

Ligands of the mitochondrial 18 kDa translocator protein attenuate apoptosis of human glioblastoma cells exposed to erucylphosphocholine

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Abstract. *Background:* We have previously shown that the anti-neoplastic agent erucylphosphocholine (ErPC3) requires the mitochondrial 18 kDa Translocator protein (TSPO), formerly known as the peripheral-type benzodiazepine receptor (PBR), to induce cell death via the mitochondrial apoptosis pathway.

Methods: With the aid of the dye JC-1 and cyclosporin A, applied to glioblastoma cells, we now investigated the significance of opening of the mitochondrial permeability transition pore (MPTP) for ErPC3-induced apoptosis in interaction with the TSPO ligands, PK 11195 and Ro5 4864. Furthermore, we measured cytochrome c release, and caspase-9 and -3 activation in this paradigm.

Results: The human glioblastoma cell lines, U87MG, A172 and U118MG express the MPTP-associated TSPO, voltage-dependent anion channel and adenine nucleotide transporter. Indeed, ErPC3-induced apoptosis was inhibited by the MPTP blocker cyclosporin A and by PK 11195 and Ro5 4864 in a concentration-dependent manner. Furthermore, PK 11195 and Ro5 4864 inhibited collapse of the mitochondrial membrane potential, cytochrome c release, and caspase-9 and -3 activation caused by ErPC3 treatment.

Conclusions: This study shows that PK 11195 and Ro5 4864 inhibit the pro-apoptotic function of ErPC3 by blocking its capacity to cause a collapse of the mitochondrial membrane potential. Thus, the TSPO may serve to open the MPTP in response to anti-cancer drugs such as ErPC3.

Keywords: Mitochondrial permeability transition pore, PK 11195, Ro5 4864, TSPO, erucylphosphocholine, cancer

1. Introduction

Despite advances in neurosurgery, radiation, chemotherapy, and gene therapy, prognosis for patients with glioblastoma multiforme is still poor [40,44]. Addressing this problem, we have reported previously

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that the alkylphosphocholines, erucylphosphocholine (ErPC) and its congener erucylphosphocholine (ErPC3, ErufosineTM), induce apoptosis in a variety of glioblastoma cell lines while sparing their non-tumorigenic counterparts [22,24,31,34]. We also found that ErPC and ErPC3 promote tumor cell apoptosis independent of p53 function and independent of death receptor/ligand interactions [31,33]. Instead, mitochondria, as well as Apaf-1 and caspase-3, may play major roles in ErPC-induced apoptosis [30]. Moreover, using genetic manipulation of a rat glioma cell line to induce knockdown of the 18 kDa Translocator Protein (TSPO), formerly known as the peripheral-type benzo-

diazepine receptor (PBR), we demonstrated that ErPC and ErPC3 indeed activate the mitochondrial apoptotic pathway via the TSPO [34,42,52].

TSPO are present in glial cells in the brain as well as in peripheral tissues [4,47,50]. Furthermore, TSPO levels appear to be enhanced in cancer cells, including glioma [1,26,27]. Moreover, TSPO levels in untreated glioma cells appear to be in proportion to the levels of tumorigenicity [49]. We also showed that antisense knockdown of TSPO in mouse MA-10 Leydig cells and in rat C6 glioma cells increased proliferation, enhanced tumorigenicity, reduced apoptotic rates and blocked the pro-apoptotic anticancer effects of ErPC and ErPC3 [28,34,54].

TSPO can be found in the outer mitochondrial membrane in complex with the voltage dependent anion channel (VDAC, 32 kDa) and the adenine nucleotide transporter (ANT, 30 kDa) [38,43]. VDAC and ANT are considered to form the core components of the mitochondrial permeability transition pore (MPTP) complex [18]. A broad spectrum of putative functions have been suggested for the TSPO [15,50–52]. These functions include steroid production [28,41], apoptosis [2, 34], regulation of the mitochondrial membrane potential [7,35] and cancer cell proliferation [6,28], including glial tumor cell proliferation [45,49]. By application of TSPO knockdown with genetic manipulation and by treatment with specific TSPO ligands, it was suggested that TSPO may modulate the function of the MPTP [34,35]. Extended opening of the MPTP may lead to mitochondrial swelling and subsequent release of mitochondrial cytochrome c, followed by the activation of a caspase cascade leading to apoptosis [18]. This suggests that the mitochondrial apoptotic pathway is one possibility whereby TSPO may be involved in the regulation of apoptosis [15,51,52].

The present study is aimed to determine the interaction between TSPO ligands, PK 11195 and Ro5 4864, and ErPC3 regarding activation of the mitochondrial apoptosis pathway in the human glioblastoma cell lines U87MG, A172 and U118MG. ErPC3 may induce collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) typically caused by opening of the MPTP [25], while TSPO and its ligands have been closely associated with the MPTP [52]. Therefore, we addressed the significance of MPTP opening for ErPC3-induced apoptosis in interaction with the TSPO and its ligands. To this end, we used the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) to assay collapse of the mitochondrial potential following exposure to ErPC3 and TSPO ligands.

We also studied the effects of the specific MPTP blocker, cyclosporin A (CsA) on the induction of apoptosis by ErPC3. To complete our study of the involvement of the mitochondrial apoptosis pathway in ErPC3-induced apoptotic mechanisms affected by PK 11195 and Ro5 4864, we measured the release of cytochrome c and the processing of caspase-9 and -3. Our study indicates that the TSPO ligands PK 11195 and Ro5 4864 can significantly reduce the activation of the mitochondrial pathway otherwise typically induced by ErPC3 in human glioblastoma cells.

2. Materials and methods

2.1. Cell culture

The human glioblastoma cell lines were obtained from European Collection of Cell Cultures, Salisbury, UK (A172) and generously provided by Dr. G. Bernhardt, University of Regensburg, Germany (U87MG, U118MG). Cells were cultured at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity, as described previously [30,33,49,53]. Briefly, A172 cells were maintained in RPMI-1640, U87MG cells in EMEM and U118MG cells in DMEM. To the culture media were also added 10% (A172, U87MG) or 5% (U118MG) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The DMEM and EMEM media were supplemented with 1% of sodium pyruvate and 1% of non-essential amino acids.

2.2. Drugs

ErPC3 (Erufosine™) was synthesized and generously provided by Prof. H. Eibl (Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany). CsA and 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5 4864) were obtained from Sigma-Aldrich (St. Louis, MO). FK506 (Tacrolimus) and 1-(2-chlorophenyl-N-methyl-1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) were from Alexis (Switzerland).

2.3. Antibodies

Commercially obtained antibodies: monoclonal mouse anti- β -actin (clone Ac-15) (Sigma-Aldrich, St.

Louis, MO); monoclonal mouse anti-COX IV (Molecular Probes, Eugene, OR); monoclonal anti-porin 31HL (Ab-3) (Calbiochem, Darmstadt, Germany); polyclonal rabbit anti-caspase-9 and polyclonal rabbit anti-cleaved caspase-3 (Cell Signaling, Beverly, MA); monoclonal mouse anti-cytochrome c (Pharmingen, San Diego, CA).

Antibodies against human TSPO and ANT2 were prepared according to methods applied previously [16, 21] in the laboratory of Prof. M. Gavish using peptides coding for TSPO (CWRDNHGWRGGRRLPE; NCBI AAH01110) and ANT 2 (CGGVDKRTQFWRYFA; NCBI AAB39266), which were synthesized in the laboratory of Prof. M. Fridkin, Weizmann Institute of Science, Rehovot, Israel. Antibody purification from the rabbit sera were carried out on Sulfolink[®] Coupling Gel (Pierce, Rockford, IL, USA). All procedures with the animals were designed to minimize their suffering and the number of animals used, as approved by the Institutional Animal Care Committee.

2.4. Assay material

WST-1 reagent, Cell Proliferation ELISA, BrdU (colorimetric) kit and Cell Death Detection ELISA^{PLUS} (Cell Death Kit) were from Roche Applied Science (Mannheim, Germany). Bicinchoninic acid (BCA) protein assay reagents kit was from Pierce (Rockford, IL, USA). The fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Calbiochem (Merck, Darmstadt, Germany), carbonyl cyanide 3-chlorophenylhydrazone (CCCP) from Sigma-Aldrich (Rehovot, Israel). The Qproteome mitochondria isolation kit was from Qiagen (Hilden, Germany). Standard chemicals for the assays described below were obtained from various commercial sources.

2.5. Western blot protein analysis of TSPO, VDAC and ANT2

The presence of TSPO, VDAC and ANT2 protein in the U87MG, A172, and U118MG cell lines was verified with Western blot according to methods described previously [31,34]. The primary antibodies were used at the following concentrations: anti-TSPO, 1:1000; anti-VDAC (porin), 1:4000; anti-ANT2, 1:3000; and anti- β -actin from Sigma, 1:15,000. Horseradish peroxidase linked anti-rabbit IgG (1:3000) from donkey or horseradish peroxidase linked anti-mouse IgG (1:5000) from sheep was used as secondary anti-

body (both from GE Healthcare, Little Chalfont, UK). Biocapt-[Biocap1] Version 97.05 s for Windows and Bio-Profile, Bio-1D, Windows, Application V97.04 (Vilber Lourmat, France) were used for determination of relative labeling intensities. Labeling of β -actin protein was used to confirm even loading and transfer of protein.

2.6. TSPO binding characteristics

The specific, radioactively labeled TSPO ligand [³H]PK 11195 was also used to verify the presence of TSPO. In addition, the binding assays were used to assess TSPO binding density and affinity in the U87MG, A172 and U118MG human glioblastoma cell lines, according to methods described previously [3,34, 49]. Six concentrations from 0.02 to 6 nM of [³H]PK 11195 were used in the absence (total binding) or presence (nonspecific binding) of 75 μ l unlabeled PK 11195 (10 μ M final concentration), for analysis of TSPO binding characteristics. Scatchard analysis of [³H]PK 11195 binding assays was used to determine the maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d) in the U87MG, A172 and U118MG cell lines, as described previously by us [34,49].

2.7. Measurement of cell proliferation and cell viability

For the quantitative determination of the DNA synthesis rate, the Cell Proliferation ELISA – BrdU (colorimetric) method was used and cell viability was determined by the WST-1 reagent, according to the instructions provided by the manufacturers as described previously [30,31]. Briefly, human glioblastoma cells were seeded in flat-bottom 96-well microtiter plates (100 μ l/well), allowed to attach overnight and treated with increasing concentrations of PK 11195 and Ro5 4864 for 48 hours. PK 11195 and Ro5 4864 were dissolved in ethanol, and control cells were treated correspondingly with this vehicle (1% ethanol). BrdU incorporation and WST-1 reactivity were measured with the ELISA reader (TECAN, Zurich, Switzerland) at 450 nm (reference at 690 nm). Nonlinear regression analysis was performed to generate curves for LC₅₀ calculation determination from the WST-1 test using GraphPad Prism[®] 4.0 software (GraphPad Software, Inc., San Diego, CA).

2.8. Quantitative analysis of apoptosis

Cytoplasmic histone-associated DNA fragments indicative of ongoing apoptosis were quantitatively measured using the Cell Death Kit (Roche), following the manufacturer's instructions, as described previously [31,49]. ErPC3 was applied at concentrations of 45 μM (A172, U87MG) or 15 μM (U118MG), which were known to result in pronounced apoptosis. The concentrations of the other compounds, PK 11195, Ro5 4864, CsA [20] and FK506 [46], as well as the various time periods of the treatments, are given in the appropriate Result sections. Absorption indicative of apoptosis was measured at 405 nm, reference 490 nm.

2.9. Mitochondrial membrane potential assay

For mitochondrial membrane potential ($\Delta\psi_m$) measurements cells were seeded in 24 well plates (2×10^5 cells per well). After achieving confluence the medium was changed to medium with PK 11195 at various concentrations (0, 25, 50, 75, 100 μM) with or without ErPC3 (15 μM for U118MG, 30 μM for A172 and 45 μM for U87MG, concentrations that we found to be optimal for inducing apoptosis and collapse of the $\Delta\psi_m$). Cell cultures containing 1% ethanol, the vehicle for both PK 11195 and ErPC3, but not treated with PK 11195 are called "control". As a positive control for depolarization of the mitochondrial membrane potential we treated the cells with the proton ionophor CCCP (50 μM) in each experiment. Floating and trypsinized adherent cells were collected for $\Delta\psi_m$ determination by fluorescence assisted cell sorting (FACS). The cell suspensions were centrifuged at $500 \times g$ and the cell pellets were resuspended in 500 μl of phosphate buffered saline (PBS) at 4°C and transferred to FACS tubes (Falcon, Bedford, MA) by filtering through MASH paper (Sinun, Petach Tikva, Israel). Using FACS, the mitochondrial membrane potential was analyzed using the fluorescent lipophilic dye JC-1, according to the manufacturer's instructions (Calbiochem). In healthy cells, the negative charge established by an intact (high) $\Delta\psi_m$ allows the JC-1 molecules, bearing a delocalized positive charge, to enter the mitochondrial matrix, where they form J-aggregates. In cells subject to $\Delta\psi_m$ collapse, JC-1 does not accumulate within the mitochondria, but remains in the cytoplasm in its monomeric form. The monomer form emits at 527 nm (green fluorescence), whereas J-aggregates emit at 590 nm (orange-red fluorescence). The relative reduction of the emis-

sion at 527 nm can be used as an indication for the maintenance of the mitochondrial membrane potential [7].

2.10. Cytochrome c release and caspase cleavage

U118MG cells were treated for 12 h with ErPC3 and TSPO ligands, control cells were treated with vehicle only. Following treatment, cells were harvested, washed, transferred to microvials and resuspended in 200 μl digitonin (0.02%) lysis buffer (250 mM sucrose, 80 mM KCl, 5 mM EDTA). After 5 min on ice, cell lysates were centrifuged ($700 \times g$, 10 min, 4°C). The supernatants were transferred to fresh microvials, centrifuged again for 25 min at $10,000 \times g$, 4°C, with the resulting supernatants containing the cytosolic fractions. The mitochondrial fraction was prepared using the Qproteome mitochondria isolation kit, according to the manufacturer's recommendations (Qiagen). Cytosolic and mitochondrial fractions were used to assay mitochondrial cytochrome c release, as well as caspase-9 and -3 processing by Western blot protein analysis [31,34]. The COX IV antibody was used as a quality control for cell separation. Primary antibodies were used at the indicated dilutions: 1:2000 of the anti-caspase-9 antibody, 1:2000 of the anti-cleaved caspase-3 antibody, 1:1000 of the anti-cytochrome c antibody and 1:2000 of the anti-COX IV antibody. Immunoblots were visualized using the Immobilon Western detection reagents (Millipore, Billerica, MA). The protein content of the cytosolic and mitochondrial fractions was quantified using BCA protein assay reagents, according to the manufacturer's manual (Pierce). Loading and transfer of even amounts of protein was confirmed by Ponceau S staining.

2.11. Data analysis

Data were expressed as means \pm SD. For the determination of statistically significant differences, Kruskal–Wallis non-parametric, one-way analysis of variance (ANOVA) test was carried out, since standard deviations typically differed significantly between groups, as indicated by Bartlett's test for homogeneity of variance. As the sample sizes were relatively small, Mann–Whitney was used as a post-hoc test. $p < 0.05$ was considered to indicate statistically significant differences. The program used for statistical analysis was InStatGraphPad™ (GraphPad Software, San Diego, CA).

3. Results

3.1. TSPO in the U87MG, A172 and U118MG human glioblastoma cell lines

Western blot protein analysis showed the presence of TSPO in the U87MG, A172 and U118MG cell lines (Fig. 1(a)). The [³H]PK 11195 binding assay also indicated that U87MG, A172 and U118MG all possess TSPO capable to bind to its specific ligand PK 11195 (Fig. 1(b)).

As additional information, we found that TSPO levels appeared to differ between the three cell lines (Fig. 2). Using densitometry of our Western blots, we normalized the relative protein levels by setting at 1 the average levels of TSPO in the U87MG cell line, which showed the lowest levels of TSPO compared to the A172 and U118MG cell lines (Fig. 2(a)). In this way, relative protein levels for TSPO were 1 ± 0.25 in the U87MG cell line, 1.6 ± 0.41 in the A172 cell line and 2.7 ± 0.57 in the U118MG cell line ($n = 8$ for each cell line). These levels of TSPO protein expression differed significantly (U87MG vs. A172, $p < 0.05$; A172 vs. U118MG, $p < 0.001$; U87MG vs. U118MG, $p < 0.001$). The levels of VDAC and ANT2 did not ap-

pear to differ significantly between the three cell lines. Labeling of β -actin did not show variation, indicating that differences in TSPO levels found were not due to uneven loading or transfer of protein.

Scatchard analysis of [³H]PK 11195 binding assay indicated that the average B_{\max} 's for TSPO ligand binding of the U87MG, A172 and U118MG cells were also significantly different from one another ($p < 0.05$ for each comparison). The average B_{\max} 's for TSPO ligand binding of the U87MG, A172 and U118MG cells were, respectively, 1016 ± 257 ($n = 5$), 3946 ± 611 ($n = 4$) and 5338 ± 568 ($n = 4$) fmol/mg protein (Fig. 2(b)). The K_d 's of the TSPO ligand binding for the U87MG, A172 and U118MG cells were respectively, 3.2 ± 0.9 ($n = 5$), 4.7 ± 1.6 ($n = 4$) and 2.33 ± 0.6 ($n = 4$) nM. These K_d 's were not significantly different from one another.

To see whether ErPC3 may bind to TSPO, we applied ErPC3 (10^{-9} – 10^{-4} M) to binding assays of TSPO in rat kidney membranes with [³H]PK 11195 (2 nM final concentration). No effect on [³H]PK 11195 binding was found, suggesting that ErPC3 does not compete with TSPO ligand binding (data not shown).

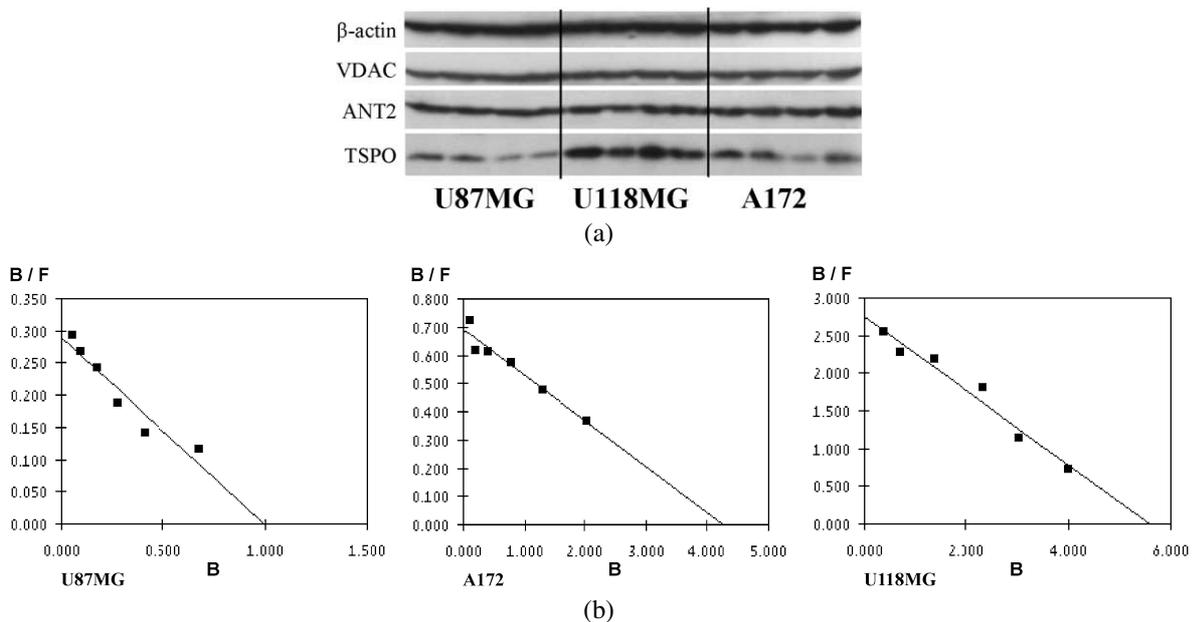


Fig. 1. (a) Examples of Western blots showing the presence of TSPO, ANT2 and VDAC in the U87MG, U118MG and A172 cell lines. β -actin was used as a reference protein. Each lane presents a different sample, i.e. cells taken from one culture flask. (b) Representative Scatchard plots demonstrating the binding of the specific TSPO ligand [³H]PK 11195 to membrane homogenates of U87MG, A172 and U118MG human glioblastoma cells. The X-axis indicates the amount of bound [³H]PK 11195 in fmol/mg protein and the Y-axis indicates the ratio of bound over free [³H]PK 11195. Abbreviations in the figure: B = bound; B/F = bound/free.

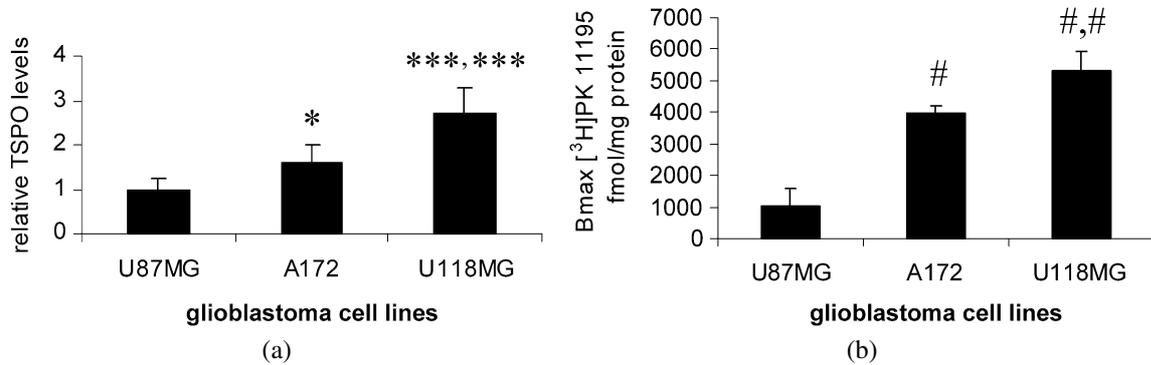


Fig. 2. (a) Densitometric measurements of average TSPO levels detected with Western blot showed statistically significant differences between the U87MG, U118MG and A172 cell lines ($n = 8$, for each comparison). No statistically significant differences in protein levels of β -actin, VDAC and ANT2 were observed ($n = 8$, for each comparison). (b) The average B_{max} 's of [³H]PK 11195 binding, were statistically significant different between the U87MG, U118MG and A172 cell lines ($n = 5$, for U87MG and $n = 4$ for U118MG and A172). Explanation of symbols: * $p < 0.05$ vs. U87MG; ***,** $p < 0.001$ vs. U87MG and A172; # $p < 0.05$ vs. U87MG; #,# $p < 0.05$ vs. U87MG and U118MG.

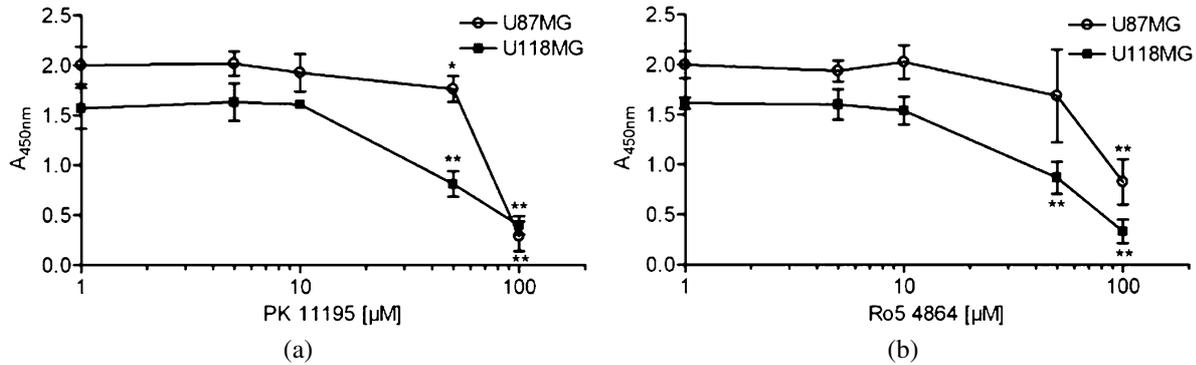


Fig. 3. Increasing concentrations of PK 11195 (a) and Ro5 4864 (b) were used to study the effects on proliferation of the cell lines U87MG and U118MG, assayed with BrdU incorporation. Absorption of 450 nm ($A_{450\text{nm}}$) indicates cell proliferation measured after 72 h of treatment. Symbols: * $p < 0.05$ vs. vehicle control; ** $p < 0.01$ vs. vehicle control.

3.2. Effects of TSPO ligands on cell proliferation and cell viability of human glioblastoma cells

To determine which concentrations of the TSPO ligands PK 11195 and Ro5 4864 might be applicable for our study, we first studied their effects on the proliferation rates and cell viability of human glioblastoma cell lines. The relative cell proliferation rates were determined with the aid of DNA synthesis assays with BrdU in U87MG and U118MG cells. We used these two cell lines since they showed the lowest and highest levels, respectively, of TSPO expression. We found that PK 11195 and Ro5 4864 concentrations in the range from 1 to 500 nM did not affect DNA synthesis i.e. cell proliferation of U87MG and U118MG cells (data not shown). Also at concentrations of 5 μ M and 10 μ M no significant effects were observed (Fig. 3). PK 11195 at a concentration of 50 μ M caused a modest

(14%) albeit significant reduction in proliferation rates in U87MG (Fig. 3(a)). At this same concentration of 50 μ M, PK 11195 could significantly reduce proliferation of U118MG cells by 50% (Fig. 3(b)). Ro5 4864 at a concentration of 50 μ M, also could significantly reduce proliferation of U118MG cells (by 48%), but not of U87MG cells (Fig. 3(b)). At a concentration of 100 μ M, both PK 11195 and Ro5 4864 consistently and significantly resulted in reductions in cell proliferation of U87MG cells (respectively, 86% and 57%) and U118MG cells (respectively, 75% and 80%) as compared to untreated controls (Fig. 3).

We used the WST-1 test to determine cell viability of the human glioblastoma cell lines U87MG, A172 and U118MG. We applied increasing concentrations of PK 11195 or Ro5 4864 (in a range from 15 μ M till 175 μ M) and found that at concentrations of approximately 100 μ M, PK 11195 and Ro5 4864 reduced cell

Table 1

Lethal effects of TSPO ligands on human glioblastoma cell lines		
Cell line	LC ₅₀ (μM)	
	PK 11195	Ro5 4864
U87MG	118 (111–125)	126 (121–132)
A172	110 (109–112)	93 (91–96)
U118MG	102 (97–107)	117 (107–126)

LC50 values of PK 11195 and Ro5 4864 on the U87MG, A172 and U118MG cell lines were determined. The data are expressed as mean values from $n = 9$. The 95% confidence intervals are presented in brackets.

survival by 50% (LC₅₀) in these three human glioblastoma cell lines (for details see Table 1). At concentrations of 50 μM or less, PK 11195 and Ro5 4864 did not appear to have significant effects on cell viability (data not shown).

3.3. The effects of cyclosporin A (CsA) and FK506 on the induction of apoptosis by ErPC3

We analyzed the effect of the specific MPTP blocker, CsA, on ErPC3-induced apoptosis in the U87MG, A172 and U118MG, human glioblastoma cell lines. We found that 10 μM CsA protects against the pro-apoptotic activity of ErPC3 measured in these cells (Fig. 4(a)). In more detail, CsA by itself does not appear to affect basal apoptotic levels in the A172 and U118MG cell lines as compared to untreated controls. Treatment of U87MG cells with CsA may cause a modest increase in apoptotic levels as compared to untreated controls ($p < 0.05$). This increase (2.5-fold) is small compared to the apoptotic levels ErPC3 induced in this cell line in this same experiment (31-fold, Fig. 4(a)). In comparison to untreated controls, ErPC3 treatment consistently causes robust and very significant ($p < 0.01$) increases of apoptosis in U87MG, A172 and U118MG glioblastoma cells (Figs 4 and 5). For example in Fig. 4(a), we see that ErPC3 increases apoptotic levels in the U87MG, A172 and U118MG cell lines, respectively, 31 fold, 12 fold and 5.5 fold. Co-treatment of ErPC3-treated cells with CsA caused significant ($p < 0.01$) reductions in apoptosis levels compared to treatment with ErPC3 alone (U87MG by 54%; A172 by 88%; U118MG by 61%). This suggests that the MPTP partakes in apoptosis induction by ErPC3.

However, as CsA is also an inhibitor of calcineurin, we investigated whether inhibition of calcineurin could account for the CsA block of apoptosis induced by ErPC3. For this purpose, we used FK506 which in-

hibits calcineurin but not the MPTP. In a pilot study, we found that FK506 at the concentrations 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M had no influence on the pro-apoptotic activity of ErPC3 in U87MG and A172 cells (data not shown). To further study the effect of FK506 on ErPC3 induced apoptosis we decided to use FK506 at a concentration of 500 nM, typically used for published studies of calcineurin [46]. This is a concentration which is considerably higher than the IC₅₀ value of the calcineurin inhibitor FK506, which reportedly is approximately 0.1 nM [29]. FK506 at this concentration had no effect on apoptosis levels of U87MG and U118MG cells in comparison to vehicle treated controls (Fig. 4(b)). A relatively small (30%), but significant ($p < 0.01$) reduction in apoptotic levels was found for A172 cells. In this experiment, too, ErPC3 robustly and significantly enhanced apoptotic levels in the U87MG, A172 and U118MG cells. Addition of 500 μM of FK506 to treatment with ErPC3 did not cause a significant difference in apoptotic levels in comparison to ErPC3 alone, in any of the three cell lines (Fig. 4(b)). Thus, ErPC3-induced apoptosis does not appear to involve activation of calcineurin in the three glioblastoma cell lines we used.

3.4. Effects of TSPO ligands on ErPC3-induced apoptosis

Since both ErPC3 and TSPO ligands have been reported to affect apoptosis via the TSPO and the MPTP, we assayed potential interactions between ErPC3 and TSPO ligands regarding apoptosis. As mentioned above, effects of the TSPO ligands PK 11195 and Ro5 4864 on cell proliferation and cell viability of human glioblastoma cells could be detected with increasing concentrations, in particular from 50 μM to 175 μM. Therefore, we decided to use concentrations of 25, 50, 75 and 100 μM to study the effects of co-administration of these TSPO ligands with ErPC3 on apoptosis in the human glioblastoma cell lines U87MG and U118MG (Fig. 5). These two cell lines were used since they showed the lowest (U87MG) and highest (U118MG) TSPO levels with our binding and Western blot assays. At concentrations of 10^{-7} , 10^{-6} and 10^{-5} M, PK 11195 and Ro5 4864 showed no effects on basal apoptotic levels in these human glioblastoma cell lines (data not shown). We found that also at a concentration of 100 μM, PK 11195 and Ro5 4864 had little or no effect on basal apoptotic levels in U87MG and U118MG cells (Fig. 5(a)–(c)). Nonetheless, at concentrations of 25, 50, 75 and 100 μM, PK 11195 and

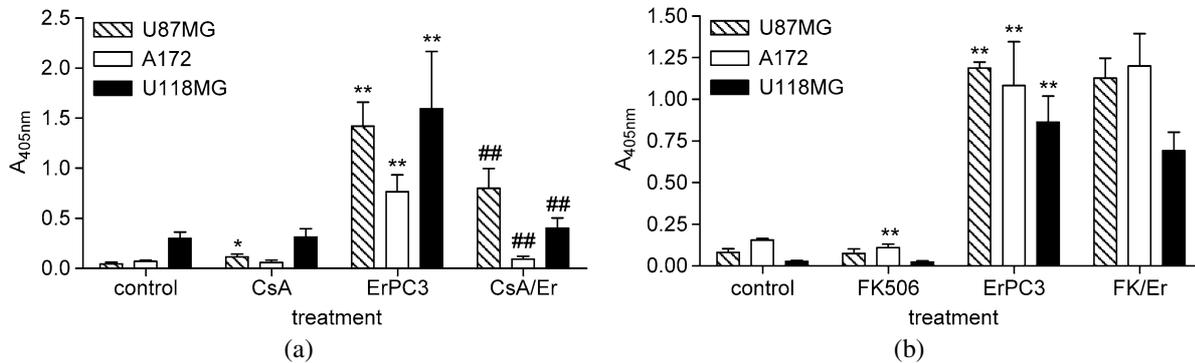


Fig. 4. The effect of the MPTP blocker, CsA (a), and of FK506 (b) on ErPC3-induced apoptosis in human glioblastoma cell lines. U87MG, A172 and U118MG cells were preincubated with 10 μ M of CsA (a) and 500 nM of FK506 (b) for 1 h prior to co-incubation with ErPC3 (CsA/Er; FK/Er) for 12 h (U87MG and U118MG) and 24 h (A172). Untreated cells (control), cells treated with CsA or FK506 alone (CsA; FK506) and cells treated with ErPC3 alone were run in parallel. Unlike CsA, FK506 had no significant effect on apoptosis induction by ErPC3 in all three cell lines. Absorbance of the 405 nm wavelength is indicative for the level of apoptosis. Symbols: * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; ### $p < 0.01$ vs. ErPC3.

Ro5 4864 appeared to be able to reduce apoptotic levels induced by ErPC3 (Fig. 5(a)–(c)). In more detail, morphologically, ErPC3 induced rounding and blebbing of human glioblastoma cells, while this was prevented by co-treatment with 100 μ M of either PK 11195 or Ro5 4864 (Fig. 5(a)). Reductions in the numbers of rounded cells and blebbing due to ErPC3 treatment could also be observed at lower concentrations of PK 11195 (1, 10, 25, 50 and 75 μ M; data not shown). Analysis with the Cell Death Kit showed in a quantitative manner that PK 11195 (Fig. 5(b)) and Ro5 4864 (Fig. 5(c)) at concentrations of 25, 50, 75 and 100 μ M appeared to be able to robustly, and significantly reduce apoptotic levels induced by ErPC3 in U87MG and U118MG cells. In numbers, PK 11195 by itself, at a concentration of 100 μ M, could cause a modest but significant increase in basal apoptotic levels (two-fold; $p < 0.05$) in U87MG cells compared to controls (Fig. 5(b)). No such effect was seen in U118MG cells (Fig. 5(b)). ErPC3 treatment caused manifold increases in apoptotic levels in U87MG and U118MG cells, compared to untreated controls (Fig. 5(b)). Compared to treatment of U87MG cells with ErPC3 alone, co-treatment of U87MG cells with 25, 50, 75 and 100 μ M of PK 11195 caused significant reductions of apoptotic levels by 32, 54, 73 and 79%, respectively (Fig. 5(b)). Also co-treatment of U118MG with 25, 50, 75 and 100 μ M of PK 11195 caused significant reductions of apoptotic levels by 80, 94, 95 and 96%, respectively (Fig. 5(b)).

At a concentration of 100 μ M, Ro5 4864 by itself does not significantly affect apoptotic levels neither of U87MG nor of U118MG, compared to untreated controls (Fig. 5(c)). Also in this experiment, ErPC3 treat-

ment resulted in manifold increases in apoptotic levels in the U87MG and U118MG cell lines, compared to untreated controls ($p < 0.01$) (Fig. 5(c)). Compared to treatment of U87MG cells with ErPC3 alone, co-treatment of U87MG cells with 25, 50, 75 and 100 μ M of Ro5 4864 caused significant reductions of apoptotic levels by 38, 56, 73 and 79%, respectively (Fig. 5(c)). Also in U118MG cells, co-treatment with 25, 50, 75 and 100 μ M of Ro5 4864 caused significant reductions of apoptotic levels by 38, 75, 91 and 93%, respectively (Fig. 5(c)).

3.5. Effects of TSPO ligands and ErPC3 on the mitochondrial potential

We also determined whether the pro-apoptotic effects of ErPC3 and the anti-apoptotic effects of TSPO ligands may involve modulation of the $\Delta\psi_m$. In comparison to untreated cells, treatment with ErPC3 caused manifold increases in the numbers of cells displaying collapse of $\Delta\psi_m$ in the glioblastoma cell lines U87MG, A172 and U118MG ($p < 0.01$, in all cases) (Fig. 6). In U87MG, all four PK 11195 concentrations used (25, 50, 75 and 100 μ M) significantly reduced this effect of ErPC3 (Fig. 6(c)). With the A172 and U118MG cell lines, PK 11195 at concentrations of 25, 50 and 75 μ M also significantly reduced the effect of ErPC3. With the A172 cell line, 100 μ M of PK 11195 did not have a significant effect on the collapse of the $\Delta\psi_m$ induced by ErPC3 (Fig. 6(d)). With the U118MG cell line, PK 11195 at a concentration of 100 μ M appeared to significantly enhance the effect of ErPC3 (Fig. 6(e)).

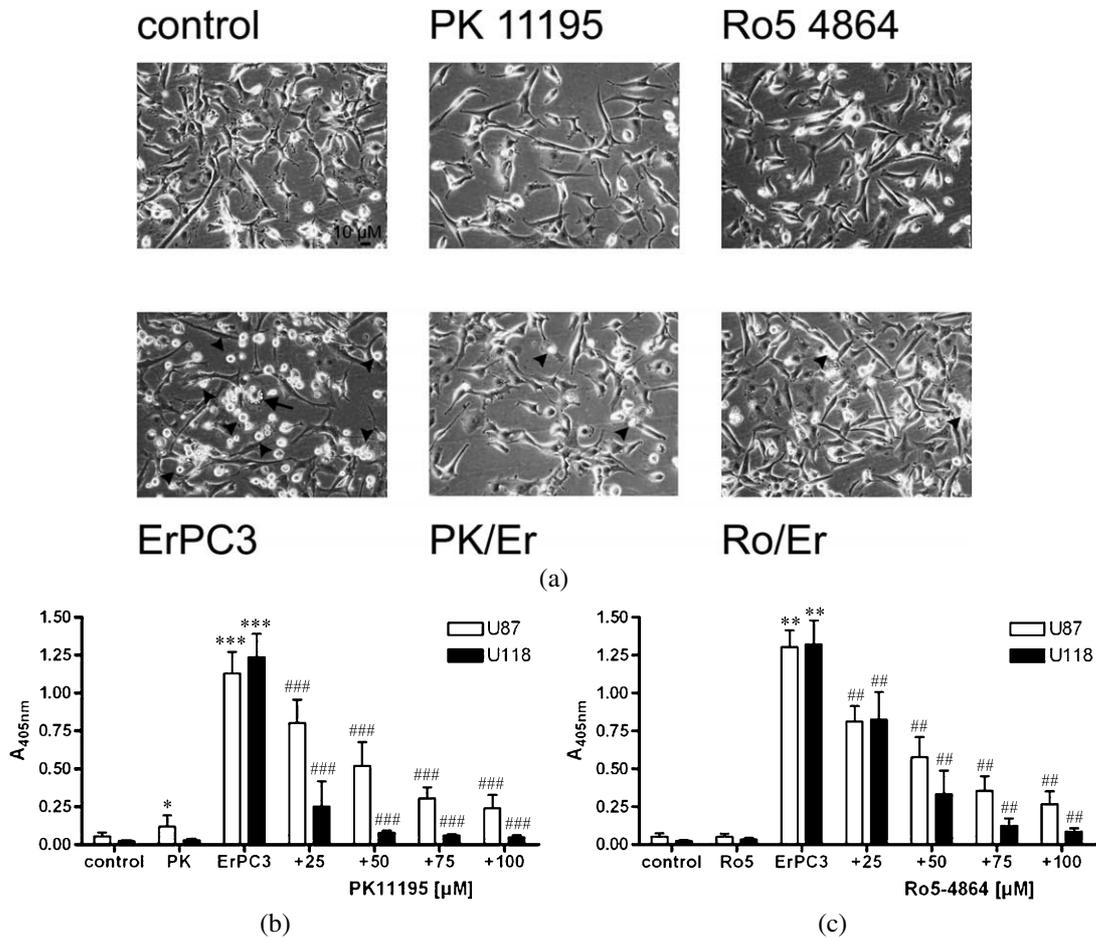


Fig. 5. Cells were treated for 12 hours with various concentrations of PK 11195 or Ro5 4864, with or without ErPC3, and apoptotic morphological changes were determined by phase-contrast microscopy (a) and quantitated by the Cell Death Kit (b, c). (a) In these examples, ErPC3 induced apoptosis in U87MG cells is observable by cell blebbing cell (arrow) and rounded cells (arrow heads). PK 11195 and Ro5 4864 showed no effects by themselves and reduced the effects of ErPC3 (PK/Er and Ro/Er). (b, c) In U87MG and U118MG cells, co-treatment of cells with PK 11195 (b) and Ro5 4864 (c) at concentrations of 25, 50, 75 and 100 μM caused significant reductions of apoptotic levels compared to treatment with ErPC3 alone. Symbols and abbreviations: scale bar is 10 μm (a). +25, +50, +75 and +100 means co-administration of ErPC3 with, respectively, 25, 50, 75 and 100 μM of PK 11195 (b) or Ro5 4864 (c); * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; *** $p < 0.001$ vs. control; ### $p < 0.01$ vs. ErPC3; ### $p < 0.001$ vs. ErPC3; Apoptotic levels are indicated by absorbance of the 405 nm wavelength ($A_{405\text{ nm}}$).

PK 11195 by itself, at concentrations of 25 and 50 μM appears to have very little or no effect on the $\Delta\psi_m$ in the U87MG and U118MG cell lines, while at higher concentrations (75 and 100 μM) PK 11195 by itself appeared to be capable to induce collapse of the $\Delta\psi_m$ in these two cell lines, particularly in U118MG cells (Fig. 6(c) and (e)). In the A172 cell line, PK 11195 by itself at concentrations of 25, 50 and 75 μM appeared to be able to prevent collapse of the mitochondrial membrane potential compared to control cells, while at a concentration of 100 μM PK 11195 had no effect on this cell line in this respect (Fig. 6(d)). Thus, concentrations of 25 and 50 μM of PK 11195 ap-

pear to be optimal to stabilize the mitochondrial membrane potential in these three glioblastoma cell lines.

3.6. TSPO ligands and activation of the mitochondrial apoptosis pathway by ErPC3

To obtain further insights into the mechanism(s) whereby the specific TSPO ligands, PK 11195 and Ro5 4864, may block ErPC3-induced apoptosis, we also measured cytochrome c release and caspase-9 and -3 cleavage in the U118MG cell line (Fig. 7). PK 11195 and Ro5 4864 at a concentration of 50 μM by themselves did not affect cytochrome c release nor caspase-9 and -3 processing, which are compo-

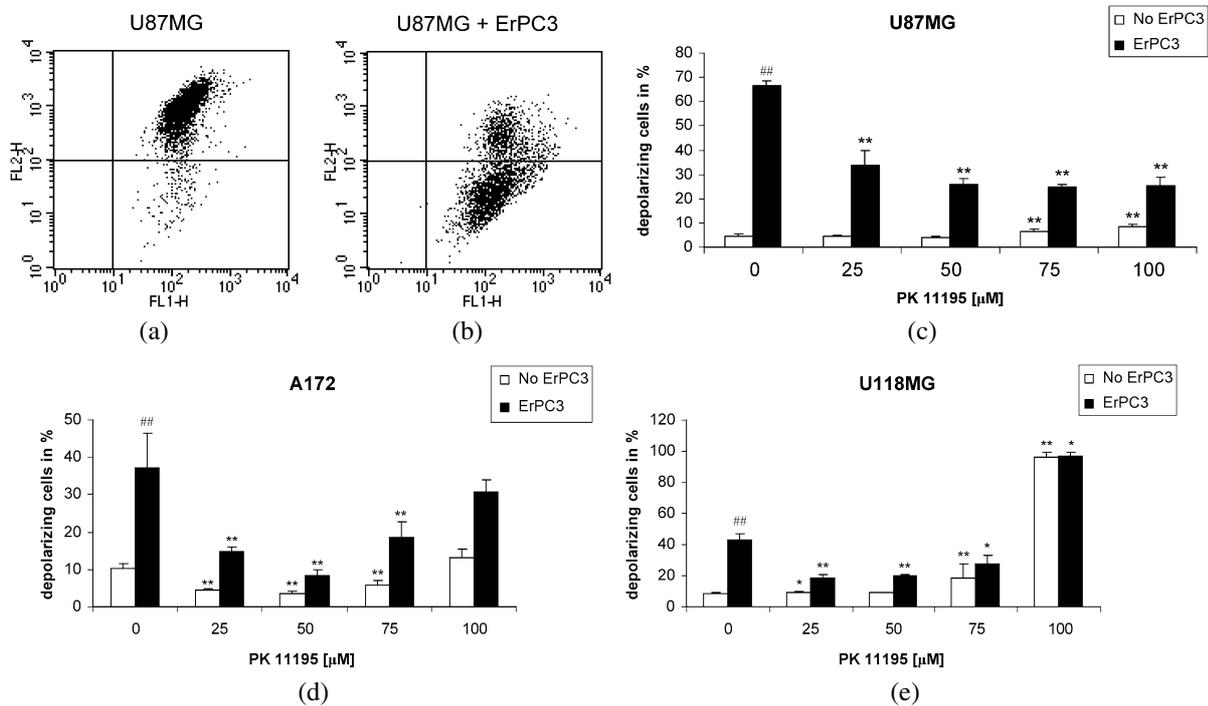


Fig. 6. Effects of ErPC3 and PK 11195 on the inner mitochondrial membrane potential ($\Delta\psi_m$) in the glioblastoma cell lines U87MG, A172 and U118MG using the dye JC-1. (a) and (b) The FACS patterns of U87MG cells labeled with JC-1 in the absence (a) or presence (b) of ErPC3 (45 μ M) are shown as examples. Cells with polarized mitochondria are found in the upper right (UR) quadrant of the plots, corresponding to high emission of fluorescence at both the 590 nm (FL2-H, orange-red) and 527 nm (FL1-H, green) wavelengths. After ErPC3 treatment, membrane depolarization becomes evident as a decrease in the 590 nm signal and an increase in the 527 nm signal, in the lower right (LR) quadrant of (b). In (c), (d) and (e), U87MG, A172 and U118MG were treated with ErPC3 (45, 30, 15 μ M, respectively) and/or PK 11195 at increasing concentrations for 24 h and $\Delta\psi_m$ was evaluated as illustrated with (a) and (b). Each column represents the mean values of cell percentages derived from each time 6 independent experiments in the LR quadrant of the $\Delta\psi_m$ analysis plot (depolarized mitochondria). In all three cell lines co-treatment with PK 11195 (at concentrations of 25, 50 and 75 μ M) reduced the incidence of $\Delta\psi_m$ collapse otherwise induced by ErPC3 itself. ## $p < 0.01$ when ErPC3 treatment is compared with no ErPC3 treatment (with PK 11195 treatment of 0 μ M). * $p < 0.05$ and ** $p < 0.01$ for the various co-treatments of PK 11195 with ErPC3 in comparison to 0 μ M PK 11195 (with ErPC3) of the cell lines in question.

nents of the mitochondrial apoptosis pathway. The same is true at concentrations of 100 μ M of PK 11195 and Ro5 4864 (data not shown). We found that a 12 hour treatment with 15 μ M of ErPC3 induced cytochrome c release and processing of caspase-9 and caspase-3 (Fig. 7). Co-treatment of ErPC3 with 50 μ M of PK 11195 or Ro5 4864 attenuated ErPC3-induced cytochrome c release as well as processing of caspase-9 and caspase-3 (Fig. 7). Similarly, co-treatment of ErPC3 with 100 μ M of PK 11195 or Ro5 4864 completely blocked ErPC3-induced cytochrome c release as well as processing of caspase-9 and caspase-3 (data not shown).

4. Discussion

In a previous study, we showed that TSPO is required for apoptosis induction by ErPC3 in rat glioma

cells [34]. In the present study we sought to determine whether ErPC3 may exert its pro-apoptotic actions via the TSPO in association with the MPTP in human glioblastoma cells. Our present study confirmed the presence of TSPO in the human glioblastoma cell lines, U87MG, A172 and U118MG. Thus, the substrate (TSPO) for the suggested mechanism of apoptosis induction by ErPC3 in rat glioma cells appears to be present in these human glioblastoma cells, as expected, and in accordance with previous studies [49]. In more detail, our ligand binding assays, as well as our Western blot protein analysis, showed relatively low levels of TSPO expression in U87MG cells, relatively high levels in U118MG cells and intermediate levels in A172 cells. TSPO is known to be closely associated with the VDAC and ANT forming the core of the MPTP [15,38,52]. While PK 11195 binds specifically to the TSPO, the benzodiazepine Ro5 4864 binds to TSPO, as well as to VDAC and ANT [38]. ANT2 is

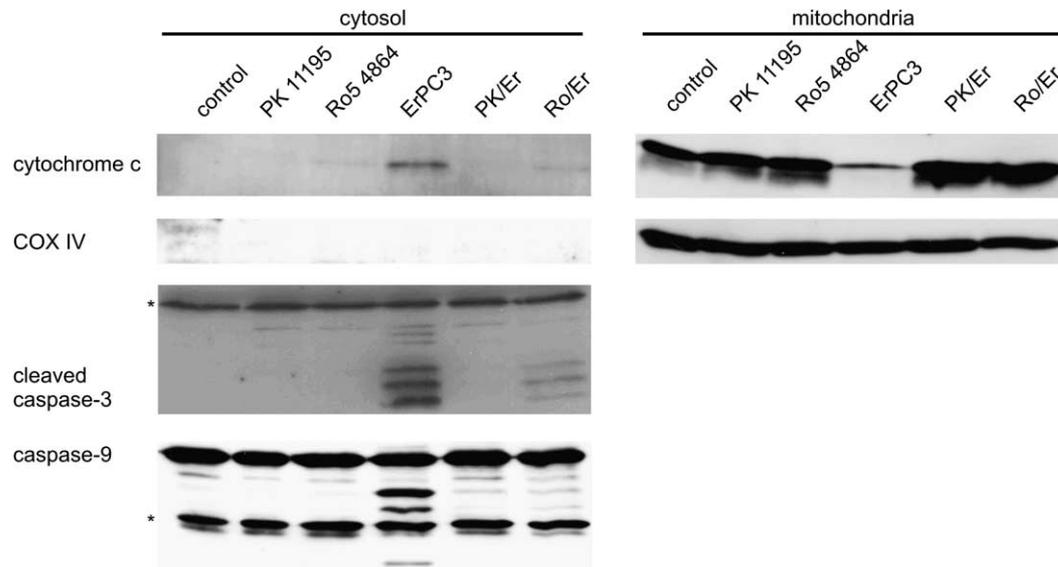


Fig. 7. Effects of TSPO ligands on ErPC3-induced cytochrome c release, and caspase-3 and -9 processing. U118MG cells were incubated for 12 h with 50 μ M of PK 11195 or Ro5 4864, or ErPC3 (15 μ M), or ErPC3 in combination with either PK 11195 or Ro5 4864 (PK/Er, Ro/Er), or treated with vehicle (control). Caspase-3 and -9 expression, including their cleavage products, and cytochrome c release, were analysed by Western blot protein analysis. Probing with a COX IV-specific antibody served as quality control for cell separation. Co-treatments with PK11195 or Ro5 4864 nearly completely blocked the effects of ErPC3 on cytochrome c release, and caspase-3 and -9 processing. Abbreviations: PK, PK 11195; Ro, Ro5-4864; Er, ErPC3; *unspecific band.

an ANT isomer which is relatively abundant in proliferating cells, including cancer cells [8,12]. While our Western blot protein analysis showed that TSPO levels differed between the three cell lines, VDAC and ANT2 levels did not. Thus, in particular the protein levels of TSPO in these three cell lines appear to account for the observed differences in their [3 H]PK 11195 binding capacity. In any case, all three proteins (TSPO, VDAC, and ANT2) are present in all three cell lines (U87MG, A172 and U118MG), potentially accounting for the apoptotic effects after treatment with ErPC3.

In a previous study, we suggested that the pre-apoptotic collapse of the mitochondrial membrane potential due to opening of the MPTP is a critical event in ErPC and ErPC3-induced apoptosis [25]. In the present study, our application of the MPTP blocker CsA [10], inhibiting the pro-apoptotic effects of ErPC3 in the U87MG, A172 and U118MG cell lines, indicates that the MPTP is required for the pro-apoptotic effects of ErPC3 in these human glioblastoma cells. Furthermore, previous studies by us, including knockdown of the TSPO by genetic manipulation in rat glioma cells, suggested that ErPC and ErPC3 induce cytochrome c release and caspase-3 processing in human and rat glioma cell lines as part of the mitochondrial apoptosis pathway, while the presence of TSPO appeared to be necessary for this [25,34]. Thus, these data suggest

that the MPTP as well as the TSPO are required for the activation of the mitochondrial apoptosis pathway in human glioblastoma cells by ErPC3.

Since our studies indicated that TSPO may be an important component in the MPTP-related pro-apoptotic mechanisms activated by ErPC3, we decided to use the well known TSPO ligands, PK 11195 and Ro5 4864, to study their potential interference with the pro-apoptotic activity of ErPC3 in human glioblastoma cells. We demonstrated that at concentrations of 25, 50, 75 and 100 μ M, PK 11195 and Ro5 4864 were able to significantly reduce the pro-apoptotic effects of ErPC3 in the U87MG and U118MG cell lines.

With the present study we found that the capability of PK 11195 and Ro5 4864 to prevent apoptosis due to ErPC3 appeared to be a consequence of their capability to prevent collapse of mitochondrial membrane potential typically induced by ErPC3 [25]. In all three cell lines, concentrations of 25, 50 and 75 μ M of the TSPO ligands appeared to be uniformly effective in preventing these actions of ErPC3. It is well known that opening of the MPTP as a cause for apoptosis induction includes collapse of the mitochondrial membrane potential, leading to the release of cytochrome c and the activation of caspase-3 and caspase-9 [18]. Indeed, with the present study we found that the TSPO ligands PK 11195 and Ro5 4864 applied at 50 μ M pre-

vented not only collapse of the mitochondrial membrane potential and cytochrome c release, but also the activation of caspase-9 and caspase-3 by ErPC3. Thus, in our model, ErPC3 appears to be able to activate the mitochondrial apoptosis pathway via collapse of the mitochondrial membrane potential and this ErPC3 function can be attenuated by TSPO ligands. This is summarized in the diagram of Fig. 8.

Regarding the inhibition of pro-apoptotic effects of ErPC3, we believe that in the present model of human glioblastoma cells, PK 11195 and Ro5 4864 bind to TSPO, thereby blocking pro-apoptotic interactions which may normally be activated by ErPC3. This would be comparable to the effects of knockdown of TSPO on the mitochondrial apoptosis pathway that we have reported previously [34]. TSPO knockdown

in the rat C6 glioma cells of this study [34] also prevented collapse of the mitochondrial membrane potential typically induced by ErPC3 (unpublished data), similar to the effects of the TSPO ligands in the present study. These data suggest that PK 11195 and Ro5 4864 act as antagonists regarding TSPO's apoptotic function. Comparable to our present study, several other studies reported anti-apoptotic effects of treatments by PK 11195 and Ro5 4864 [2,14,37,39,48]. We postulate that in these experimental models, including ours, PK 11195 and Ro5 4864 saturate the TSPO, thereby preventing the pro-apoptotic effects of other treatments, including ErPC3 treatment.

Interestingly, with the present study we also found that ErPC3 does not compete directly with the specific TSPO ligand [³H]PK 11195 for binding to TSPO. We

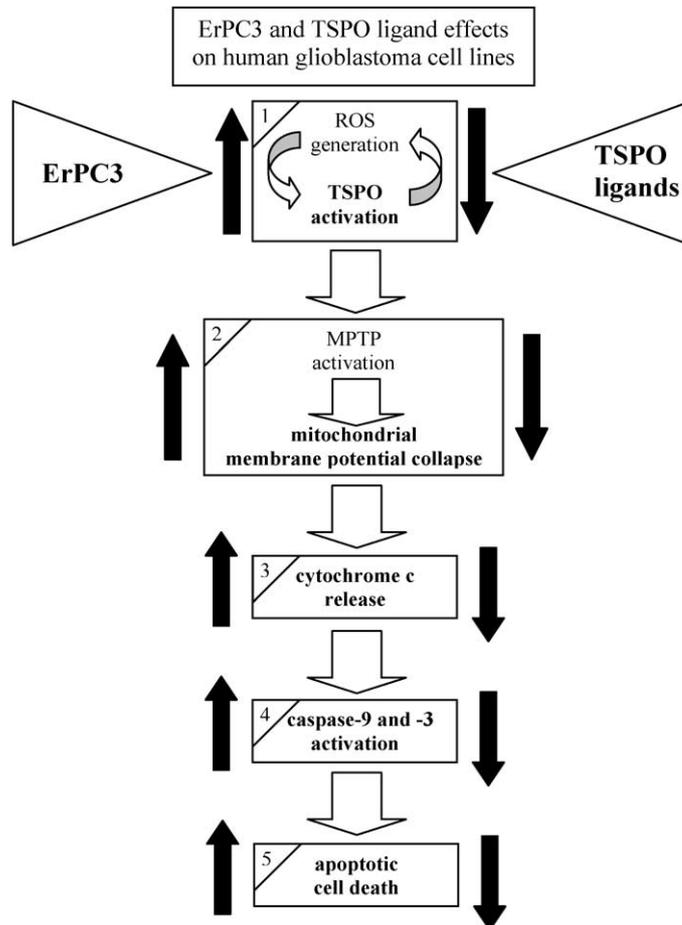


Fig. 8. The present study indicates that TSPO ligands can reduce activation by ErPC3 of the mitochondrial apoptosis cascade, including TSPO activation (blocks 1–5). The black, upward pointing arrow mark activation by ErPC3 of the steps indicated in the blocks. The black, downward pointing arrows depict counteraction of these ErPC3 effects by the TSPO ligands PK 11195 and Ro5 4864. We assume that the activation of TSPO may involve ROS generation (block 1). Our study further demonstrated unequivocally that activation of the mitochondrial apoptosis pathway by ErPC3 includes collapse of mitochondrial membrane potential (block 2), possibly due to direct interactions between ROS, the TSPO and the MPTP.

suggest that ErPC3 may affect TSPO indirectly. Recent preliminary studies by us indicate that ErPC3 generates reactive oxygen species (ROS) that may interact with TSPO (unpublished results) as indicated in Fig. 8. Furthermore, while TSPO often is found in association with the MPTP [38,43], further studies are needed to determine whether the effects of ErPC3 on TSPO regarding apoptosis are mediated directly to the MPTP or take place via an alternative route to affect the MPTP.

Further evidence for the importance of TSPO for apoptosis induction is provided by the different TSPO expression levels of our three cell lines. The effectiveness of ErPC3 to affect the mitochondrial membrane potential and apoptotic levels appeared to correlate straightforward with TSPO levels i.e. the higher the expression levels of TSPO the smaller the amount of ErPC3 that was needed to induce apoptosis and collapse of the mitochondrial membrane potential. Thus, higher levels of TSPO appeared to facilitate activation of the mitochondrial apoptosis pathway by ErPC3. Vice versa, low levels of TSPO appear to impede the capability of ErPC3 to activate the mitochondrial apoptosis pathway. This is further supported by another study, using rat C6 glioma cells, where we found that reduction of TSPO levels, by employing knockdown of TSPO by genetic manipulation, attenuates the capability of ErPC to induce apoptosis [34], as well as collapse of the mitochondrial potential (unpublished results).

We assume that relatively high levels of TSPO in cancer cells serve as a compensatory instrument for other dysregulated apoptotic mechanisms [34,49]. Such mechanisms, we assume, may include apoptotic pathways other than the mitochondrial pathway, or may form parts of events occurring downstream and/or upstream to the TSPO.

Previous studies have shown that PK 11195 and Ro5 4864 can also have pro-apoptotic effects (for a review, see [52]). It is important to note in this regard that studies employing knockdown of the TSPO or using Jurkat cells reportedly not expressing TSPO indicated that this induction of apoptosis by PK 11195 and Ro5 4864 did not require TSPO [17,58]. Presently it is unresolved by which alternative pathways PK 11195 and Ro5 4864 may induce apoptosis [52]. Our present study further indicates that PK 11195 and Ro5 4864 at concentrations higher than 50 μM may affect cell proliferation and induce cell death via pathways other than apoptosis. We believe that it is worthwhile to develop novel TSPO ligands that lack these lethal effects at relatively high concentrations, as they may be useful for novel treatments of neurodegenerative diseases [36,50,51].

We also consider the possibility that the anti-apoptotic activity of PK 11195 and Ro5 4864 may be co-treatment specific. Studies so far indicate that PK 11195 and Ro5 4864 prevent apoptosis induced by ErPC3 (the present study), by tamoxifen, and by photodynamic therapy [14,39,48]. Oxidative stress may be the common denominator for the pro-apoptotic effects of these treatments [13,19,32]. As mentioned above, preliminary studies by us suggest that ErPC3 generates ROS that may interact with the TSPO (unpublished results). We hypothesize that the anti-apoptotic effects by PK 11195 and Ro5 4864 found with the present study may be due to their binding to TSPO, which may inhibit the apoptotic effects of ROS generated by ErPC3. Indeed, it has been suggested that TSPO may serve as an oxygen sensor and may be involved in oxidative stress [5,9,56]. Furthermore, it was suggested that ROS may cause TSPO polymer formation, which may be correlated with altered TSPO function [11]. A recent preliminary study by us, suggests that TSPO knockdown in U118MG cells reduces apoptotic levels normally due to hypoxia, implying that apoptosis induced by oxidative stress indeed may require TSPO [57].

In conclusion, the TSPO ligands Ro5 4864 and PK 11195 appear to be able to inhibit apoptosis induced by the anticancer agent, ErPC3. This inhibition of apoptosis appears to be due to the capability of TSPO ligands to prevent collapse of the mitochondrial potential and as a consequence to inhibit activation of the mitochondrial apoptosis pathway i.e. prevention of cytochrome c release, and caspase-9 and caspase-3 activation (Fig. 8). This suggests that the anti-apoptotic effects of Ro5 4864 and PK 11195 may involve the MPTP. Thus, we consider the possibility that binding of Ro5 4864 and PK 11195 to TSPO may block TSPO's pro-apoptotic function, similar to the effect of TSPO knockdown [34]. These findings may have implications for our understanding of the role of TSPO in the pro-apoptotic mechanisms of anticancer agents such as ErPC3.

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References

- [1] K.L. Black, K. Ikezaki, E. Santori, D.P. Becker and H.V. Vinters, Specific high-affinity binding of peripheral benzodiazepine receptor ligands to brain tumors in rat and man, *Cancer* **65** (1990), 93–97.
- [2] F. Bono, I. Lamarche, V. Prabonnaud, G. Le Fur and J.M. Herbert, Peripheral benzodiazepine receptor agonists exhibit potent antiapoptotic activities, *Biochem. Biophys. Res. Commun.* **265** (1999), 457–461.
- [3] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* **72** (1976), 248–254.
- [4] C. Braestrup and R.F. Squires, Specific benzodiazepine receptors in rat brain characterized by high-affinity [³H]diazepam binding, *Proc. Natl. Acad. Sci. USA* **74** (1977), 3805–3809.
- [5] P. Carayon, M. Portier, D. Dussoisoy, A. Bord, G. Petitpretre, X. Canat, G. Le Fur and P. Casellas, Involvement of peripheral benzodiazepine receptors in the protection of hematopoietic cells against oxygen radical damage, *Blood* **87** (1996), 3170–3178.
- [6] I. Carmel, F.A. Fares, S. Leschiner, H. Scherubl, G. Weisinger and M. Gavish, Peripheral-type benzodiazepine receptors in the regulation of proliferation of MCF-7 human breast carcinoma cell line, *Biochem. Pharmacol.* **58** (1999), 273–278.
- [7] B. Chelli, A. Lena, R. Vanacore, E.D. Pozzo, B. Costa, L. Rossi, A. Salvetti, F. Scatena, S. Ceruti, M.P. Abbracchio, V. Gremigni and C. Martini, Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells, *Biochem. Pharmacol.* **68** (2004), 125–134.
- [8] A. Chevrollier, D. Loiseau, F. Gautier, Y. Malthiery and G. Stepien, ANT2 expression under hypoxic conditions produces opposite cell-cycle behavior in 143B and HepG2 cancer cells, *Mol. Carcinog.* **42** (2005), 1–8.
- [9] A. Courtiere, J. Reybaud, C. Camilla, P. Lobert, J. Drouet and G. Jadot, Oxygen-induced modifications of benzodiazepine receptors and D2 dopamine receptors in the rat under hyperoxia, *Free Radic. Res. Commun.* **15** (1991), 29–34.
- [10] M. Crompton, H. Ellinger and A. Costi, Inhibition by cyclosporin A of a Ca²⁺-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress, *Biochem. J.* **255** (1988), 357–360.
- [11] F. Delavoie, H. Li, M. Hardwick, J.C. Robert, C. Giatzakis, G. Peranzi, Z.X. Yao, J. Maccario, J.J. Lacapere and V. Papadopoulos, *In vivo* and *in vitro* peripheral-type benzodiazepine receptor polymerization: functional significance in drug ligand and cholesterol binding, *Biochemistry* **42** (2003), 4506–4519.
- [12] A. Torroni, G. Stepien, J.A. Hodge and D.C. Wallace, Neoplastic transformation is associated with coordinate induction of nuclear and cytoplasmic oxidative phosphorylation genes, *J. Biol. Chem.* **265** (1990), 20589–20593.
- [13] C. Ferlini, G. Scambia, M. Marone, M. Distefano, C. Gaggini, G. Ferrandina, A. Fattorossi, G. Isola, P. Benedetti Panici and S. Mancuso, Tamoxifen induces oxidative stress and apoptosis in oestrogen receptor-negative human cancer cell lines, *Br. J. Cancer* **79** (1999), 257–263.
- [14] I.E. Furre, S. Shahzidi, Z. Luksiene, M.T. Moller, E. Borgen, J. Morgan, K. Tkacz-Stachowska, J.M. Nesland and Q. Peng, Targeting PBR by hexaminolevulinate-mediated photodynamic therapy induces apoptosis through translocation of apoptosis-inducing factor in human leukemia cells, *Cancer Res.* **65** (2005), 11051–11060.
- [15] M. Gavish, I. Bachman, R. Shoukrun, Y. Katz, L. Veenman, G. Weisinger and A. Weizman, Enigma of the peripheral benzodiazepine receptor, *Pharmacol. Rev.* **51** (1999), 629–650.
- [16] I. Golani, A. Weizman, S. Leschiner, I. Spanier, N. Eckstein, R. Limor, J. Yanai, K. Maaser, H. Scherubl, G. Weisinger and M. Gavish, Hormonal regulation of peripheral benzodiazepine receptor binding properties is mediated by subunit interaction, *Biochemistry* **40** (2001), 10213–10222.
- [17] R.A. Gonzalez-Polo, G. Carvalho, T. Braun, D. Decaudin, C. Fabre, N. Laroche, J.L. Perfettini, M. Djavaheri-Mergny, I. Youlyouz-Marfak, P. Codogno, M. Raphael, J. Feuillard and G. Kroemer, PK 11195 potently sensitizes to apoptosis induction independently from the peripheral benzodiazepine receptor, *Oncogene* **24** (2005), 7503–7513.
- [18] D.R. Green and J.C. Reed, Mitochondria and apoptosis, *Science* **281** (1998), 1309–1312.
- [19] S. Gupta, N. Ahmad and H. Mukhtar, Involvement of nitric oxide during phthalocyanine (Pc4) photodynamic therapy-mediated apoptosis, *Cancer Res.* **58** (1998), 1785–1788.
- [20] D. Haouzi, I. Cohen, H.L. Vieira, D. Poncet, P. Boya, M. Castedo, N. Vadrot, A.S. Belzacq, D. Fau, C. Brenner, G. Feldmann and G. Kroemer, Mitochondrial permeability transition as a novel principle of hepatorenal toxicity *in vivo*, *Apoptosis* **7** (2002), 395–405.
- [21] V. Held-Kuznetsov, A. Premkumar, L. Veenman, W. Kugler, S. Leschiner, I. Spanier, M. Lakomek, G.W. Pasternak and M. Gavish, Knockdown of the adenine nucleotide transporter (ANT) isoform 2 (ANT2) component of the peripheral-type benzodiazepine receptor (PBR) reduces C6 glioma cell tumorigenicity, *Rev. Neurosci.* **16**(Suppl. 1) (2005), S30.
- [22] P. Hilgard, T. Klenner, J. Stekar and C. Unger, Alkylphosphocholines: a new class of membrane-active anticancer agents, *Cancer Chemother. Pharmacol.* **32** (1993), 90–95.
- [23] T. Hirsch, D. Decaudin, S.A. Susin, P. Marchetti, N. Laroche, M. Resche-Rigon and G. Kroemer, PK 11195, a ligand of the mitochondrial benzodiazepine receptor, facilitates the induction of apoptosis and reverses Bcl-2-mediated cytoprotection, *Exp. Cell Res.* **241** (1998), 426–434.

- [24] V. Jendrossek, B. Erdlenbruch, A. Hunold, W. Kugler, H. Eibl and M. Lakomek, Erucylphosphocholine, a novel antineoplastic ether lipid, blocks growth and induces apoptosis in brain tumor cell lines *in vitro*, *Int. J. Oncol.* **14** (1999), 15–22.
- [25] V. Jendrossek, W. Kugler, B. Erdlenbruch, H. Eibl, F. Lang and M. Lakomek, Erucylphosphocholine-induced apoptosis in chemoresistant glioblastoma cell lines: involvement of caspase activation and mitochondrial alterations, *Anticancer Res.* **21** (2001), 3389–3396.
- [26] Y. Katz, G. Ben-Baruch, Y. Kloog, J. Menczer and M. Gavish, Increased density of peripheral benzodiazepine-binding sites in ovarian carcinomas as compared with benign ovarian tumours and normal ovaries, *Clin. Sci. (London)* **78** (1990), 155–158.
- [27] Y. Katz, A. Eitan, Z. Amiri and M. Gavish, Dramatic increase in peripheral benzodiazepine binding sites in human colonic adenocarcinoma as compared to normal colon, *Eur. J. Pharmacol.* **148** (1988), 483–484.
- [28] E. Kelly-Herschkovitz, R. Weizman, I. Spanier, S. Leschiner, M. Lahav, G. Weisinger and M. Gavish, Effects of peripheral-type benzodiazepine receptor antisense knockout on MA-10 Leydig cell proliferation and steroidogenesis, *J. Biol. Chem.* **273** (1998), 5478–5483.
- [29] T. Kino, H. Hatanaka, S. Miyata, N. Inamura, M. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki et al., FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 *in vitro*, *J. Antibiot. (Tokyo)* **40** (1987), 1256–1265.
- [30] W. Kugler, F. Buchholz, F. Köhler, H. Eibl, M. Lakomek and B. Erdlenbruch, Downregulation of Apaf-1 and caspase-3 by RNA interference in human glioma cells: Consequences for erucylphosphocholine-induced apoptosis, *Apoptosis* **10** (2005), 1163–1174.
- [31] W. Kugler, B. Erdlenbruch, A. Jünemann, D. Heinemann, H. Eibl and M. Lakomek, Erucylphosphocholine-induced apoptosis in glioma cells: involvement of death receptor signaling and caspase activation, *J. Neurochem.* **82** (2002), 1160–1170.
- [32] W. Kugler, K. Linnemannstöns, L. Veenman, M. Gavish and M. Lakomek, Erucylphosphocholine-induced cell death in human glioma cells: Role of reactive oxygen species, *Neur. Plast.* **1** (2007), doi:10.1155/2007/73079 (abstract).
- [33] W. Kugler, B. Erdlenbruch, K. Otten, V. Jendrossek, H. Eibl and M. Lakomek, MAP kinase pathways involved in glioblastoma response to erucylphosphocholine, *Int. J. Oncol.* **25** (2004), 1721–1727.
- [34] E. Levin, A. Premkumar, L. Veenman, W. Kugler, S. Leschiner, I. Spanier, G. Weisinger, M. Lakomek, A. Weizman, S.H. Snyder, G.W. Pasternak and M. Gavish, The peripheral-type benzodiazepine receptor and tumorigenicity: isoquinoline binding protein (IBP) antisense knockdown in the C6 glioma cell line, *Biochemistry* **44** (2005), 9924–9935.
- [35] K. Maaser, M. Hopfner, A. Jansen, G. Weisinger, M. Gavish, A.P. Kozikowski, A. Weizman, P. Carayon, E.O. Riecken, M. Zeitz and H. Scherubl, Specific ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in human colorectal cancer cells, *Br. J. Cancer* **85** (2001), 1771–1780.
- [36] I. Maniv, L. Veenman, A. Shterenberg, I. Marek, W. Kugler, M. Lakomek and M. Gavish, Drug development for the prevention of secondary brain injury, *Neur. Plast.* **1** (2007), 66; doi:10.1155/2007/73079 (abstract).
- [37] L. Marselli, L. Trincavelli, C. Santangelo, R. Lupi, S. Del Guerra, U. Boggi, A. Falleni, V. Gremigni, F. Mosca, C. Martini, F. Dotta, U. Di Mario, S. Del Prato and P. Marchetti, The role of peripheral benzodiazepine receptors on the function and survival of isolated human pancreatic islets, *Eur. J. Endocrinol.* **51** (2004), 207–214.
- [38] M.W. McEnery, A.M. Snowman, R.R. Trifiletti and S.H. Snyder, Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier, *Proc. Natl. Acad. Sci. USA* **89** (1992), 3170–3174.
- [39] R.L. Morris, M.E. Varnes, M.E. Kenney, Y.S. Li, K. Azizuddin, M.W. McEnery and N.L. Oleinick, The peripheral benzodiazepine receptor in photodynamic therapy with the phthalocyanine photosensitizer Pc 4, *Photochem. Photobiol.* **75** (2002), 652–661.
- [40] H. Ohgaki and P. Kleihues, Epidemiology and etiology of gliomas, *Acta Neuropathol. (Berlin)* **9** (2005), 93–108.
- [41] V. Papadopoulos, H. Amri, N. Boujrad, C. Cascio, M. Culty, M. Garnier, M. Hardwick, H. Li, B. Vidic, A.S. Brown, J.L. Reversa, J.M. Bernassau and K. Drieu, Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis, *Steroids* **62** (1997), 21–28.
- [42] V. Papadopoulos, M. Baraldi, T.R. Guilarte, T.B. Knudsen, J.J. Lacapere, P. Lindemann, M.D. Norenberg, D. Nutt, A. Weizman, M.R. Zhang and M. Gavish, Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function, *Trends Pharmacol. Sci.* **27** (2006), 402–409.
- [43] V. Papadopoulos, N. Boujrad, M.D. Ikonovic, P. Ferrara and B. Vidic, Topography of the Leydig cell mitochondrial peripheral-type benzodiazepine receptor, *Mol. Cell. Endocrinol.* **104** (1994), R5–R9.
- [44] N.G. Rainov, A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme, *Hum. Gene Ther.* **11** (2000), 2389–2401.
- [45] E.K. Richfield, B.J. Ciliax, S.R. Starosta-Rubinstein, P.E. McKeever, J.B. Penney and A.B. Young, Comparison of [¹⁴C]-deoxyglucose metabolism and peripheral benzodiazepine receptor binding in rat C₆ glioma, *Neurology* **38** (1988), 1255–1262.
- [46] A. Rivera and S.A. Maxwell, The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway, *J. Biol. Chem.* **280** (2005), 29346–29354.
- [47] H. Schoemaker, R.G. Boles, W.D. Horst and H.I. Yamamura, Specific high-affinity binding sites for [³H]Ro 5-4864 in rat brain and kidney, *J. Pharmacol. Exp. Ther.* **225** (1983), 61–69.
- [48] R. Strohmeier, M. Roller, N. Sanger, R. Knecht and H. Kuhl, Modulation of tamoxifen-induced apoptosis by peripheral benzodiazepine receptor ligands in breast cancer cells, *Biochem. Pharmacol.* **64** (2002), 99–107.
- [49] L. Veenman, E. Levin, G. Weisinger, S. Leschiner, I. Spanier, S.H. Snyder, A. Weizmann and M. Gavish, Peripheral-type benzodiazepine receptor density and *in vitro* tumorigenicity of glioma cell lines, *Biochem. Pharmacol.* **68** (2004), 689–698.

- [50] L. Veenman and M. Gavish, Peripheral-type benzodiazepine receptors: Their implication in brain disease, *Drug Dev. Res.* **50** (2000), 355–370.
- [51] L. Veenman and M. Gavish, The peripheral-type benzodiazepine receptor and the cardiovascular system: Implications for drug development, *Pharmacol. Ther.* **10** (2006), 503–524.
- [52] L. Veenman, V. Papadopoulos and M. Gavish, Channel-like functions of the 18-kDa translocator protein (TSPO): Regulation of apoptosis and steroidogenesis as part of the host-defense response, *Curr. Pharm. Design* **13** (2007), 2385–2405.
- [53] W. Vogelhuber, T. Spruss, G. Bernhardt, A. Buschauer and A. Gopferich, Efficacy of BCNU and paclitaxel loaded subcutaneous implants in the interstitial chemotherapy of U-87 MG human glioblastoma xenografts, *Int. J. Pharm.* **238** (2002), 111–121.
- [54] G. Weisinger, E. Kelly-Herskovitz, L. Veenman, I. Spanier, S. Leschiner and M. Gavish, Peripheral benzodiazepine receptor antisense knockout increases tumorigenicity of MA-10 Leydig cells *in vivo* and *in vitro*, *Biochemistry* **43** (2004), 12315–12321.
- [55] W. Xia, S. Spector, L. Hardy, S. Zhao, A. Saluk, L. Alemane and N.L. Spector, Tumor selective G2/M cell cycle arrest and apoptosis of epithelial and hematological malignancies by BBL22, a benzazepine, *Proc. Natl. Acad. Sci. USA* **97** (2000), 7494–7499.
- [56] A.A. Yeliseev, K.E. Krueger and S. Kaplan, A mammalian mitochondrial drug receptor functions as a bacterial “oxygen” sensor, *Proc. Natl. Acad. Sci. USA* **94** (1997), 5101–5106.
- [57] S. Zeno, L. Veenman, S. Leschiner, M. Zaarur and M. Gavish, The correlation between the Translocator Protein (TSPO) and apoptosis in human glioma cells under hypoxia, in: *Abstracts of the 16th Annual Meeting of the Israel Society for Neuroscience (ISFN)*, Eilat, Israel, 2007.
- [58] S.J. Zunino and D.H. Storms, Resveratrol-induced apoptosis is enhanced in acute lymphoblastic leukemia cells by modulation of the mitochondrial permeability transition pore, *Cancer Lett.* **240** (2006), 123–134.



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