protein Drp1 impairs mitochondrial biogenesis and causes defective axonal transport and synaptic degeneration in Huntington's disease," *Human Molecular Genetics*, vol. 21, no. 2, pp. 406–420, 2012.

[44] D. H. Cho, T. Nakamura, J. Fang et al., "β-Amyloid-related mitochondrial fission and neuronal injury," *Science*, vol. 324, no. 5923, pp. 102–105, 2009.

[45] B. Bossy, A. Petrilli, E. Klinglmayr et al., "S-nitrosylation of DRP1 does not affect enzymatic activity and is not specific to Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 20, no. 2, pp. 513–526, 2010.

[46] Z. Jin and W. S. El-Deiry, "Overview of cell death signaling pathways," *Cancer Biology and Therapy*, vol. 4, no. 2, pp. 139–163, 2005.

[47] I. Lavrik, A. Golks, and P. H. Krammer, "Death receptor signaling," *Journal of Cell Science*, vol. 118, no. 2, pp. 265–267, 2005.

[48] H. Li, H. Zhu, C. J. Xu, and J. Yuan, "Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis," *Cell*, vol. 94, no. 4, pp. 491–501, 1998.

[49] X. Luo, I. Budihardjo, H. Zou, C. Slaughter, and X. Wang, "Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors," *Cell*, vol. 94, no. 4, pp. 481–490, 1998.

[50] A. Schinzel, T. Kaufmann, and C. Borner, "Bcl-2 family members: integrators of survival and death signals in physiology and pathology," *Biochimica et Biophysica Acta*, vol. 1644, no. 2-3, pp. 95–105, 2004.

[51] B. Leber, J. Lin, and D. W. Andrews, "Embedded together: the life and death consequences of interaction of the Bcl-2 family with membranes," *Apoptosis*, vol. 12, no. 5, pp. 897–911, 2007.

[52] Y. Zaltsman, L. Shachnai, N. Yivgi-Ohana et al., "MTCH2/MIMP is a major facilitator of tBID recruitment to mitochondria," *Nature Cell Biology*, vol. 12, no. 6, pp. 553–562, 2010.

[53] T. Moldoveanu, Q. Liu, A. Tocilj, M. Watson, G. Shore, and K. Gehring, "The X-ray structure of a BAK homodimer reveals an inhibitory zinc binding site," *Molecular Cell*, vol. 24, no. 5, pp. 677–688, 2006.

[54] A. P. Gilmore, A. D. Metcalfe, L. H. Romer, and C. H. Streuli, "Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization," *Journal of Cell Biology*, vol. 149, no. 2, pp. 431–445, 2000.

[55] E. H. Cheng, T. V. Sheiko, J. K. Fisher, W. J. Craigen, and S. J. Korsmeyer, "VDAC2 inhibits BAK activation and mitochondrial apoptosis," *Science*, vol. 301, no. 5632, pp. 513–517, 2003.

[56] M. Lazarou, D. Stojanovski, A. E. Frazier et al., "Inhibition of Bak activation by VDAC2 is dependent on the Bak transmembrane anchor," *Journal of Biological Chemistry*, vol. 285, no. 47, pp. 36876–36883, 2010.

[57] S. S. Roy, A. M. Ehrlich, W. J. Craigen, and G. Hajnóczky, "VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting BAK to the mitochondria," *EMBO Reports*, vol. 10, no. 12, pp. 1341–1347, 2009.

[58] K. Setoguchi, H. Otera, and K. Mihara, "Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins," *The EMBO Journal*, vol. 25, no. 24, pp. 5635–5647, 2006.

[59] F. Edlich, S. Banerjee, M. Suzuki et al., "Bcl-x(L) retrotranslocates Bax from the mitochondria into the cytosol," *Cell*, vol. 145, pp. 104–116, 2011.

[60] M. Karbowski, Y. J. Lee, B. Gaume et al., "Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis," *Journal of Cell Biology*, vol. 159, no. 6, pp. 931–938, 2002.

[61] M. Karbowski, D. Arnoult, H. Chen, D. C. Chan, C. L. Smith, and R. J. Youle, "Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis," *Journal of Cell Biology*, vol. 164, no. 4, pp. 493–499, 2004.

[62] C. Brooks, Q. Wei, L. Feng et al., "Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 28, pp. 11649–11654, 2007.

[63] S. Wasiak, R. Zunino, and H. M. McBride, "Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death," *Journal of Cell Biology*, vol. 177, no. 3, pp. 439–450, 2007.

[64] S. Frank, B. Gaume, E. S. Bergmann-Leitner et al., "The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis," *Developmental Cell*, vol. 1, no. 4, pp. 515–525, 2001.

[65] Y. J. Lee, S. Y. Jeong, M. Karbowski, C. L. Smith, and R. J. Youle, "Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, Opa1 in apoptosis," *Molecular Biology of the Cell*, vol. 15, no. 11, pp. 5001–5011, 2004.

[66] S. Gandre-Babbe and A. M. van der Bliek, "The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells," *Molecular Biology of the Cell*, vol. 19, no. 6, pp. 2402–2412, 2008.

[67] T. Rival, M. MacChi, L. Arnauné-Pelloquin et al., "Inner-membrane proteins PMI/TMEM11 regulate mitochondrial morphogenesis independently of the DRP1/MFN fission/fusion pathways," *EMBO Reports*, vol. 12, no. 3, pp. 223–230, 2011.

[68] A. Cassidy-Stone, J. E. Chipuk, E. Ingerman et al., "Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization," *Developmental Cell*, vol. 14, no. 2, pp. 193–204, 2008.

[69] S. Montessuit, S. P. Somasekharan, O. Terrones et al., "Membrane remodeling induced by the dynamin-related protein Drp1 stimulates bax oligomerization," *Cell*, vol. 142, no. 6, pp. 889–901, 2010.

[70] O. Y. Pletjushkina, K. G. Lyamzaev, E. N. Popova et al., "Effect of oxidative stress on dynamics of mitochondrial reticulum," *Biochimica et Biophysica Acta*, vol. 1757, no. 5-6, pp. 518–524, 2006.

[71] L. Scorrano, M. Ashiya, K. Buttle et al., "A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis," *Developmental Cell*, vol. 2, no. 1, pp. 55–67, 2002.

[72] A. Olichon, L. Baricault, N. Gas et al., "Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis," *Journal of Biological Chemistry*, vol. 278, no. 10, pp. 7743–7746, 2003.

[73] C. Frezza, S. Cipolat, O. Martins de Brito et al., "OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion," *Cell*, vol. 126, no. 1, pp. 177–189, 2006.

[74] M. G. Sun, J. Williams, C. Munoz-Pinedo et al., "Correlated three-dimensional light and electron microscopy reveals transformation of mitochondria during apoptosis," *Nature Cell Biology*, vol. 9, no. 9, pp. 1057–1065, 2007.

[75] D. Tondera, S. Grandemange, A. Jourdain et al., "SlP-2 is required for stress-induced mitochondrial hyperfusion," *The EMBO Journal*, vol. 28, no. 11, pp. 1589–1600, 2009.

[76] A. S. Ramold, B. Kostelecky, N. Elia, and J. Lippincott-Schwartz, "Tubular network formation protects mitochondria from autophagosomal deradation during nutrient starvation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 25, pp. 10190–10195, 2011.

[77] L. C. Gomes, G. D. Benedetto, and L. Scorrano, "During autophagy mitochondria elongate, are spared from degradation and sustain cell viability," *Nature Cell Biology*, vol. 13, no. 5, pp. 589–598, 2011.

[78] D. Narendra, A. Tanaka, D. F. Suen, and R. J. Youle, "Parkin is recruited selectively to impaired mitochondria and promotes their autophagy," *Journal of Cell Biology*, vol. 183, no. 5, pp. 795–803, 2008.

[79] D. P. Narendra, S. M. Jin, A. Tanaka et al., "PINK1 is selectively stabilized on impaired mitochondria to activate Parkin," *Plos Biology*, vol. 8, no. 1, Article ID e1000298, 2010.

[80] N. Matsuda, S. Sato, K. Shiba et al., "PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy," *Journal of Cell Biology*, vol. 189, no. 2, pp. 211–221, 2010.

[81] E. Deas, H. Plun-Faureau, and N. W. Wood, "PINK1 function in health and disease," *EMBO Molecular Medicine*, vol. 1, no. 3, pp. 152–165, 2009.

[82] J. C. Fitzgerald and H. Plun-Favreau, "Emerging pathways in genetic Parkinson's disease: autosomal-recessive genes in Parkinson's disease—a common pathway?" *The FEBS Journal*, vol. 275, no. 23, pp. 5758–5766, 2008.

[83] R. J. Youle and D. P. Narendra, "Mechanisms of mitophagy," *Nature Reviews Molecular Cell Biology*, vol. 12, no. 1, pp. 9–14, 2011.

[84] D. C. Rubinsztein, G. Mariño, and G. Kroemer, "Autophagy and aging," *Cell*, vol. 146, no. 5, pp. 682–695, 2011.

[85] D. R. Green, L. Galluzzi, and G. Kroemer, "Mitochonria and the autophagy-inflammation-cell death axis in organismal aging," *Science*, vol. 333, no. 6046, pp. 1109–1112, 2011.

[86] J. Tschopp, "Mitochondria: sovereign of inflammation?" *European Journal of Immunology*, vol. 41, no. 5, pp. 1196–1202, 2011.

[87] R. A. Merrill, R. K. Dagda, A. S. Dickey et al., "Mechanism of neuroprotective mitochondrial remodeling by pka/akap1," *Plos Biology*, vol. 9, no. 4, Article ID e1000612, 2011.

[88] B. Kornmann, E. Currie, S. R. Collins et al., "An ER-mitochondria tethering complex revealed by a synthetic biology screen," *Science*, vol. 325, no. 5939, pp. 477–481, 2009.

[89] D. A. Stroud, S. Oeljeklaus, S. Wiese et al., "Composition and topology of the endoplasmic reticulum-mitochondria encounter structure," *Journal of Molecular Biology*, vol. 413, no. 4, pp. 743–750, 2011.

[90] B. Kornmann, C. Osman, and P. Walter, "The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 34, pp. 14151–14156, 2011.

[91] O. M. de Brito and L. Scorrano, "Mitofusin 2 tethers endoplasmic reticulum to mitochondria," *Nature*, vol. 456, no. 7222, pp. 605–610, 2008.

[92] C. Cerqua, V. Anesti, A. Pyakurel et al., "Trichoplein/mitostatin regulates endoplasmic reticulum-mitochondria juxtaposition," *EMBO Reports*, vol. 11, no. 11, pp. 854–860, 2010.

[93] M. Germain, J. P. Mathai, H. M. McBride, and G. C. Shore, "Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis," *The EMBO Journal*, vol. 24, no. 8, pp. 1546–1556, 2005.

[94] Y. Yoon, E. W. Krueger, B. J. Oswald, and M. A. McNiven, "The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1," *Molecular and Cellular Biology*, vol. 23, no. 15, pp. 5409–5420, 2003.

[95] D. I. James, P. A. Parone, Y. Mattenberger, and J. C. Martinou, "hFis1, a novel component of the mammalian mitochondrial fission machinery," *Journal of Biological Chemistry*, vol. 278, no. 38, pp. 36373–36379, 2003.

[96] E. Alirol, D. James, D. Huber et al., "The mitochondrial fission protein hFis1 requires the endoplasmic reticulum gateway to induce apoptosis," *Molecular Biology of the Cell*, vol. 17, no. 11, pp. 4593–4605, 2006.

[97] R. Iwasawa, A. L. Mahul-Mellier, C. Datler, E. Pazarentzos, and S. Grimm, "Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction," *The EMBO Journal*, vol. 30, pp. 556–568, 2010.

[98] J. E. Chipuk, G. P. McStay, A. Bharti et al., "Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis," *Cell*, vol. 148, no. 5, pp. 988–1000, 2012.

[99] J. R. Friedman, L. L. Lackner, M. West, J. R. DiBenedetto, J. Nunnari, and G. K. Voeltz, "ER tubules mark sites of mitochondrial division," *Science*, vol. 334, no. 6054, pp. 358–362, 2011.

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 317645, 14 pages
doi:10.1155/2012/317645

Review Article

Novel Insights into the Interplay between Apoptosis and Autophagy

Hidemi Rikiishi

Department of Microbiology and Immunology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

Correspondence should be addressed to Hidemi Rikiishi, riki@m.tains.tohoku.ac.jp

Received 27 October 2011; Accepted 31 December 2011

Academic Editor: Lina Ghibelli

For several decades, apoptosis has taken center stage as the principal mechanism of programmed cell death (type I cell death) in mammalian tissues. Autophagic cell death (type II) is characterized by the massive accumulation of autophagic vacuoles in the cytoplasm of cells. The autophagic process is activated as an adaptive response to a variety of extracellular and intracellular stresses, including nutrient deprivation, hormonal or therapeutic treatment, pathogenic infection, aggregated and misfolded proteins, and damaged organelles. Increasing evidence indicates that autophagy is associated with a number of pathological processes, including cancer. The regulation of autophagy in cancer cells is complex since it can enhance cancer cell survival in response to certain stresses, while it can also act to suppress the initiation of cancer growth. This paper focused on recent advances regarding autophagy in cancer and the techniques currently available to manipulate autophagy.

1. Introduction

Current cancer therapies are based on the surgical removal of solid tumor masses, usually combined with a series of chemical (chemotherapy) or physical (radiotherapy) treatments; however, chemotherapy has reached a plateau of efficacy as a treatment modality with the emergence of resistant tumors. Despite the wide variety of mechanisms, most new drugs are thought to ultimately induce apoptosis of tumor cells through mitochondrial and/or death-receptor pathways, although these pathways are often defective in cancer. More recently, other mechanisms of cell death have emerged as potential novel mechanisms for cancer therapies to induce cell death.

Macroautophagy (hereafter referred to autophagy) is a eukaryotic, evolutionarily conserved homeostatic process in which organelles and bulk proteins are turned over by lysosomal activity [1]. Autophagy and apoptosis may be interconnected and even simultaneously regulated by the same trigger in tumor cells. In periods of metabolic stress, autophagy provides ATP and other macromolecules as energy sources to enable cell survival; however, if the intensity or duration of metabolic stress is excessive, cells may progress to autophagic programmed cell death, which is distinct from apoptosis [2]. In contrast, whether autophagy contributes to the antitumor effect of chemotherapeutic drugs or to drug resistance is largely unknown. In addition, there is no current consensus on how to manipulate autophagy to improve clinical outcomes. This paper describes the role of autophagy with a particular focus on the roles of cytoplasmic organelles and presents newly recognized approaches to pharmacologically exploit these mechanisms for improved antitumor outcomes.

2. Autophagy Networks

2.1. Degradation System. Eukaryotic cells respond to changes in their environment and the intracellular milieu by altering their anabolic and catabolic pathways. As one of the responses, living organisms from yeast to humans are capable of eating parts of themselves in order to survive. The so-called autophagy is a self-degradative process which ensures the regular turnover of cellular components by sequestering damaged organelles and misfolded proteins, targeting them for lysosomal degradation [3]. While the ubiquitin-proteasomal system is generally used for the degradation of

short-lived proteins, autophagy degrades and recycles long-lived proteins and organelles. After its discovery, autophagy was considered a kind of disposal mechanism aimed at recycling of cellular components [4], but now autophagy is implicated in more diverse physiological processes such as development, proliferation, remodeling, aging, tumor suppression, neurodegeneration, antigen presentation, innate immunity, regulation of organismal lifespan, and cell death [5]. In most of these situations, autophagy has both beneficial and harmful effects on cell functions. One aspect of this complexity probably reflects the dual role of autophagy, which is both cell-protective and -destructive. Autophagy can be a survival response in situations of stress, allowing the elimination of toxic metabolites, intracellular pathogens, and damaged proteins and organelles and providing energy and amino acids necessary for vital functions during metabolic stress; however, in some sense the induction of excessive autophagy can lead to cell death. In addition, autophagy provides signals for removal of apoptotic cells and genomic stability [6]. Hence, autophagy can be generally considered as a cell protector against various types of injuries or continuous cellular wear and tear and is expected to play a protective role in diverse types of cellular stress. Paradoxically, autophagy can also lead to a form of nonapoptotic cell death, which is called type II programmed cell death [7]. Thus, autophagy could either promote cell death or protect cells from diverse types of injuries depending on the cellular and environmental context.

2.2. Signaling System. The serine/threonine kinase, mammalian target of rapamycin (mTOR), is a major negative regulator of autophagy. The phosphoinositide 3-kinase- (PI3K-) activated serine/threonine kinase Akt phosphorylates the mTOR repressor TSC2 (tuberous sclerosis complex 2), thus leading to activation of mTOR and subsequent blockade of expression and function of autophagy-inducing Atg proteins. In addition to Akt, one of the main mTOR regulators is AMP-activated protein kinase (AMPK), a principal energy-sensing intracellular enzyme activated in various cellular and environmental stress conditions. In response to increases in AMP/ATP ratio, AMPK preserves energy by switching off ATP-requiring processes, while switching on ATP-generating catabolic pathways. AMPK maintains energy homeostasis by inducing autophagy and blocking protein synthesis and cell proliferation mainly through phosphorylation of its downstream target Raptor and consequent inhibition of mTOR. In addition to a major role of the AMPK/mTOR pathway in regulation of intracellular and whole body metabolism, recent findings point to its potential involvement in controlling proliferation, survival, and death of cancer cells [8]. Namely, pharmacological activation or overexpression of AMPK in a variety of cancer cell types caused an mTOR inhibition-associated cell cycle arrest and apoptotic death both *in vitro* and *in vivo* [9]. On the other hand, AMPK activity in certain conditions protected normal and cancer cells from metabolic stress and/or chemotherapy-induced apoptosis [10]. Autophagic digestion of intracellular proteins triggered by mTOR-downregulation in tumor cells seems to play an important role both in AMPK-mediated cytotoxicity and cytoprotection [11, 12], the final outcome possibly depending on the nature of the cytotoxic stimulus. The physiological role of autophagy in the mammalian system has been shown in several *in vivo* animal models. Disruption of the autophagic process leads to failure of cavitation during embryogenesis or accumulation of abnormal organelles such as mitochondria or endoplasmic reticulum (ER) in adult tissues [13, 14]. In addition to the physiological roles, dysregulated autophagy has been suggested to play pathogenetic roles in a variety of disease processes, particularly when cellular stress is increased, which is probably because of the accumulation of damaged molecules and organelles. It has been suggested that the role of autophagy in degenerative changes is associated with ageing or diverse degenerative disorders [15].

3. Programmed Cell Death

The term programmed cell death refers to controlled or regulated forms of cell death associated with a series of biochemical and morphological changes. Programmed cell death is an evolutionarily conserved process to decide cell fate, which has therefore been drawing increasing attention in cancer treatment. Programmed cell death can be divided into several categories: type I (apoptosis), type II (autophagic cell death), and others (necrosis, senescence, and mitotic catastrophe).

3.1. Type I Programmed Cell Death. In type I, the typical morphology of apoptosis is largely the end result of caspase-mediated destruction of the cellular structure [16]. There are two core pathways inducing apoptosis: the extrinsic and intrinsic pathways. The extrinsic pathway is triggered by the Fas death receptor (DR), which is mainly dependent on the initiation of the combination between FasL and Fas. The intrinsic pathway is another process leading to apoptosis, in which mitochondria play a central role. When cells sense extracellular stimuli or intracellular signals, the outer mitochondrial membrane becomes permeable, releasing cytochrome *c*. The ER, lysosomes, and the *trans*-Golgi-network also play important roles [17]. Each organelle possesses sensors that detect specific alterations, locally activate signal transduction pathways, and emit signals that ensure interorganellar cross-talk. It is often assumed that apoptosis is initiated if the damage is too severe to be repaired, or if the time-scale for complete repair is too long.

3.2. Type II Programmed Cell Death. Autophagy has been associated with a particular mode of cell death that is characterized by the appearance of double- or multiple-membrane cytoplasmic vesicles engulfing bulk cytoplasm and/or cytoplasmic organelles such as mitochondria and ER; however, many studies failed to demonstrate a causal link between autophagy and cell death. Autophagy is probably not an executor of cell death *per se*, but it is a required process in certain settings in combination with other prodeath signals. Cells undergoing autophagic cell death were engulfed by

human macrophages as well as vital cells [18]. To distinguish it from apoptosis (type I cell death), this has been termed autophagic or type II cell death [19]. The machineries for apoptosis, autophagy, and necrosis (type III cell death) are interconnected and somehow coordinated between the two. For example, the elimination of damaged mitochondria by autophagy would prevent the release of proapoptotic substances from mitochondria, thus preventing apoptosis. In the absence of such clean up, the release of molecules like cytochrome *c* and apoptosis-inducing factor (AIF) from damaged mitochondria would lead to apoptosis and in the case of extreme damage and ATP depletion, to necrotic cell death.

4. Autophagy in Cancer

There is an accumulation of evidence that highlights the important function of autophagy in cancer. Although it is still controversial as to whether autophagy kills cancer cells or sustains their survival under stressful conditions, more reports provide data to support that autophagy promotes cancer cell survival after chemotherapy or radiation therapy [20]. The high rate of autophagy observed in undifferentiated colon cancer cells is compatible with the expression of Beclin 1 and PTEN (phosphatase and tensin homolog) in these cells. Cancer cells are frequently exposed to inherent metabolic stress owing to hypoxia and lack of nutrient supplies. Hypoxia-inducible factor 1α (HIF-1α), a key transcription factor encoding a plethora of genes responsible for altered metabolism, angiogenesis, invasion, metastasis, or therapy-resistance in hypoxic tumors [21], is a positive regulator of autophagy. Small-interfering-RNA- (siRNA-) mediated depletion of Atg proteins sensitizes cancer cells to radio- and chemotherapy, and the autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin A1 cause radiosensitization of malignant glioma cells [20]. Thus, cancer was one of the first diseases genetically linked to impaired autophagy.

The tumor-suppressive role of autophagy was first shown in mice heterozygous for the Beclin 1 autophagy protein. These mice (*beclin* $1^{+/-}$) showed reduced autophagy and increased cellular proliferation, which translated into increased incidence of spontaneous malignancies, such as lymphomas, lung, and liver cancers [22]. Incidentally, *beclin* $1^{-/-}$ mutant mice died early in embryonic development. Liang et al. reported *beclin 1* as a potential tumor suppressor gene, the expression of which is frequently decreased in human breast epithelial carcinoma cell lines and tissue compared to the higher level in normal tissue [23]. Low expression of Beclin 1 in 115 node-positive colon cancer specimens was associated with a significantly worse 5-year overall survival (47% versus 67%) [24]. The PI3K/Akt/mTOR axis plays a decisive role in the negative regulation of autophagy, and the constitutive activation of this pathway has been implicated in many human cancers [25]. Autophagy is thought to mediate Sqstm1/p62 elimination and to suppress tumorigenesis; however, defective autophagy allows overexpression of p62 that promotes tumorigenesis. The sustained p62 overexpression in tumor cells with defective autophagy appears to dysregulate NF-κB signaling, and altered NF-κB regulation resulting from p62 overexpression is proposed to be a primary mechanism for enhancing tumorigenesis [26]. Persistent DNA damage induced by the increased levels of reactive oxygen species (ROS), which are associated with the accumulated mutant proteins and dysfunctional mitochondria found in autophagy-deficient tissues, is also proposed as a causative link between autophagy inhibition and enhanced tumor formation [27]. Thus, the inability of autophagy-defective tumor cells to eliminate p62 contributes to oxidative stress and likely to DNA damage. These observations suggest that autophagy is important for tumor suppression.

Ultimately, autophagy in cancer has different effects in different ways in different cancer cell lines at different stages of tumorigenesis and progression. The early stages of tumor development require cancer cells to undergo a higher level of protein synthesis than protein degradation for the tumor to grow. Thus, autophagy probably functions to prevent cancer initially, but once a tumor develops, the cancer cells utilize autophagy for their own cytoprotection. Elucidating this complex physiological process is crucial for development of anticancer therapies with maximum efficacy and minimum drug resistance.

5. Cross-Talk between Apoptosis and Autophagy in Cancer

Expanding our knowledge of the molecular cross-talk among pathways that regulate tumor cell death is crucial in guiding the successful design of future anticancer therapeutics. In particular, apoptosis and autophagy can act as partners to induce cell death in a coordinated or cooperative fashion. Autophagy proteins can also play a role in cellular events that occur during apoptosis. For example, Atg5 may be an independent key player in both apoptosis and autophagy. The low levels of Atg5 cleavage product may have significant effects on apoptosis, but not the intact Atg5 that participates in autophagy [28]. Bcl-2 phosphorylation may not only be a mechanism for regulating apoptosis and a mechanism for regulating autophagy, but perhaps also a mechanism for regulating the switch between the two pathways [29]. JNK (c-Jun NH_2-terminal kinase) is able to trigger autophagy by targeting Bcl-2/Bcl-xL proteins and abrogating their binding to Beclin 1. Recently, Beclin 1 has been shown to be among the substrates of death-associated protein kinase (DAPK), a proapoptotic serine/threonine kinase, and its phosphorylation reduces its binding to the Bcl-2 family members, thus suggesting a possible mechanism by which DAPK may also induce autophagy [30]. A complex role in the regulation of autophagy is played by p53, one of the most important tumor suppressor proteins. In fact, p53 regulates autophagy both in a positive and in a negative fashion, depending on its subcellular localization [31]. p53 and DRAM (damage-regulated autophagy modulator) can induce accumulation of autophagosomes. In addition, p53 affects autophagy by modulating signaling through the mTOR nutrient-sensing kinase which controls autophagy at the initiation stage [32].

Inhibition of autophagy leads in most cases to an increase susceptibility to apoptotic stimuli. Moreover, a growing number of proteins that play a negative regulatory role in both events have been identified [33]. Autophagy and apoptosis may be triggered by common upstream signals. Recent reports have shown that Akt inhibits apoptosis by phosphorylation of the Bcl-2 protein family member, Bad, allowing for cell survival. In addition, activation of the PI3K/Akt/mTOR pathway can cause inhibitory effects of Akt on apoptosis and mTOR on autophagy and enhance survival capacity in neoplastic cells [34].

In addition, several Atg proteins, including Atg5 and Beclin 1 (Atg6), can be cleaved by calpain or caspases that are activated during apoptosis [35]. Caspase-dependent cleavage of Beclin 1 occurred in HeLa cells treated with a death receptor ligand [36]. Chemotherapy-induced apoptosis suppresses autophagy at the execution stage following cytochrome *c* release, at least in part through caspase 8-mediated cleavage of Beclin 1 at Asp^{133} and Asp^{146} [37]. Caspase 3 cleaved Beclin 1 at Asp^{149} in apoptosis induced by Bax overexpression [38]. Caspase-9 can also cleave Beclin 1, thereby destroying its proautophagic activity [39]. Moreover, the C-terminal fragment of Beclin 1 that results from this cleavage acquires a new function, which can amplify mitochondrion-mediated apoptosis. These findings provide important insights into the molecular cross-talk between autophagy and apoptosis. Apoptosis can begin with autophagy, which can end with apoptosis. Some links between the two types of cell death are indicated via mitochondria. Induction of mitochondrial membrane permeabilization at a low level, below the threshold required for induction of apoptosis, results in sequestering damaged mitochondria in autophagic vacuoles. When mitochondrial membrane permeabilization is sufficiently high to sustain the active execution of cell death, apoptosis is induced [40]. Therefore, it is likely that induction of apoptotic or autophagic cell death may depend on the level of mitochondrial membrane permeabilization.

6. ER and Autophagy

ER is an essential intracellular organelle providing apparatus for synthesizing nascent proteins as well as their further modification and correct folding, such as the formation of N-linked glycans and disulphide bonds. Disruption of any of these processes, such as with glucose depletion that prevents the proper glycosylation of proteins or with alterations in calcium homeostasis such that calcium-dependent chaperones cannot function properly, leads to the accumulation of misfolded proteins that triggers ER stress. ER stress has been implicated in different stages of tumor development. The ER has by now been established as an essential site for the regulation of apoptotic pathways and has recently been recognized as an important component of autophagic signaling. ER stress appears to signal autophagy by pathways that all depend on eIF2α (eukaryotic initiation factor 2α). How eIF2α regulates autophagy is presently unknown, but the induction of Atg12 expression via ATF4 (activating transcription factor 4) is likely to participate in this process. In cancer cells, when the amount of unfolded or misfolded proteins exceeds the capacity of the proteasome-mediated degradation system, autophagy is triggered to remove these proteins.

Bcl-2 has been mainly studied in the context of cell death, but it is also known to carry out other functions in different cellular processes, such as cell cycle progression, glucose homeostasis, transcriptional repression by p53, and autophagy. Beclin 1 has been identified as a novel BH3- (Bcl-2-homology-3-) only protein. Beclin 1 induces autophagy by promoting autophagosome formation when in complex with hVps34 (vacuolar protein sorting 34)/class III PI3K. Beclin 1-Bcl-2 (Bcl-xL) complexes normally inhibit autophagy, which points to organelle-specific regulation of autophagy [41]. Beclin 1 binding with Bcl-2 has been suggested to interfere with the ability of Beclin 1 to form a complex with hVps34/PI3K, thus resulting in a loss of Beclin 1-associated PI3K autophagy-inducing activity. The interaction between Beclin 1 and Bcl-2 is abolished when key residues within the BH3 domain of Beclin 1 or the BH3-binding groove of Bcl-2 are mutated. ER-localized Bcl-2, but not mitochondria-localized Bcl-2, has been shown to be important for the inhibition of autophagy [42]. The ER functions to synthesize proteins that store high intracellular calcium that can activate calcium-dependent chaperone proteins that assist in protein folding. The autophagy inhibition by Bcl-2 is evident only when Bcl-2 resides in the ER, where it has been suggested to regulate cellular Ca^{2+} homeostasis. Ca^{2+} regulates autophagy via a signaling pathway involving AMPK and mTOR, and ER-located Bcl-2 effectively inhibits this pathway. ER-located Bcl-2 inhibits autophagy induced by Ca^{2+} mobilizing agents by regulating the Ca^{2+} homeostasis in a Beclin 1-independent manner [43].

7. Golgi and Autophagy

Eukaryotic cells possess an internal endomembrane system that compartmentalizes the cell for various different cellular functions. The main components of this membrane system are the ER, Golgi complex, vesicles, plasma membrane, vacuole/lysosome, and nuclear envelope. Autophagy displays unique membrane dynamics, distinct from classical membrane trafficking. The Golgi complex is required for double-membrane cytoplasm to vacuole targeting vesicle and autophagosome formation. A number of Rab GTPases which regulate secretory and endocytic membrane traffic have been shown to play either critical or accessory roles in autophagy. Rab33B, a member of the Rab small GTPase family, which was originally described as a Golgi-resident protein involved in Golgi-to-ER transport [44], directly interacts with an autophagosome precursor marker, Atg16L, in a GTP-dependent manner, and the activation and inactivation of Rab33B modulate autophagy. Based on these results, the possible role of the interaction between Rab33B and Atg16L was suggested in autophagosome formation [45]. The autophagosomes are not formed by Golgi-derived vesicles/membranes alone because single-membrane structures, the so-called isolation membranes,

and autophagosome membranes are composed of membranes derived from rough ER. ER and Golgi complex-derived vesicles could be delivered to the lysosome/vacuole by a pathway that is different from canonical autophagy. Under certain conditions, these two organelles might not require sequestration by a double membrane for delivery and subsequent degradation. Furthermore, it has been reported that one of the Atg proteins, Atg9, a membrane protein with unknown function, shuttled between the *trans*-Golgi network and late endosomes. Bax-interacting factor 1 (Bif-1) binds the proapoptotic Bcl-2 family protein Bax. Bif-1 overexpression promotes Bax activation and apoptosis. Bif-1 also regulates the fission of Golgi membranes and the trafficking of Atg9 from the Golgi complex to autophagosomes during starvation [46]. Therefore, isolation membranes and autophagosome membranes may be made up of membranes from several different sources, including the Golgi; however, there is little evidence that the Golgi complex contributes directly to the formation of the autophagic membrane.

8. Mitochondria and Autophagy

Mitochondria are key players in several cancer cellular functions, including growth, division, energy metabolism, and apoptosis, and play a central role in cell survival and death. Mitochondria also play an important role in the formation of autophagosome and its subsequent docking and fusion with lysosome and could contribute to the increased autophagy and autophagic flux in metastatic cancer. The ability of tumor mitochondria to increase autophagy may help cancer cells to fulfill high anabolic needs during rapid growth; however, mitochondrial functions (such as respiration) can block autophagic cell death [47]. Autophagy often occurs when the mitochondria fail to maintain ATP levels, during starvation, or when the mitochondria are damaged. A process known as mitophagy selectively eliminates the damaged mitochondria. Furthermore, blocking mitochondria-mediated apoptosis through the elimination of mouse *Bax* and *Bak* (*Bak1*) expression promotes autophagy-induced cell death. Arsenic-trioxide- (As_2O_3-) induced cell death in human malignant glioma cell lines was accompanied by involvement of an autophagy-specific marker, LC3, and damage to mitochondrial membrane integrity. Arsenic trioxide induces the mitochondrial localization of the pro-cell-death Bcl-2 family member BNIP3 (Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3), which determines the on/off state of the mitochondrial permeability transition (MPT) pore [48]. The reciprocal relationship between mitochondrial metabolism and the activity of the rapamycin-sensitive pathway has been described before [49]. Mitochondria dysfunction leads to inhibition of mTOR, and inactivation of p70S6K (ribosomal protein S6 kinase) results in dephosphorylation of Bad, potentially leading to increased interaction between Bad and Bcl-xL or Bcl-2, to mitochondrial damage and cell death [50]. In addition, the promotion of MPT also contributed to increased autophagy. These results suggested that mitochondria from a highly metastatic breast cancer cell line can promote homeostatic autophagy of cancer by opening low-conductance MPT pores. The activation of autophagy by tumor mitochondria was reduced by cyclosporine A, which is an inhibitor that blocks the low-conductance state of MPT pores [47]. ROS could be the mediator leading to the loss of mitochondria membrane potential, possibly by triggering the opening of the MPT pore that subsequently leads to cell death. ROS were recently shown to activate starvation-induced autophagy, antibacterial autophagy, and autophagic cell death. Short-chain fatty acids were shown to induce ROS production, which led to AMPK activation and consequential mTOR inhibition [51]. Current findings implicate ROS in the regulation of autophagy through distinct mechanisms, depending on the cell type and stimulation conditions; conversely, autophagy can also suppress ROS production.

9. Experimental Manipulation of Autophagy

For a better understanding of the regulation of autophagy, many studies have used chemical modulators of autophagy: inhibitors and inducers. The different modulators are an important tool in studying the functions of autophagy, although it is difficult to define the mechanism closely. Appropriate modification of autophagy, that is, inhibition of cytoprotective autophagy or promotion of cyto-killing autophagy could mitigate the cytotoxicity caused by anticancer therapy in tumor cells.

9.1. ProAutophagics (Table 1)

9.1.1. Beclin 1. The mechanism by which a small molecule Bcl-2/Bcl-xL antagonist *ABT-737* can potentiate autophagy may be related to its ability to competitively disrupt the binding of Bcl-2/Bcl-xL to the autophagic protein Beclin 1 (Atg6) [52]. Treatment with *fenretinide* (RT-101), a synthetic derivative of retinoic acid, resulted in an increase in Beclin 1 expression, the conversion to the form LC3-II form, and its shift from diffuse to punctate staining and finally an increase in lysosomes/autophagosomes [53]. In addition, HIF-1α is required for *fenretinide*-induced protective autophagy under hypoxia. The inositol 1,4,5-trisphosphate receptor (IP_3R) antagonist *xestospongin B* induces autophagy by disrupting a molecular complex formed by IP_3R and Beclin 1. *Xestospongin B*-induced autophagy was inhibited by overexpression of the IP_3R ligand-binding domain, which co-immunoprecipitated with Beclin 1 [54]. Autophagy can be generally induced via several distinct pathways, such as inhibition of mTOR or activation of Beclin 1. *Okadaic acid*, a protein phosphatase 2A inhibitor, increased both the mTOR and Beclin 1 pathways simultaneously, which suggests that autophagy in *okadaic acid*-treated neurons is induced mainly via the Beclin 1 pathway, and less so via mTOR inhibition [55].

9.1.2. DNA Damage. Exogenous environmental agents such as *ultraviolet* (UV) and *ionizing radiation*, genotoxic chemicals, and endogenous byproducts of metabolism, including *ROS*, can cause alterations in DNA structure (DNA damage).

Table 1: Proautophagics.

Class (target)	Compounds	Autophagic mechanisms	Reference
Beclin 1	ABT-737	Disruption of Bcl-2/Bcl-xL binding to Beclin 1	[52]
	Fenretinide	Increase in Beclin 1 expression	[53]
	Xestospongin B	Disruption of the complex formed by IP_3R and Beclin 1	[54]
	Okadaic acid	Increase in Beclin 1 pathways	[55]
DNA damage	UV	p53 activation	[56]
	ROS	PTEN upregulation	
	Camptothecin	mTOR suppression	
ER stressors	A23187	GRP78 induction	[57] [58] [59]
	Thapsigargin	Increase of Ca^{2+} in cytosol	
	Tunicamycin	PERK/elF2α pathway	
	Brefeldin A	Induction of GRP78 and GRP94	
	Bortezomib	Unfolded protein response	
	Sorafenib	Inhibition of mTOR signaling	
Farnesyltransferase inhibitors	Manumycin A	Akt downregulation and mTOR phosphorylation	[60]
	FTI-276		
	Lonafarnib		
Golgi-associated agents	Brefeldin A	Interruption of trafficking of N-linked glycoproteins	[61]
	Paclitaxel	Fragmentation of Golgi apparatus	[62]
HDAC inhibitors	SAHA	Downregulation of Akt and mTOR activity	[63] [64] [65] [66]
	Valproic acid	Increase in mitochondrial ROS	
	FK228	Nuclear translocation of AIF	
Mitochondrial agents	Selenite	Degradation of mitochondrial proteins	[7]
	Resveratrol	Inhibition of mitochondrial function	[67]
mTOR inhibitors	Rapamycin	mTORC1 inhibition	[68]
	Berberine	Akt suppression and p38 MAPK upregulation	[69]
	Torin1	Inhibition of mTORC1 and mTORC2	[70]
	Ku-0063794	Dephosphorylation of mTORC1 substrate 4E-BP1	[71]
	PM02734	DAPK activation	[72]
Pathogens	Bacteria	Ubiquitin-p62-NDP52 pathway	[73]
	HSV-1	PKR and eIF2α activation	[74]
Proteasome inhibitors	MG-132	ER stress induced by misfolded proteins	[75]
	Glidobactin A	Catalytic subunits of 20S proteasome	[76]

DNA damage induces autophagy, but its role in the DNA damage response is still unclear. Recent reports using the DNA-damaging agents, *camptothecin*, *etoposide* (VP-16), *tomozolomide*, and *p-anilioaniline* (*p*-aminodiphenylamine), demonstrate that cells, in addition to initiating cell cycle arrest, also initiate autophagy. p53 is a central regulator of apoptosis induced by DNA damage. Interestingly, p53 is a bidirectional regulator of autophagy. p53 activation also leads to upregulation of the PTEN, an inhibitor of the PI3K/Akt signaling pathway, and TSC2, at the transcriptional level, which may contribute to the long-term suppression of mTOR [56].

9.1.3. ER Stressors. Autophagy was induced by chemicals, such as *A23187*, *tunicamycin*, *thapsigargin*, and *brefeldin A* that cause ER stress [57]. The calcium ionophore *A23187* induced the expression of BiP/GRP78 protein, which is an ER stress marker protein. GRP78, through maintenance of ER structure and homeostasis, facilitates autophagy. Disturbances in normal ER processes lead to an evolutionarily conserved cell stress response, referred to as the unfolded protein response (UPR). *Tunicamycin*, an inhibitor of N-glycosylation, is a potent stimulator of ER stress. The link between *tunicamycin*-induced ER stress and autophagy was the PERK/elF2α pathway. *Thapsigargin*, an inhibitor of the ER calcium transporters, generates Ca^{2+}-store depletion within the ER and simultaneously increases the Ca^{2+} level in the cytosol. *Brefeldin A* (an inhibitor of vesicle transport between the ER and Golgi) can increase the expression of both glucose-regulated proteins GRP78 and 94, which disrupt some functions of the ER. The inhibition of the 26S proteasome by the dipeptide boronic acid *bortezomib* (velcade) leads to the accumulation of misfolded proteins, resulting in ER stress followed by a coordinated cellular UPR response [58]. Induction of autophagy by *bortezomib* is dependent on the proteasomal stabilisation of ATF4 and upregulation of LC3B by ATF4. *Sorafenib* (nexavar), a potent multikinase inhibitor, inhibited phosphorylation of Stat3 and expression of cyclins, D and E, and exerted significant antitumor activity through inhibition of mTOR signaling. In addition, *sorafenib* induced autophagy in human hepatocellular carcinoma cells through mechanisms that involved ER

stress and was independent of the MEK1/2-ERK1/2 pathway [59].

9.1.4. Farnesyltransferase Inhibitors. Farnesylation is a post-translational modification of proteins in which farnesyltransferase catalyzes the attachment of the isoprenoid group from farnesyl pyrophosphate to a cysteine residue at the C-terminus of proteins. The farnesyltransferase inhibitors *manumycin A*, *FTI-276*, and *lonafarnib* (sarasar) induced autophagy in human cancer cell lines [60]. Treatment with a combination of oridonin and *manumycin A* (a natural product of *Streptomyces parvulus*) downregulated phosphorylation of Akt, downstream of PI3K. Oridonin triggers apoptosis of cancer cells and is partially effective through mitochondrial depolarization. *Lonafarnib* decreased phosphorylation of mTOR and S6 kinase, which is downstream of mTOR, in a dose-dependent manner. *FTI-276* is a CAAX peptidomimetic of the carboxyl terminal of Ras proteins. *FTI-276* was identified as a highly selective suppressor of Ras-dependent oncogenicity, and *FTI-276* inhibited Akt phosphorylation.

9.1.5. Golgi-Associated Agents. Brefeldin A inhibits the activation and membrane-binding properties of most ADP-ribosylation factors and causes the redistribution of Golgi proteins into the ER. *Brefeldin A* leads to dramatic changes in the structure of the Golgi apparatus causing the conversion of the staked cisternae into vesicular/cisternal remnants. *Brefeldin A* even when acting alone, increases the volume fraction of autophagic vacuoles. The destabilization of the Golgi apparatus by *brefeldin A* interrupts the intracellular trafficking of N-linked glycoproteins along the secretory pathway [61]. Treatment of cells with *paclitaxel* (taxol) results in polymerization of microtubules. After incubation with *paclitaxel*, the Golgi apparatus is fragmented and is conspicuously present in areas of the cytoplasm enriched in microtubules. *Paclitaxel* treatment could lead to the formation of acidic vesicular organelles, the induction of Atg5, Beclin 1, and LC3 expressions, and an increase in punctate fluorescent signals in A549 cells pretransfected with GFP-tagged LC3 [62].

9.1.6. HDAC Inhibitors. Histone acetylation is mediated by histone acetyltransferases and deacetylases (HDACs), which influence chromatin dynamics, protein turnover, and the DNA damage response. The HDAC inhibitor *SAHA* (suberoylanilide hydroxamic acid, vorinostat) can induce both caspase-dependent apoptosis and caspase-independent autophagic cell death, so it has clear therapeutic implications [63]. *SAHA* induced autophagy through downregulation of Akt/mTOR signaling and induction of the ER stress response. Moreover, *SAHA* treatment upregulated expression of Beclin 1 and Atg7 and promoted formation of the Atg5-Atg12 conjugate [64]. *Valproic acid* was identified as a potent selective histone deacetylase inhibitor, which induces autophagy in glioma cells. *Valproic acid* is capable of producing elevated levels of mitochondrial ROS in glioma cells. ROS production results in autophagosome development and autolysosomal degradation [65]. *FK228* (romidepsin) is a unique cyclic peptide and is among the most potent inhibitors of both Class I and II HDACs. *FK228* converted LC3-I to LC3-II and induced localization of LC3 to autophagosomes. *FK228*-mediated autophagy in rhabdomyosarcoma cells coincided with nuclear translocation of AIF, while knockdown of AIF abrogated autophagy following *FK228* exposure [66].

9.1.7. Mitochondrial Agents. An essential trace element, *selenite*, is preferentially cytotoxic to various human glioma cells over normal astrocytes via autophagic cell death. Before *selenite*-induced cell death in glioma cells, disruption of the mitochondrial cristae, loss of mitochondrial membrane potential, and subsequent entrapment of disorganized mitochondria within autophagosomes or autophagolysosomes, along with degradation of mitochondrial proteins, were noted, showing that *selenite* induces autophagy in which mitochondria serve as the main target [7]. The phytoalexin *resveratrol* (trans-3,5,4′-trihydroxystilbene), a defensive substance produced by plants in response to infection by pathogenic microorganisms, displays a wide range of biological effects in mammalian cells. *Resveratrol* induced autophagy through any of numerous mechanisms that involve activation of mitochondria, downregulation of cell survival proteins, inhibition of cell survival kinases, and survival transcription factors [67].

9.1.8. mTOR Inhibitors. Rapamycin (sirolimus) and its analogues (such as *CCI-779*, *RAD001*, and *AP23573*) inhibit mTOR (mTORC1), the kinase that normally suppresses both apoptosis and autophagy. *Rapamycin* activates the autophagic process, and silencing of mTOR with siRNA increases the inhibitory effect of *rapamycin* on tumor cell viability by stimulating autophagy [68]. *Berberine*, a small molecule derived from *Coptidis rhizome*, can induce both autophagy and apoptosis in hepatocellular carcinoma cells. *Berberine* may also induce autophagic cell death in HepG2 and MHCC97-L cells through activation of Beclin 1 and inhibition of the mTOR-signaling pathway by suppressing the activity of Akt and upregulating p38 MAPK signaling [69]. Treatment with *torin1* significantly enhanced lysosomal accumulation of mTOR and Raptor. *Torin1* inhibits mTOR by directly competing with ATP for binding to the kinase domain. Thus, *torin1* inhibits both mTORC1 and mTORC2 [70]. The small molecule *Ku-0063794*, which inhibits both mTORC1 and mTORC2 with an IC_{50}, induced a much greater dephosphorylation of the mTORC1 substrate 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) than *rapamycin* [71]. *PM02734* (elisidepsin) causes cell death by a complex mechanism that involves increased autophagosome content, due for the most part to impairment of autophagic flux, inhibition of the Akt/mTOR pathway, and activation of DAPK. This unique mechanism of action justifies the continued development of this agent for the treatment of lung cancer [72].

9.1.9. Pathogens. Autophagy has recently emerged as an important mechanism for controlling intracellular pathogens.

A variety of different bacteria, including *Mycobacteria*, *Salmonella*, *Shigella*, and *Listeria*, are recognized by autophagy, yet the specific signals that mediate recognition of intracellular pathogens by the autophagy machinery have not been defined thus far. It is currently thought that ubiquitin associated with intracellular pathogens promotes targeted autophagosome formation and pathogen destruction. In addition, p62 and NDP52 mediate different pathways of selective autophagy for *Shigella* and *Listeria* and provide novel insight into the mechanisms by which adaptor proteins target bacteria to autophagy [73]. Infection with *Herpes simplex virus type 1* (*HSV-1*) provoked autophagy under conditions that inhibited viral gene expression. The induction of autophagy occurs very early after infection with *HSV-1*, and *de novo* protein synthesis is not required for the response [74]. Autophagy can be induced by the presence of foreign DNA within cells. The interferon-inducible, dsRNA-dependent protein kinase R (PKR) plays an important role in innate immunity against viral infections. PKR activation leads to phosphorylation of eIF2α and a subsequent shutdown of host and viral protein synthesis and viral replication. PKR and eIF2α phosphorylation regulate another fundamental cellular process, the lysosomal degradation pathway of autophagy.

9.1.10. Proteasome Inhibitors. The ubiquitin-proteasome system and lysosome-dependent autophagy are two major intracellular pathways for protein degradation. The ubiquitin-proteasome pathway is a novel therapeutic target for cancer treatment. Inhibition of proteasome function leads to the accumulation of polyubiquitinated proteins and likely causes the accumulation of misfolded proteins in the ER due to the blockage of ER-associated degradation. Thus, proteasome inhibitors activate autophagy via ER stress. Proteasome inhibitors have been potent anticancer agents for various cancers. Proteasome inhibitor *MG-132* increased the protein expression of LC3-I and -II in a time-dependent manner. 3-MA significantly abolished the formation of $LC3^+$ autophagic vacuoles and the expression of LC3-II, but not LC3-I, induced by *MG-132* [75]. A new class of proteasome inhibitors such as *glidobactin A* treatment also induced autophagy as judged by the presence of the lipidated form of LC3 and autophagosomes [76]. The eukaryotic 20S proteasome contains three catalytic subunits (β1, β2, and β5) conferring caspase-like, trypsin-like, and chymotrypsin-like proteolytic activities, respectively. *Glidobactin A* blocked the chymotrypsin-like activity irreversibly at low concentrations, whereas the trypsin-like activity was less sensitive and the caspase-like activity was not inhibited at the concentrations tested (up to 20 μM).

9.2. AntiAutophagics (Table 2)

9.2.1. Atg Proteins. The regulation of the autophagic and apoptotic response has been shown to require components that are involved in both processes. *Caspases*, a family of cystinyl aspartate-requiring proteases, play a central role in apoptosis, a well-studied pathway of programmed cell death. *Calpain* is a calcium-dependent intracellular cysteine protease that plays a crucial role in the regulation of cell spreading, cell migration, programmed cell death, and cell cycle progression. The majority of human Atg proteins can be cleaved by *caspases* and *calpains*, which are activated in some apoptotic paradigms [77]. For example, Atg3 is cleaved by *caspase-3*, *-6,* and *-8*, while Atg9, Atg7, and Atg4 homologues can be cleaved by *caspase-3*. Beclin 1 (Atg6) is a substrate of *caspase-3* with two cleavage sites at positions 124 and 149, respectively. Cleavage of Beclin 1 was also seen in apoptosis of HeLa cells induced by staurosporine and TRAIL. The cleavage of Beclin 1 resulted in abrogation of the interaction between Bcl-2 with Beclin 1, which could be blocked by z-VAD-fmk. Atg5 is a gene product required for the formation of autophagosomes. *Calpains-1* and *-2* activation and Atg5 cleavage are general phenomena in apoptotic cells. Truncated Atg5 translocated from the cytosol to mitochondria, associated with the antiapoptotic molecule Bcl-xL, and triggered cytochrome *c* release and *caspase* activation. *Caspase* cleavage of autophagy-related proteins can affect the autophagic process.

9.2.2. Autophagosome Formation. Verteporfin (visudyne) is a benzoporphyrin derivative used in photodynamic therapy, and *verteporfin* inhibited drug- and starvation-induced autophagic degradation and the sequestration of cytoplasmic materials into autophagosomes [78]. *Verteporfin* inhibited autophagy stimulated by serum starvation, a physiological stimulus, and by rapamycin, the chemical inhibitor of mTORC1. *Verteporfin* binds to the membrane of expanding phagophores or to a factor involved in phagophore expansion and prevents expanding phagophores from adopting their characteristic cup shape, so they are unable to capture cytoplasmic cargo and only form empty single-membrane vesicles. *3-Methyladenine* (*3-MA*), as an autophagy inhibitor, was first discovered via screening of purine-related substances using isolated hepatocytes from starved rats. *3-MA*, which interferes with the formation of autophagosomes in mammalian cells via inhibition of the class III PI3K activity that controls the autophagic pathway [79], has been widely used in studies on autophagy. *3-MA* has a dual role in modulation of autophagy; although it is capable of suppressing autophagy induced by starvation, its prolonged treatment in full medium induces autophagy. Under specific treatment conditions, *3-MA* acts similar to rapamycin, a well-established autophagy inducer via suppression of mTOR function.

9.2.3. Bcl-2 Family. Apoptosis and autophagy are both closely regulated biological processes that play a central role in tissue homeostasis, development, and disease. The anti-apoptotic protein *Bcl-2* functions not only as an antiapoptotic protein but also as an anti-autophagy protein via its inhibitory interaction with Beclin 1 [80]. Beclin 1 functions in the lysosomal degradation pathway of autophagy and induces autophagic cell death in cancer cells. *Bcl-2*/*Bcl-xL* can bind Beclin 1 and inhibit Beclin 1-dependent autophagic cell death in cancer cells. The BH3 domain of Beclin 1 also binds

Table 2: Antiautophagics.

Class (target)	Compounds	Antiautophagic mechanisms	Reference
Atg proteins	Calpain-1, -2 Caspase-3, -6, -8	Defect in autophagic process and apoptotic enhancement	[77]
Autophagosome formation	Verteporfin 3-Methyladenine	Inhibition of autophagic degradation Inhibition of class III PI3K activity	[78] [79]
Bcl-2 family	Bcl-2/Bcl-xL	Inhibitory interaction with Beclin 1	[80] [41]
Beclin 1	Metformin	Decrease in Beclin 1 expression and AMPK activation	[81]
Chemokines	CCL2	Survivin upregulation	[82]
Golgi-associated agents	Monensin Nocodazole	Dilatation of Golgi apparatus Disconnection of Golgi stacks and dispersal into fragments	[83] [84]
Heat shock proteins	Geldanamycin PES	Promotion of Beclin 1 degradation Impairment of the autophagy-lysosome system	[85] [86]
Lysosomal function	Bafilomycin A1 Lucanthone Chloroquine	Prevention of fusion of autophagosomes with lysosomes Lysosomal membrane permeabilization Blocking of lysosyme acidification	[87] [88] [89]
Small regulatory RNAs	Beclin 1 Lamp-2 mi-30a	Potentiation of apoptotic death, autophagosome degradation Impairment of autolysosome formation Beclin 1 translational suppression	[90] [91] [92]

to the BH3 binding groove of *Bcl-xL*. When the BH3 binding groove of *Bcl-xL* is mutated, the interaction between *Bcl-xL* and Beclin 1 is disrupted. Furthermore, the interaction between Beclin 1 and *Bcl-xL* can be inhibited by ABT737 at low doses, which stimulates autophagy without inducing cell apoptosis. A novel Bcl-xL inhibitor, Z36, efficiently induces autophagic cell death in HeLa cells. Z36 can competitively inhibit the interaction between *Bcl-xL* and Beclin 1 *in vitro*, and thus it is likely that Z36 induces autophagy by blocking the interaction between *Bcl-xL*/*Bcl-2* and Beclin 1 [41]. *Bcl-2* phosphorylation resulted in increased dissociation of the Bcl-2-Beclin 1 complex and increased Beclin 1-dependent autophagy. The activation of JNK upregulated Beclin 1 expression and mediated *Bcl-2* phosphorylation, thereby promoting cellular survival.

9.2.4. Beclin 1. *Metformin*, a widely used antidiabetic agent, should potentially induce autophagy as an activator of AMPK and an inhibitor of mTOR. *Metformin*'s action is mainly mediated by AMPK activation. 2-Deoxyglucose (2DG) is an inhibitor of glucose metabolism, since it inhibits hexokinase, the first rate-limiting enzyme of glycolysis. Treatment with 2DG leads to intracellular ATP depletion and induces autophagy in prostate cancer cells. *Metformin* inhibits 2DG-induced autophagy, reduces Beclin 1 expression, and triggers a switch from a survival process to cell death [81], suggesting that, depending on cell type, activation of AMPK is not automatically associated with the induction of autophagy.

9.2.5. Chemokines. The CC chemokine, *CCL2* (MCP-1), is one of the most frequently observed chemokines in the microenvironment of tumors. *CCL2* has been demonstrated to play a significant role in prostate cancer neoplasia and invasion. *CCL2* protects prostate cancer PC3 cells from autophagic death via the PI3K/Akt/survivin pathway and showed survivin to be a critical molecule in this survival mechanism [82]. *CCL2* stimulation of PC3 cells, upon serum deprivation, results in Akt hyperphosphorylation of the two key regulatory sites (Ser^{473} and Thr^{308}) required for its full activation. Survivin is a 16.5-kDa protein that belongs to the inhibitor of apoptosis protein (IAP) family that functions in mitotic progression and antagonizes caspases, thereby inhibiting apoptosis. Survivin is highly expressed in cancer tissues and its high level of expression is associated with poor prognosis and survival in many cancer types. *CCL2*-mediated survivin upregulation was shown to be PI3K/Akt-dependent; inhibition of this pathway not only abrogated survivin expression but also dramatically reduced cell survival. Strong evidence for interaction between survivin and LC3 was detected by co-immunoprecipitation experiments [82]. As LC3 plays an important role in the formation and expansion of autophagosomal membranes, the survivin-LC3 interaction could inhibit this process, which is then reflected in changes in LC3 localization as observed when starved cells are treated with *CCL2*.

9.2.6. Golgi-Associated Agents. Chemical inhibition of autophagy by incubations with *monensin* (coban) significantly increases the extent of apoptosis, which takes place via the mitochondrial pathway, and shortens the time in which the apoptotic markers are detectable. *Monensin* causes an accumulation of early forms of autophagic vacuoles and blocks the swelling of lysosomes seen in the presence of methylamine. Incubation with *monensin* at higher concentrations (10 and 100 μM) resulted in severe mitochondrial damage and marked dilatation of the Golgi apparatus and rough ER cisternae [83]. Interruption of microtubules with microtubule destabilizing *nocodazole* impairs the conversion of LC3-I to LC3-II but does not block the degradation

of LC3-II-associated autophagosomes. Strong inhibition of autophagic vacuole accumulation was found in *nocodazole*-arrested pseudoprometaphase cells. The loss of microtubules induced by *nocodazole* treatment results in the disconnection of Golgi stacks and dispersal into 70–100 smaller fragments [84].

9.2.7. Heat Shock Proteins. Heat shock protein 90 (Hsp90) forms a protein complex to maintain the stability of its client proteins. Disruption of this protein complex with specific Hsp90 inhibitors leads to proteolytic degradation of the client proteins, usually through the ubiquitin-proteasome pathway. Hsp90 forms a complex with Beclin 1 through an evolutionarily conserved domain to maintain the stability of Beclin 1. *Geldanamycin*, an Hsp90 inhibitor, effectively promoted proteasomal degradation of Beclin 1 in a concentration-dependent and time-dependent manner [85]. A small molecule called *2-phenylethynesulfonamide* (synonym, also called pifithrin-μ, PES) interacts selectively with Hsp70 and leads to a disruption of the association between Hsp70 and several of its cochaperones and substrate proteins. *PES* was identified as a novel Hsp70 inhibitor. *PES*-mediated inhibition of Hsp70 family proteins in tumor cells results in impairment of the autophagy-lysosome system. *PES* impaired the mitochondrial localization of p53 [86]. The *p14/p19*^{ARF} tumor suppressor gene is frequently mutated in human cancer. ARF (alternative reading frame) has been shown to localize to mitochondria and to induce autophagy. Treatment of cells with *PES* blocks the trafficking of ARF to mitochondria.

9.2.8. Lysosomal Function. Bafilomycin A1 is in the pleco-macrolide subclass of macrolide antibiotics, and a highly specific inhibitor of V-ATPase, whereas other types (F-type, P-type, Ca^{2+}, and K^{+}) of ATPases are not affected by this antibiotic. The anticancer effect of *bafilomycin A1* is well known and is attributed mainly to the inhibition of autophagy by preventing the fusion of autophagosomes with dysfunctional lysosomes, consequently triggering apoptosis [87]. *Lucanthone* (miracil D) has been extensively used as an antischistome agent. The drug also blocks topoisomerase II activity and has been reported to inhibit AP (apurinic/apyrimidinic) endonuclease, an important enzyme in DNA base excision repair. *Lucanthone* inhibits autophagy, caused by induction of lysosomal membrane permeabilization. *Lucanthone* is a novel autophagic inhibitor that induces apoptosis via cathepsin D accumulation. In addition, *lucanthone* enhanced the anticancer activity of the histone deacetylase inhibitor vorinostat [88]. *Lucanthone* is currently being investigated as a sensitizer to chemotherapy and radiation due to its ability to interfere with DNA repair. *Chloroquine* (avloclor) is a well-known 4-aminoquinoline class drug that is widely used for prophylaxis treatment against malaria. Pharmacologically, *chloroquine* is a weak base and is trapped in acidic organelles like lysosomes, resulting in increased vacuolar pH6.0. *Chloroquine* and its analog *hydroxychloroquine* (plaquenil) are the only clinically relevant autophagy inhibitors that block lysosyme acidification and degradation of autophagosomes [89]. *Chloroquine*, through its lysosomotropic properties, as well as its autophagy inhibition ability, may be a promising agent to be used in combination with chemotherapeutic agents to improve clinical results.

9.2.9. Small Regulatory RNAs. Small regulatory RNAs have become a specific and powerful tool to turn off the expression of target genes. Their actions include the suppression of overexpressed oncogenes, retarding cell division by interfering with cyclins and related genes or enhancing apoptosis by inhibiting anti-apoptotic genes. Paclitaxel, which stabilizes microtubules and causes apoptosis, offers both symptomatic and survival benefits for lung adenocarcinoma. Both autophagy and apoptosis are induced in cancer cells during the course of paclitaxel treatment. Paclitaxel treatment could lead to the formation of acidic vesicular organelles and the induction of Atg5, Beclin 1, and LC3 expression. Paclitaxel-mediated apoptotic cell death was further potentiated by pretreatment with *Beclin 1 siRNA* [90]. Lysosomal-associated membrane protein 2 (Lamp-2) is a ubiquitous lysosomal membrane protein that is highly expressed in normal human pancreatic tissue and is required for the proper fusion of lysosomes with autophagosomes in the late stage of the autophagic process. Silencing with a specific *Lamp-2 siRNA* abolished immunofluorescence particles for Lamp-2, confirming depletion of Lamp-2 protein. LC3-positive immunofluorescence puncta significantly increased in *Lamp-2*-silenced cells, suggesting that Lamp-2 depletion correlated with the accumulation of autophagosomes and a relative paucity of autolysosomes in pancreatic acinar cells. Lamp-2 depletion causes a "traffic jam" that culminates in the accumulation of autophagosomes [91]. MicroRNAs (miRNAs) are a class of endogenous, 22–24 nucleotide RNA molecules with the ability to induce mRNA degradation, translational repression, or both, via pairing with partially complementary sites in the 3′ UTR of the targeted genes. miRNAs can control the expression of autophagic genes, thereby modulating autophagic activity. Indeed, inhibition of the expression by *beclin 1 miR-30a* leads to suppression of autophagic activity [92].

10. Technical Errors in Autophagy Study

One of the technical problems in the study of autophagy is the lack of convenient and reliable methods to detect it. Uncertainty in the confirmation of the fully functional autophagic process could be one of the causes of confusion related to the role of autophagy in cell death. These types of confusion and difficulties are more pronounced in animal experiments that are critical in the study of cancers. LC3 is now widely used to monitor autophagy. One approach is to detect LC3 conversion (LC3-I to LC3-II) by immunoblot analysis because the amount of LC3-II is clearly correlated with the number of autophagosomes. Despite a higher molecular weight than LC3-I,

LC3-II migrates more rapidly in SDS-PAGE compared to LC3-I, likely due to its higher hydrophobicity associated with the phosphatidylethanolamine group. However, LC3-II itself is degraded by autophagy, making interpretation of LC3 immunoblotting results problematic. Furthermore, the amount of LC3 at a certain time point does not indicate autophagic flux. Monitoring of the natural autophagic substrate p62 (also called sequestosome 1) has been widely used to assess autophagic flux. The degradation of GFP-LC3 was clearly less sensitive for inducers and activators of autophagy than GFP-p62 [93]. A reduction in the abundance of p62 due to its sequestration into autophagosomes, which results from the direct molecular interaction between LC3 and the LC3 interacting motifs (LIR) of p62, a 22-amino-acid acidic peptide motif, is interpreted as a sign of increased autophagy [94]; however, endogenous p62 is not produced at a constant level because some cellular stress conditions lead to induction of p62 at the transcriptional level, meaning that the level of p62 proteins is not solely determined by its turnover. Earlier autophagy studies relied on cell staining and fluorescent microscopy. In particular, the overexpression of GFP-LC3, in which GFP (green fluorescent protein) is expressed as a fusion protein at the amino terminus of LC3, was widely used to measure autophagy. These studies are limited, however, by several issues as follows: incorporation into protein that aggregates independent of autophagy, induction of autophagy by transfection procedures, sensitivity of GFP-LC3 to acid pH, and cessation of fluorescence [95]. GFP is more resistant than LC3 in response to lysosomal degradation. When analyzing autophagic flux based on GFP-LC3 degradation, the accumulation of free GFP and GFP-LC3-II in the absence and presence of a lysosomal inhibitor should be compared. These may represent a serious problem in experiments testing the autophagic process.

Many compounds to regulate autophagy are limited by the uncertainty as to whether they specifically target one step of the autophagy process. It is of interest to compare relative drug effects obtained under different settings, including conditions, time points, and concentrations. The compound 3-MA has been touted as an autophagy inhibitor but requires mM concentrations to inhibit class III PI3Ks involved in autophagy. 3-MA not only arrests autophagy but also prevents apoptosis by inhibiting the release of cytochrome *c* and cathepsin B. Rapamycin, commonly applied to induce autophagy, enhanced autophagy under full medium but had a surprising inhibitory effect under nutrient deprivation conditions. Bafilomycin A1 is also commonly employed to suppress autophagic flux due to its inhibition of the vacuolar type H^+-ATPase (V-ATPase), blocking acidification of lysosomes and endosomes. However, long-term treatment (>4 h) of cells with bafilomycin A1 also interferes with the trafficking of proteosomes, endosomes, and other cellular processes [96]. Therefore, a need exists for chemicals that target specific components of the autophagy machinery for use as research tools, to address questions about autophagy mechanisms and investigate the role of autophagy in cancers.

11. Concluding Remarks

In this paper, the molecular players and mechanisms associated with autophagy have been described. Although autophagy is an important mechanism used by tumor cells to tolerate metabolic stress and is involved in nearly all stages of tumorigenesis and tumor progression, the issue of whether autophagy is aimed primarily at cell survival or at cell death is likely to remain a subject of debate for some time. As autophagy is a dynamic process that is difficult to measure and quantify, new techniques for assessing autophagy need to be developed to ensure progress in this area of investigation. The multifaceted nature of autophagy and its diverse cross-talk with other biological processes must be carefully considered when the autophagic system is targeted for anticancer benefit.

Acknowledgments

The author apologizes to all colleagues whose work may not have been cited for space reasons. He is grateful to D. Mrozek for editing the manuscript.

References

[1] B. Levine, "Cell biology: autophagy and cancer," *Nature*, vol. 446, no. 7137, pp. 745–747, 2007.

[2] O. Kepp, L. Galluzzi, M. Lipinski, J. Yuan, and G. Kroemer, "Cell death assays for drug discovery," *Nature Reviews Drug Discovery*, vol. 10, no. 3, pp. 221–237, 2011.

[3] N. Mizushima and B. Levine, "Autophagy in mammalian development and differentiation," *Nature Cell Biology*, vol. 12, no. 9, pp. 823–830, 2010.

[4] Z. Yang and D. J. Klionsky, "Eaten alive: a history of macroautophagy," *Nature Cell Biology*, vol. 12, no. 9, pp. 814–822, 2010.

[5] T. Shintani and D. J. Klionsky, "Autophagy in health and disease: a double-edged sword," *Science*, vol. 306, no. 5698, pp. 990–995, 2004.

[6] B. Levine and G. Kroemer, "Autophagy in the pathogenesis of disease," *Cell*, vol. 132, no. 1, pp. 27–42, 2008.

[7] E. H. Kim, S. Sohn, H. J. Kwon et al., "Sodium selenite induces superoxide-mediated mitochondrial damage and subsequent autophagic cell death in malignant glioma cells," *Cancer Research*, vol. 67, no. 13, pp. 6314–6324, 2007.

[8] Z. Luo, A. K. Saha, X. Xiang, and N. B. Ruderman, "AMPK, the metabolic syndrome and cancer," *Trends in Pharmacological Sciences*, vol. 26, no. 2, pp. 69–76, 2005.

[9] R. Okoshi, T. Ozaki, H. Yamamoto et al., "Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress," *Journal of Biological Chemistry*, vol. 283, no. 7, pp. 3979–3987, 2008.

[10] H. U. Park, S. Suy, M. Danner et al., "AMP-activated protein kinase promotes human prostate cancer cell growth and survival," *Molecular Cancer Therapeutics*, vol. 8, no. 4, pp. 733–741, 2009.

[11] I. Papandreou, A. L. Lim, K. Laderoute, and N. C. Denko, "Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L," *Cell Death and Differentiation*, vol. 15, no. 10, pp. 1572–1581, 2008.

[12] L. Harhaji-Trajkovic, U. Vilimanovich, T. Kravic-Stevovic, V. Bumbasirevic, and V. Trajkovic, "AMPK-mediated autophagy

inhibits apoptosis in cisplatin-treated tumour cells," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 9 B, pp. 3644–3654, 2009.

[13] X. Qu, Z. Zou, Q. Sun et al., "Autophagy gene-dependent clearance of apoptotic cells during embryonic development," *Cell*, vol. 128, no. 5, pp. 931–946, 2007.

[14] M. Komatsu, S. Waguri, T. Ueno et al., "Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice," *Journal of Cell Biology*, vol. 169, no. 3, pp. 425–434, 2005.

[15] A. R. Winslow and D. C. Rubinsztein, "Autophagy in neurodegeneration and development," *Biochimica et Biophysica Acta*, vol. 1782, no. 12, pp. 723–729, 2008.

[16] S. E. Logue and S. J. Martin, "Caspase activation cascades in apoptosis," *Biochemical Society Transactions*, vol. 36, no. 1, pp. 1–9, 2008.

[17] P. Pizzo and T. Pozzan, "Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics," *Trends in Cell Biology*, vol. 17, no. 10, pp. 511–517, 2007.

[18] G. Petrovski, G. Zahuczky, K. Katona et al., "Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes," *Cell Death and Differentiation*, vol. 14, no. 6, pp. 1117–1128, 2007.

[19] L. Galluzzi, M. C. Maiuri, I. Vitale et al., "Cell death modalities: classification and pathophysiological implications," *Cell Death and Differentiation*, vol. 14, no. 7, pp. 1237–1243, 2007.

[20] A. Apel, I. Herr, H. Schwarz, H. P. Rodemann, and A. Mayer, "Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy," *Cancer Research*, vol. 68, no. 5, pp. 1485–1494, 2008.

[21] J. A. Bertout, S. A. Patel, and M. C. Simon, "The impact of O_2 availability on human cancer," *Nature Reviews Cancer*, vol. 8, no. 12, pp. 967–975, 2008.

[22] X. Qu, J. Yu, G. Bhagat et al., "Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene," *Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1809–1820, 2003.

[23] C. Liang, P. Feng, B. Ku et al., "Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG," *Nature Cell Biology*, vol. 8, no. 7, pp. 688–699, 2006.

[24] B. X. Li, C. Y. Li, R. Q. Peng et al., "The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers," *Autophagy*, vol. 5, no. 3, pp. 303–306, 2009.

[25] K. M. Nicholson and N. G. Anderson, "The protein kinase B/Akt signalling pathway in human malignancy," *Cellular Signalling*, vol. 14, no. 5, pp. 381–395, 2002.

[26] R. Mathew, C. M. Karp, B. Beaudoin et al., "Autophagy Suppresses Tumorigenesis through Elimination of p62," *Cell*, vol. 137, no. 6, pp. 1062–1075, 2009.

[27] R. Mathew, V. Karantza-Wadsworth, and E. White, "Role of autophagy in cancer," *Nature Reviews Cancer*, vol. 7, no. 12, pp. 961–967, 2007.

[28] S. Yousefi, R. Perozzo, I. Schmid et al., "Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis," *Nature Cell Biology*, vol. 8, no. 10, pp. 1124–1132, 2006.

[29] Y. Wei, S. Sinha, and B. Levine, "Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation," *Autophagy*, vol. 4, no. 7, pp. 949–951, 2008.

[30] E. Zalckvar, H. Berissi, L. Mizrachy et al., "DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy," *EMBO Reports*, vol. 10, no. 3, pp. 285–292, 2009.

[31] D. R. Green and G. Kroemer, "Cytoplasmic functions of the tumour suppressor p53," *Nature*, vol. 458, no. 7242, pp. 1127–1130, 2009.

[32] A. V. Budanov and M. Karin, "p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling," *Cell*, vol. 134, no. 3, pp. 451–460, 2008.

[33] A. Eisenberg-Lerner, S. Bialik, H. U. Simon, and A. Kimchi, "Life and death partners: apoptosis, autophagy and the cross-talk between them," *Cell Death and Differentiation*, vol. 16, no. 7, pp. 966–975, 2009.

[34] L. Moretti, Y. I. Cha, K. J. Niermann, and B. Lu, "Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress?" *Cell Cycle*, vol. 6, no. 7, pp. 793–798, 2007.

[35] M. Djavaheri-Mergny, M. C. Maiuri, and G. Kroemer, "Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1," *Oncogene*, vol. 29, no. 12, pp. 1717–1719, 2010.

[36] D. H. Cho, Y. K. Jo, J. J. Hwang, Y. M. Lee, S. A. Roh, and J. C. Kim, "Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells," *Cancer Letters*, vol. 274, no. 1, pp. 95–100, 2009.

[37] H. Li, P. Wang, Q. Sun et al., "Following cytochrome c release, autophagy is inhibited during chemotherapy-induced apoptosis by caspase 8-mediated cleavage of beclin 1," *Cancer Research*, vol. 71, no. 10, pp. 3625–3634, 2011.

[38] S. Luo and D. C. Rubinsztein, "Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL," *Cell Death and Differentiation*, vol. 17, no. 2, pp. 268–277, 2010.

[39] D. Furuya, N. Tsuji, A. Yagihashi, and N. Watanabe, "Beclin 1 augmented cis-diamminedichloroplatinum induced apoptosis via enhancing caspase-9 activity," *Experimental Cell Research*, vol. 307, no. 1, pp. 26–40, 2005.

[40] S. P. Elmore, T. Qian, S. F. Grissom, and J. J. Lemasters, "The mitochondrial permeability transition initiates autophagy in rat hepatocytes," *The FASEB Journal*, vol. 15, no. 12, pp. 2286–2287, 2001.

[41] M. C. Maiuri, G. Le Toumelin, A. Criollo et al., "Functional and physical interaction between Bcl-XL and a BH3-like domain in Beclin-1," *EMBO Journal*, vol. 26, no. 10, pp. 2527–2539, 2007.

[42] S. Pattingre, A. Tassa, X. Qu et al., "Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy," *Cell*, vol. 122, no. 6, pp. 927–939, 2005.

[43] M. Hoyer-Hansen, L. Bastholm, P. Szyniarowski et al., "Control of Macroautophagy by Calcium, Calmodulin-Dependent Kinase Kinase-β, and Bcl-2," *Molecular Cell*, vol. 25, no. 2, pp. 193–205, 2007.

[44] S. Jiang and B. Storrie, "Cisternal rab proteins regulate Golgi apparatus redistribution in response to hypotonic stress," *Molecular Biology of the Cell*, vol. 16, no. 5, pp. 2586–2596, 2005.

[45] T. Itoh, N. Fujita, E. Kanno, A. Yamamoto, T. Yoshimori, and M. Fukuda, "Golgi-resident small GTPase Rab33B interacts with Atg16L and modulates autophagosome formation," *Molecular Biology of the Cell*, vol. 19, no. 7, pp. 2916–2925, 2008.

[46] Y. Takahashi, C. L. Meyerkord, T. Hori et al., "Bif-1 regulates Atg9 trafficking by mediating the fission of Golgi membranes during autophagy," *Autophagy*, vol. 7, no. 1, pp. 61–73, 2011.

[47] Y. F. Tu, B. A. Kaipparettu, Y. Ma, and L. J. C. Wong, "Mitochondria of highly metastatic breast cancer cell line MDA-MB-231 exhibits increased autophagic properties," *Biochimica et Biophysica Acta*, vol. 1807, no. 9, pp. 1125–1132, 2011.

[48] T. Kanzawa, L. Zhang, L. Xiao, I. M. Germano, Y. Kondo, and S. Kondo, "Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3," *Oncogene*, vol. 24, no. 6, pp. 980–991, 2005.

[49] B. N. Desai, B. R. Myers, and S. L. Schreiber, "FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 7, pp. 4319–4324, 2002.

[50] H. Harada, J. S. Andersen, M. Mann, N. Terada, and S. J. Korsmeyer, "p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 17, pp. 9666–9670, 2001.

[51] Y. Tang, Y. Chen, H. Jiang, and D. Nie, "The role of short-chain fatty acids in orchestrating two types of programmed cell death in colon cancer," *Autophagy*, vol. 7, no. 2, pp. 235–237, 2011.

[52] S. Huang and F. A. Sinicrope, "Celecoxib-induced apoptosis is enhanced by ABT-737 and by inhibition of autophagy in human colorectal cancer cells," *Autophagy*, vol. 6, no. 2, pp. 256–269, 2010.

[53] B. Fazi, W. Bursch, G. M. Fimia et al., "Fenretinide induces autophagic cell death in caspase-defective breast cancer cells," *Autophagy*, vol. 4, no. 4, pp. 435–441, 2008.

[54] J. M. Vicencio, C. Ortiz, A. Criollo et al., "The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1," *Cell Death and Differentiation*, vol. 16, no. 7, pp. 1006–1017, 2009.

[55] S. Y. Yoon, J. E. Choi, H. S. Kweon et al., "Okadaic acid increases autophagosomes in rat neurons: implications for Alzheimer's disease," *Journal of Neuroscience Research*, vol. 86, no. 14, pp. 3230–3239, 2008.

[56] H. Rodriguez-Rocha, A. Garcia-Garcia, M. I. Panayiotidis, and R. Franco, "DNA damage and autophagy," *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 711, no. 1-2, pp. 158–166, 2011.

[57] W. X. Ding, H. M. Ni, W. Gao et al., "Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival," *Journal of Biological Chemistry*, vol. 282, no. 7, pp. 4702–4710, 2007.

[58] M. Milani, T. Rzymski, H. R. Mellor et al., "The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with Bortezomib," *Cancer Research*, vol. 69, no. 10, pp. 4415–4423, 2009.

[59] Y. H. Shi, Z. -B. Ding, J. Zhou et al., "Targeting autophagy enhances sorafenib lethality for hepatocellular carcinoma via ER stress-related apoptosis," *Autophagy*, vol. 7, no. 10, pp. 1159–1172, 2011.

[60] J. Pan, B. Chen, C. H. Su et al., "Autophagy induced by farnesyltransferase inhibitors in cancer cells," *Cancer Biology and Therapy*, vol. 7, no. 10, pp. 1679–1684, 2008.

[61] F. C. M. Zoppino, R. D. Militello, I. Slavin, C. Álvarez, and M. I. Colombo, "Autophagosome formation depends on the small GTPase rab1 and functional ER exit sites," *Traffic*, vol. 11, no. 9, pp. 1246–1261, 2010.

[62] K. H. Eum and M. Lee, "Crosstalk between autophagy and apoptosis in the regulation of paclitaxel-induced cell death in v-Ha-ras-transformed fibroblasts," *Molecular and Cellular Biochemistry*, vol. 348, no. 1-2, pp. 61–68, 2011.

[63] H. Rikiishi, "Possible role of autophagy in the treatment of pancreatic cancer with histone deacetylase inhibitors," *Cancers*, vol. 2, no. 4, pp. 2026–2043, 2010.

[64] Q. Cao, C. Yu, R. Xue et al., "Autophagy induced by suberoylanilide hydroxamic acid in Hela S3 cells involves inhibition of protein kinase B and up-regulation of Beclin 1," *International Journal of Biochemistry and Cell Biology*, vol. 40, no. 2, pp. 272–283, 2008.

[65] J. Fu, C. J. Shao, F. R. Chen, H. K. Ng, and Z. P. Chen, "Autophagy induced by valproic acid is associated with oxidative stress in glioma cell lines," *Neuro-Oncology*, vol. 12, no. 4, pp. 328–340, 2010.

[66] M. Watanabe, S. Adachi, H. Matsubara et al., "Induction of autophagy in malignant rhabdoid tumor cells by the histone deacetylase inhibitor FK228 through AIF translocation," *International Journal of Cancer*, vol. 124, no. 1, pp. 55–67, 2009.

[67] D. Delmas, E. Solary, and N. Latruffe, "Resveratrol, a phytochemical inducer of multiple cell death pathways: apoptosis, autophagy and mitotic catastrophe," *Current Medicinal Chemistry*, vol. 18, no. 8, pp. 1100–1121, 2011.

[68] F. Lefranc, V. Facchini, and R. Kiss, "Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas," *Oncologist*, vol. 12, no. 12, pp. 1395–1403, 2007.

[69] N. Wang, Y. Feng, M. Zhu et al., "Berberine induces autophagic cell death and mitochondrial apoptosis in liver cancer cells: the cellular mechanism," *Journal of Cellular Biochemistry*, vol. 111, no. 6, pp. 1426–1436, 2010.

[70] S. Schenone, C. Brullo, F. Musumeci, M. Radi, and M. Botta, "ATP-competitive inhibitors of mTOR: an update," *Current Medicinal Chemistry*, vol. 18, no. 20, pp. 2995–3014, 2011.

[71] J. M. García-Martínez, J. Moran, R. G. Clarke et al., "Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR)," *Biochemical Journal*, vol. 421, no. 1, pp. 29–42, 2009.

[72] Y. H. Ling, M. Aracil, Y. Zou et al., "PM02734 (elisidepsin) induces caspase-independent cell death associated with features of autophagy, inhibition of the Akt/mTOR signaling pathway, and activation of death-associated protein kinase," *Clinical Cancer Research*, vol. 17, no. 16, pp. 5353–5366, 2011.

[73] S. Mostowy, V. Sancho-Shimizu, M. A. Hamon et al., "p62 and NDP52 proteins target intracytosolic Shigella and Listeria to different autophagy pathways," *Journal of Biological Chemistry*, vol. 286, no. 30, pp. 26987–26995, 2011.

[74] Z. Tallóczy, H. W. Virgin, and B. Levine, "PKR-dependent autophagic degradation of herpes simplex virus type 1," *Autophagy*, vol. 2, no. 1, pp. 24–29, 2006.

[75] W. K. K. Wu, Y. C. Wu, L. Yu, Z. J. Li, J. J. Y. Sung, and C. H. Cho, "Induction of autophagy by proteasome inhibitor is associated with proliferative arrest in colon cancer cells," *Biochemical and Biophysical Research Communications*, vol. 374, no. 2, pp. 258–263, 2008.

[76] C. R. Archer, D. L. T. Koomoa, E. M. Mitsunaga et al., "Syrbactin class proteasome inhibitor-induced apoptosis and autophagy occurs in association with p53 accumulation and Akt/PKB activation in neuroblastoma," *Biochemical Pharmacology*, vol. 80, no. 2, pp. 170–178, 2010.

[77] J. M. Norman, G. M. Cohen, and E. T. W. Bampton, "The in vitro cleavage of the hAtg proteins by cell death proteases," *Autophagy*, vol. 6, no. 8, pp. 1042–1056, 2010.

[78] E. Donohue, A. Tovey, A. W. Vogl et al., "Inhibition of autophagosome formation by the benzoporphyrin derivative verteporfin," *Journal of Biological Chemistry*, vol. 286, no. 9, pp. 7290–7300, 2011.

[79] A. Petiot, E. Ogier-Denis, E. F. C. Blommaart, A. J. Meijer, and P. Codogno, "Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells," *Journal of Biological Chemistry*, vol. 275, no. 2, pp. 992–998, 2000.

[80] S. Sinha and B. Levine, "The autophagy effector Beclin 1: a novel BH3-only protein," *Oncogene*, vol. 27, no. 1, supplement 1, pp. S137–S148, 2008.

[81] I. B. Sahra, K. Laurent, S. Giuliano et al., "Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells," *Cancer Research*, vol. 70, no. 6, pp. 2465–2475, 2010.

[82] H. Roca, Z. Varsos, and K. J. Pienta, "CCL2 protects prostate cancer PC3 cells from autophagic death via phosphatidylinositol 3-kinase/AKT-dependent survivin Up-regulation," *Journal of Biological Chemistry*, vol. 283, no. 36, pp. 25057–25073, 2008.

[83] A. C. Souza, F. S. Machado, M. R. N. Celes et al., "Mitochondrial damage as an early event of monensin-induced cell injury in cultured fibroblasts L929," *Journal of Veterinary Medicine. Series A*, vol. 52, no. 5, pp. 230–237, 2005.

[84] Y. F. Fuchs, S. A. Eisler, G. Link et al., "A Golgi PKD activity reporter reveals a crucial role of PKD in nocodazole-induced Golgi dispersal," *Traffic*, vol. 10, no. 7, pp. 858–867, 2009.

[85] C. Xu, J. Liu, L. -C. Hsu, Y. Luo, R. Xiang, and T. H. Chuang, "Functional interaction of Heat Shock protein 90 and beclin 1 modulates toll-like receptor-mediated autophagy," *The FASEB Journal*, vol. 25, no. 8, pp. 2700–2710, 2011.

[86] J. I. J. Leu, J. Pimkina, A. Frank, M. E. Murphy, and D. L. George, "A Small Molecule Inhibitor of Inducible Heat Shock Protein 70," *Molecular Cell*, vol. 36, no. 1, pp. 15–27, 2009.

[87] Y. C. Wu, W. K. K. Wu, Y. Li et al., "Inhibition of macroautophagy by bafilomycin A1 lowers proliferation and induces apoptosis in colon cancer cells," *Biochemical and Biophysical Research Communications*, vol. 382, no. 2, pp. 451–456, 2009.

[88] J. S. Carew, C. M. Espitia, J. A. Esquivel II et al., "Lucanthone is a novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis," *Journal of Biological Chemistry*, vol. 286, no. 8, pp. 6602–6613, 2011.

[89] P. Boya, R. A. González-Polo, N. Casares et al., "Inhibition of macroautophagy triggers apoptosis," *Molecular and Cellular Biology*, vol. 25, no. 3, pp. 1025–1040, 2005.

[90] G. Xi, X. Hu, B. Wu et al., "Autophagy inhibition promotes paclitaxel-induced apoptosis in cancer cells," *Cancer Letters*, vol. 307, no. 2, pp. 141–148, 2011.

[91] F. Fortunato, H. Bürgers, F. Bergmann et al., "Impaired autolysosome formation correlates with lamp-2 depletion: role of apoptosis, autophagy, and necrosis in pancreatitis," *Gastroenterology*, vol. 137, no. 1, pp. 350–360, 2009.

[92] H. Zhu, H. Wu, X. Liu et al., "Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells," *Autophagy*, vol. 5, no. 6, pp. 816–823, 2009.

[93] K. B. Larsen, T. Lamark, A. Overvatn, I. Harneshaug, T. Johansen, and G. Bjorkoy, "A reporter cell system to monitor autophagy based on p62/SQSTM1," *Autophagy*, vol. 6, no. 6, pp. 784–793, 2010.

[94] S. Pankiv, T. H. Clausen, T. Lamark et al., "p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy," *Journal of Biological Chemistry*, vol. 282, no. 33, pp. 24131–24145, 2007.

[95] A. Kuma, M. Matsui, and N. Mizushima, "LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization," *Autophagy*, vol. 3, no. 4, pp. 323–328, 2007.

[96] S. Barth, D. Glick, and K. F. Macleod, "Autophagy: assays and artifacts," *Journal of Pathology*, vol. 221, no. 2, pp. 117–124, 2010.

Hindawi Publishing Corporation
International Journal of Cell Biology
Volume 2012, Article ID 424072, 6 pages
doi:10.1155/2012/424072

Research Article

Effects of Cisplatin in Neuroblastoma Rat Cells: Damage to Cellular Organelles

Giada Santin,[1] Luigi Scietti,[2] Paola Veneroni,[2] Sergio Barni,[3] Graziella Bernocchi,[2] and Maria Grazia Bottone[1,2]

[1] *Istituto di Genetica Molecolare, CNR, 27100 Pavia, Italy*
[2] *Laboratorio di Biologia Cellulare e Neurobiologia, Dipartimento di Biologia Animale, Università di Pavia, Via Ferrata, 27100 Pavia, Italy*
[3] *Laboratorio di Anatomia Comparata e Citologia, Dipartimento di Biologia Animale, Università di Pavia, 27100 Pavia, Italy*

Correspondence should be addressed to Maria Grazia Bottone, bottone@unipv.it

Received 28 October 2011; Accepted 12 December 2011

Academic Editor: Lina Ghibelli

Cisplatin (cisPt) is a chemotherapy agent used as a treatment for several types of cancer. The main cytotoxic effect of cisplatin is generally accepted to be DNA damage. Recently, the mechanism by which cisPt generates the cascade of events involved in the apoptotic process has been demonstrated. In particular it has been shown that some organelles are cisPt target and are involved in cell death. This paper aims to describe the morphological and functional changes of the Golgi apparatus and lysosomes during apoptosis induced in neuronal rat cells (B50) by cisplatin. The results obtained show that the cellular organelles are the target of cisPt, so their damage can induce cell death.

1. Introduction

From many years, cisplatin (cisPt) was used in chemotherapy of different cancers because of its apoptotic effects. It binds DNA-generating adduct cisPt/DNA that provokes the distortion of double helix blocking the transcription and the replication processes [1]. We reported that, in B50 neuroblastoma rat cells, cisPt induces cytotoxic cell death mediated by activation of death receptor-mediated apoptotic signaling mechanisms as well as mitochondrial pathways [2, 3]. Nevertheless, organelles damage cisPt induced is today not so well elucidated. The cytotoxic action of this drug can be initiated by cytoplasmic events thus determining organelle damage. Yu et al. [4] demonstrated, in fact, that cisplatin initiates apoptosis from the cytoplasm and suggested that nuclear events may not be critical for the initiation of cisplatin-induced cytotoxicity, at least, not in immortalized mouse kidney proximal tubule epithelial (TKPTS) cells [4].

Apoptosis is a genetically controlled cell death program consisting of several essential steps that are critical checkpoints, as well as nonessential steps depending on the cell type, context, or pathophysiological stimuli [5]. Moreover, the intricate network of relationships and communication (i.e., crosstalk in which multiple organelles emit signals and receive responses) occurs between all the cellular organelles. During apoptosis, the cytoskeleton undergoes disassembly, bringing with him the different organelles, such as the Golgi vesicles, which in apoptosis following the reorganization of microtubules [6–8] wasting a clear spatial organization in the cell [9, 10]. Mitochondria and endoplasmic reticulum are, respectively, involved in intrinsic apoptotic pathway and in the pathway mediated by caspase 12 [11].

We have demonstrated that in B50 neuronal rat cells cisplatin induces apoptosis, morphological and functional modification of cytoskeleton, mitochondria [12], and endoplasmic reticulum [2], yet. In this work we applied histochemical techniques in confocal microscopy and electron microscopy to the analyses of changes of Golgi apparatus and lysosomes in B50 cells after treatment with cisPt.

2. Material and Methods

2.1. Cells and Treatments. B50 neuroblastoma rat cells (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, cat. n. BS TCL 115) were cultured in

"75 cm^2" flasks in Dulbecco's minimal essential medium supplemented with "10%" fetal bovine serum, "1%" glutamine, "100 U" penicillin, and streptomycin (Celbio) in a "5% CO_2" humidified atmosphere. "24 h" before experiments, cells were seeded on glass coverslips for fluorescence microscopy, or grown in "75 cm^2" plastic flasks for electron microscopy analysis.

Cells are submitted to a continued exposure to cisPt (Teva Pharma) "40 μM" for "48 h" at "37°C". This concentration was chosen considering our in vivo experimental design (i.e., a single injection of "5 μg/g" b. w. in "10-day-old" rats); this dose corresponds to the dose most commonly used in chemotherapy [13, 14]. For immunocytochemical analysis, after the treatments, the samples grown on coverslips were fixed with "4%" formalin and postfixed with "70%" ethanol for "30 min" each, at "−20°C."

2.2. Double Immunocytochemical Detection of Golgi Proteins and Cytoskeletal Components. Cells were incubated with a solution of anti-Golgi proteins (autoimmune serum recognizing the Golgi proteins, a kind gift of the IRCCS San Matteo, Pavia, Italy) diluted "1 : 400" in PBS and another primary antibody: Alexa 594-Phalloidin (Molecular Probes, Invitrogen) diluted "1 : 40" in PBS. After "60 min" at room temperature, coverslips were incubated with the secondary antibody: Alexa 488-conjugated anti-human antibody (Molecular Probes, Invitrogen) diluted "1 : 200" in PBS for Golgi protein for "1 h." Sections were counterstained for DNA with "0.1 μg/mL" Hoechst "33258," washed with PBS, and mounted in a drop of Mowiol (Calbiochem), for confocal microscopy analysis. Three independent experiments were carried out.

2.3. Double Immunocytochemical Detection of Golgi Proteins and Endoplasmic Reticulum. Cells were incubated with a solution of anti-Golgi proteins (autoimmune serum recognizing the Golgi proteins, a kind gift of the IRCCS San Matteo, Pavia, Italy) diluted "1 : 400" in PBS and another primary antibody: antireticulum proteins (Abcam) diluted "1 : 100" in PBS. After "60 min" at room temperature, coverslips were incubated with the secondary antibodies: Alexa 488-conjugated anti-human antibody (Molecular Probes, Invitrogen) diluted "1 : 200" in PBS for Golgi protein and Alexa-594-coniugated anti-rabbit antibody for reticulum proteins for "1 h." Sections were counterstained for DNA with "0.1 μg/mL" Hoechst "33258," washed with PBS and mounted in a drop of Mowiol (Calbiochem), for confocal microscopy analysis. Three independent experiments were carried out.

2.4. Golgi Apparatus and Endoplasmic Reticulum Morphological Features at Transmission Electron Microscopy (TEM). For TEM analysis, the cells grown in "75 cm^2" plastic flasks for electron microscopy harvested by mild trypsinization ("0.25%" trypsin in PBS containing "0.05%" EDTA), immediately fixed with "2%" glutaraldehyde in the culture medium ("1 h" at "4°C") and postfixed in "1% OsO_4" in PBS for "1 h" at room temperature. The cell pellets were embedded in "2%" agar, thoroughly rinsed with Sörensen buffer ("pH 7.2") and dehydrated in ethanol. Finally, the pellets were embedded in Epon resin and polymerized at "60°C" for "24 h." Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss EM900 transmission electron microscope.

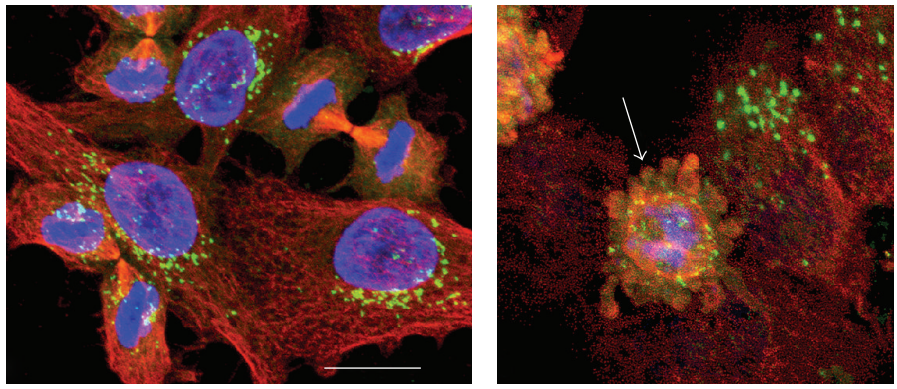
2.5. Double Immunocytochemical Detection of Lysosomes Proteins and Mitochondria. Cells were incubated with a solution of antilysosomes proteins (autoimmune serum recognizing the lysosome proteins, a kind gift of the IRCCS San Matteo, Pavia, Italy) diluted "1 : 500" in PBS and another primary antibody: mitochondrial anti-HSP70 (Molecular Probes, Invitrogen) diluted "1 : 50" in PBS. After "60 min" at room temperature, coverslips were incubated with the secondary antibody: Alexa 594-conjugated anti-human antibody (Molecular Probes, Invitrogen) diluted "1 : 200" in PBS for lysosome proteins and Alexa 488-coniugated anti-mouse for HSP70 for "1 h." Sections were counterstained for DNA with "0.1 μg/mL" Hoechst "33258", washed with PBS, and mounted in a drop of Mowiol (Calbiochem), for confocal microscopy analysis. Three independent experiments were carried out. Confocal microscope software was used to obtain bar charts of colocalization; eight fields of cells were considered to count number of lysosomes per cells, both in control and in treated sample, and the graphic was done with excel program.

2.6. Confocal Fluorescence Microscopy. For confocal laser scanning microscopy, Leica TCS-SP system (Leica, Heidelberg, Germany) mounted on a Leica DMIRBE-inverted microscope was used. For fluorescence excitation, an Ar/UV laser at "364 nm" was used for Hoechst "33258", an Ar/Vis laser at "488 nm" was used for FITC, and an He/Ne laser at "543 nm" was used for Alexa 594. Spaced ("0.5 μm") optical sections were recorded using a "63×" oil immersion objective. Images were collected in the "1024 × 1024 pixel" format, stored on a magnetic mass memory and processed by Leica confocal software.

3. Results

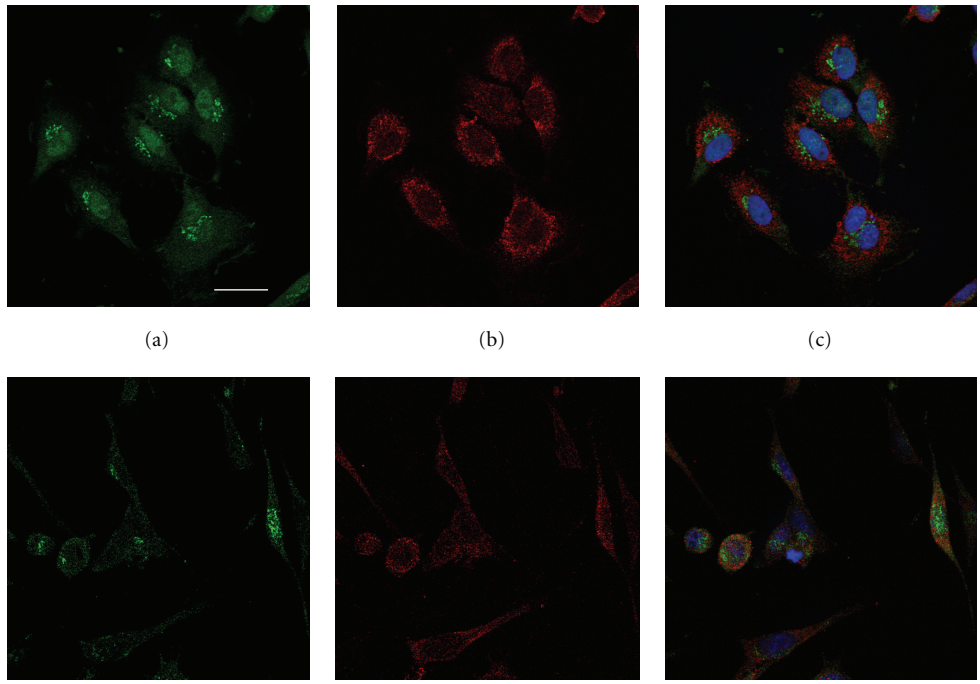
Our experiments revealed that cisPt treatment entails an organellar degradation and a reorganization in the cytoplasm. In particular, Golgi apparatus, endoplasmic reticulum, and lysosomes are the main target of the drug.

Figure 1 shows the Golgi apparatus (green fluorescence) in control cells (a) and in 48 h cisPt-treated cells (b); in control cells, Golgi exhibits its typical shape "ribbon like," with flattened cisternae, according to its function as a proteins sorter. It mostly has a perinuclear localization in the cytoplasm with a progressive decreased concentration towards the periphery of the cell. In cisPt treated cells, a strong production of vesicles is visible, accompanied by a spatial redistribution and dense masses. After treatment, the tubulin cytoskeleton (red fluorescence) shows morphological changes, too. Soldani et al. [15] demonstrated that the morphological alterations of the Golgi apparatus may be



(a) (b)

Figure 1: Double immunolabeling of Golgi apparatus (green fluorescence) and α-tubulin (red fluorescence) in B50 control cells (a) and in 48 h cisPt-treated cells (b). In (b), arrow indicates an apoptotic cell. DNA is counterstained with Hoechst 33258 (blue fluorescence). Bar: 20 μm.



(a) (b) (c)

(a′) (b′) (c′)

Figure 2: Double immunolabeling of Golgi apparatus (green fluorescence) and endoplasmic reticulum (red fluorescence) in B50 control cells (a, b, and c) and in 48 h cisPt-treated cells (a′, b′, and c′). In (c) and (c′), DNA is counterstained with Hoechst 33258 (blue fluorescence). Bar: 20 μm.

related to structural changes in cytoskeletal apparatus [15]; in the treated cells, in fact, it can be observed that the vesiculation of this organelle is localized mainly at the periphery of the cell (arrow).

It is well known that the endoplasmic reticulum and Golgi apparatus are closely linked in terms of both organizational and functional point of view in the process of synthesis and posttranslational maturation of proteins through an intense vesicular traffic. In Figure 2, the double immunoexpression of Golgi apparatus and endoplasmic reticulum is shown both in control cells (a, b, and c) and in treated cells (a′, b′, and c′). In the first condition, Golgi apparatus maintains the perinuclear location (a) as it is above described in Figure 1, and endoplasmic reticulum is homogeneously arranged in the whole cytoplasm (b), where it is main concentrated near the nucleus. In the second

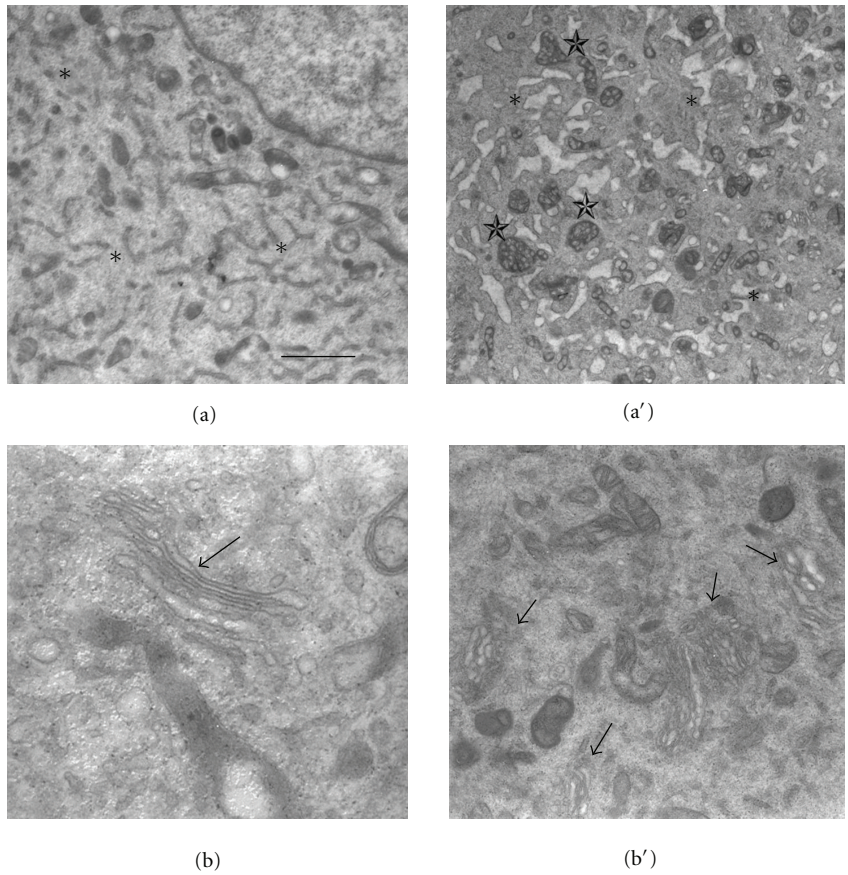


Figure 3: Cellular morphology at TEM in B50 control cells (a and b) and in 48 h cisPt-treated cells (a′ and b′). In (a and a′), asterisks indicatesendoplasmic reticulum; in (a′), stars indicate damaged mitochondria. In (b and b′), arrows indicate Golgi apparatus. Bar: 6 μm.

condition, Golgi apparatus undergoes a strong disruption losing the typical semilunar shape (a′), while endoplasmic reticulum is less thickened and quite disrupted, too (b′).

Figure 3 depicts the ultrastructural morphology of B50 control (a and b) and treated (a′ and b′) cells at TEM. In (a) it is visible a cellular area with the intact endoplasmic reticulum (asterisks) properly distributed in the cytoplasm, but after treatment (a′) it results heavily dilated (asterisks). In (a′) it is also appreciable the mitochondrial damage (stars) cisPt induced [12]. In (b) Golgi apparatus in physiological condition is shown (arrow), but after treatment (b′) it results suffering, because it loses the regular shape of cisternae which becomes in some case dilated and in other more thickened (arrows).

Lysosomes are cisPt target, too. In Figure 4 the double immunolabeling of lysosomes (red fluorescence) and mitochondria (green fluorescence) is reported, compared to a control situation (a), where lysosomes are numerous, and mitochondria presents their typical cytoplasmic distribution, after cisPt injury (b) cells show a decrease of lysosomes number (c: from 83 ± 20.44 to 50.5 ± 6.99; a reduction of about 30%) and mitochondria form dense perinuclear masses (b: triangle). Moreover, it has to be noted that lysosomal and mitochondrial expressions do not colocalized in treated cells (see bar charts of colocalization b′ and b″ for the selected point of noncolocalization asterisk in b).

4. Discussion

It is generally accepted that the damage induced upon binding of cisPt with the DNA may inhibit transcription and/or DNA replication mechanisms [16]. The process of cisplatin-induced apoptosis is complex and involves multiple pathways [2, 12], and in literature several studies demonstrate that apoptosis induced by cisPt can also be DNA injury independent [17]. Nevertheless, in cisPt-induced cell death mitochondrial are considered perhaps a more important target than nuclear DNA damage [18], due to hydrolyzed cisPt that generates a positively charged metabolite which preferentially accumulates within the negatively charged mitochondria. Thus, the sensitivity of cells to cisplatin appears to correlate with both the density of mitochondria [19] and the mitochondrial membrane potential [20]. We have already demonstrated that cisPt targets, in addition to DNA, included the cytoskeleton (actin and tubulin) and mitochondria [12]. Recent data have shown that cisPt is able to induce cell death in different cell lines and that this implies the involvement of cytoplasmic organelles [11]. In addition,

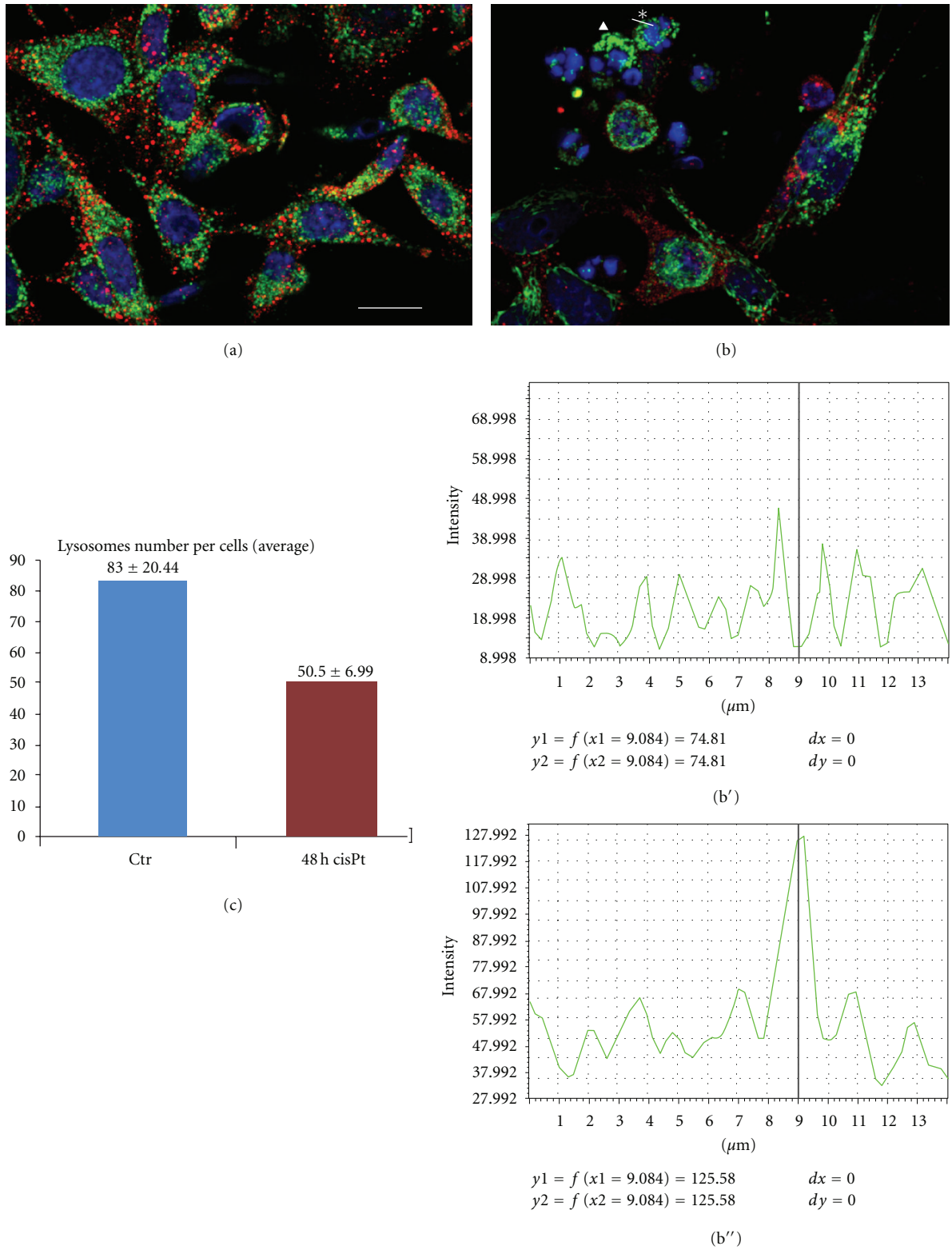


Figure 4: Double immunolabeling of lysosomes (red fluorescence) and mitochondria (green fluorescence) in B50 control cells (a) and in 48 h cisPt-treated cells (b). DNA is counterstained with Hoechst 33258 (blue fluorescence). Bar: 20 μm. In (c), bar chart of lysosomes number per cells (average). In b, triangle represents dense mitochondrial masses, asterisk represents the selected point of noncolocalization. In (b′, b″), bar charts of colocalization, the (b′) bar chart is for red fluorescence, the (b″) is for green fluorescence.

Nakagomi et al. [21] have identified the fragmentation of the Golgi apparatus during apoptosis as a marker of some neurodegenerative diseases [21]. The results obtained have allowed us to demonstrate the onset of organelles alterations after cisPt treatment in B50 neuroblastoma rat cells. In particular, we focused on Golgi apparatus and endoplasmic reticulum injury, organelles that make up the digestive vacuole of the cell [7]. Both of them have been called "stress sensor" as it has been shown that proteins on their membranes are able to trigger the caspase cascade by activating caspase-12 [2, 6, 8]. Our obtained results, both in confocal microscopy and in TEM, indicate that these organelles undergo strong rearrangement due to the 48 h cisPt treatment and that these alterations are linked to the reorganization of cytoskeletal tubulin. This evidence suggests that the whole process is also on the base of the generation of apoptotic bodies, designed for the ultimate phagocytosis.

The effect of cisPt has been also evident in lysosomes that become smaller and less numerous in treated cells. Because lysosomes are involved also in autophagy mechanisms, we applied a double immunolabeling of lysosomes and mitochondria in order to analyze if there was a colocalization between the two respective fluorescence. The absence of this colocalization after 48 h treatment indicates that cisPt does not induce any autophagic process as alternative response to the treatment. So, we can conclude that immunocytochemical techniques and the TEM are useful to demonstrate that B50 neuroblastoma cells undergo apoptosis through organelles injury, and not only as a consequent of DNA damage.

Acknowledgments

The research was supported by the following Italian Institutions: Fondazione Banca del Monte di Lombardia and University of Pavia (Fondi di Ateneo per la Ricerca, (FAR) 2010). Santin G. is supported by funding from "Superpig Program," project cofinanced by Lombardy Region through the "Fund for promoting institutional agreements." Special thanks to Dott. Ivana Scovassi for helpful suggestions.

References

[1] R. C. Todd and S. J. Lippard, "Inhibition of transcription by platinum antitumor compounds," *Metallomics*, vol. 1, no. 4, pp. 280–291, 2009.

[2] G. Santin, V. M. Piccolini, P. Veneroni, S. Barni, G. Bernocchi, and M. G. Bottone, "Different patterns of apoptosis in response to cisplatin in B50 neuroblastoma rat cells," *Histology and Histopathology*, vol. 26, no. 7, pp. 831–842, 2011.

[3] G. Bernocchi, M. G. Bottone, V. M. Piccolini et al., "Developing central nervous system and vulnerability to platinum compounds," *Chemotherapy Research and Practice*, vol. 2011, Article ID 315418, 14 pages, 2011.

[4] F. Yu, J. Megyesi, and P. M. Price, "Cytoplasmic initiation of cisplatin cytotoxicity," *American Journal of Physiology*, vol. 295, no. 1, pp. F44–F52, 2008.

[5] M. Le Bras, I. Rouy, and C. Brenner, "The modulation of inter-organelle cross-talk to control apoptosis," *Medicinal Chemistry*, vol. 2, no. 1, pp. 1–12, 2006.

[6] T. Nakagawa and J. Yuan, "Cross-talk between two cysteine protease families: activation of caspase-12 by calpain in apoptosis," *Journal of Cell Biology*, vol. 150, no. 4, pp. 887–894, 2000.

[7] J. D. Lane, J. Lucocq, J. Pryde et al., "Caspase-mediated cleavage of the stacking protein GRASP65 is required for Golgi fragmentation during apoptosis," *Journal of Cell Biology*, vol. 156, no. 3, pp. 495–509, 2002.

[8] R. Chiu, L. Novikov, S. Mukherjee, and D. Shields, "A caspase cleavage fragment of p115 induces fragmentation of the Golgi apparatus and apoptosis," *Journal of Cell Biology*, vol. 159, no. 4, pp. 637–648, 2002.

[9] C. Soldani, A. C. Croce, M. G. Bottone et al., "Apoptosis in tumour cells photosensitized with Rose Bengal acetate is induced by multiple organelle photodamage," *Histochemistry and Cell Biology*, vol. 128, no. 5, pp. 485–495, 2007.

[10] M. G. Bottone, C. Soldani, A. Fraschini et al., "Enzyme-assisted photosensitization activates different apoptotic pathways in Rose Bengal acetate treated HeLa cells," *Histochemistry and Cell Biology*, vol. 131, no. 3, pp. 391–399, 2009.

[11] K. F. Ferri and G. Kroemer, "Organelle-specific initiation of cell death pathways," *Nature Cell Biology*, vol. 3, no. 11, pp. E255–E263, 2001.

[12] M. G. Bottone, C. Soldani, P. Veneroni, D. Avella, M. Pisu, and G. Bernocchi, "Cell proliferation, apoptosis and mitochondrial damage in rat B50 neuronal cells after cisplatin treatment," *Cell Proliferation*, vol. 41, no. 3, pp. 506–520, 2008.

[13] D. L. Bodenner, P. C. Dedon, and P. C. Keng, "Selective protection against cis-diamminedichloroplatinum(II)-induced toxicity in kidney, gut, and bone marrow by diethyldithiocarbamate," *Cancer Research*, vol. 46, no. 6, pp. 2751–2755, 1986.

[14] J. Dietrich, R. Han, Y. Yang, M. Mayer-Pröschel, and M. Noble, "CNS progenitor cells and oligodendrocytes are targets of chemotherapeutic agents *in vitro* and *in vivo*," *Journal of Biology*, vol. 5, article 22, 2006.

[15] C. Soldani, M. G. Bottone, A. C. Croce, A. Fraschini, G. Bottiroli, and C. Pellicciari, "The Golgi apparatus is a primary site of intracellular damage after photosensitization with Rose Bengal acetate," *European Journal of Histochemistry*, vol. 48, no. 4, pp. 443–448, 2004.

[16] V. M. González, M. A. Fuertes, C. Alonso, and J. M. Perez, "Is cisplatin-induced cell death always produced by apoptosis?" *Molecular Pharmacology*, vol. 59, no. 4, pp. 657–663, 2001.

[17] A. Mandic, J. Hansson, S. Linder, and M. C. Shoshan, "Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling," *The Journal of Biological Chemistry*, vol. 278, no. 11, pp. 9100–9106, 2003.

[18] K. J. Cullen, Z. Yang, L. Schumaker, and Z. Guo, "Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer," *Journal of Bioenergetics and Biomembranes*, vol. 39, no. 1, pp. 43–50, 2007.

[19] W. Qian, M. Nishikawa, A. M. Haque et al., "Mitochondrial density determines the cellular sensitivity to cisplatin-induced cell death," *American Journal of Physiology*, vol. 289, no. 6, pp. C1466–C1475, 2005.

[20] M. Hirama, S. Isonishi, M. Yasuda, and H. Ishikawa, "Characterization of mitochondria in cisplatin-resistant human ovarian carcinoma cells," *Oncology Reports*, vol. 16, no. 5, pp. 997–1002, 2006.

[21] S. Nakagomi, M. J. Barsoum, E. Bossy-Wetzel, C. Sütterlin, V. Malhotra, and S. A. Lipton, "A Golgi fragmentation pathway in neurodegeneration," *Neurobiology of Disease*, vol. 29, no. 2, pp. 221–231, 2008.