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# Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells

Beatrice Chelli[a,1], Annalisa Lena[b,1], Renato Vanacore[c], Eleonora Da Pozzo[a], Barbara Costa[a], Leonardo Rossi[b], Alessandra Salvetti[b], Fabrizio Scatena[c], Stefania Ceruti[d], Maria P. Abbracchio[d], Vittorio Gremigni[b], Claudia Martini[a,*]

[a]*Department of Psychiatry, Neurobiology, Pharmacology and Biotechnology, University of Pisa, Pisa, Italy*
[b]*Department of Human Morphology and Applied Biology, University of Pisa, Pisa, Italy*
[c]*UO Immunohaematology 2, "Cisanello" Hospital, Pisa, Italy*
[d]*Department of Pharmacological Sciences, University of Milan, Milan, Italy*

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**Abstract**

The peripheral benzodiazepine receptor (PBR) is a component of a multiprotein complex, located at the contact site between the inner and outer mitochondrial membranes, which constitutes the mitochondrial permeability transition (MPT)-pore. The opening of the MPT-pore, leading to the transmembrane mitochondrial potential ($\Delta\Psi_m$) dissipation, is a critical event in the mechanism of apoptosis. In the present work, we investigated the ability of the specific PBR ligands, PK 11195 or Ro5-4864, to affect mitochondrial potential and to induce apoptotic cell death in rat C6 glioma cells. Both specific ligands inhibited cell survival in a dose- and time-dependent manner, as assessed by MTS conversion assay, whereas the non-site selective ligand Diazepam or the low-affinity benzodiazepine Clonazepam showed no significant effects. After cell exposure to PK 11195 or Ro5-4864 we evidenced typical alterations of apoptotic cell death such as DNA fragmentation and chromatin condensation assessed by flow cytometric and transmission electron microscopy (TEM) analysis, respectively. Activation of the ''effector'' caspase-3 confirmed the ability of specific PBR ligands to induce apoptosis. Moreover, PK 11195 and Ro5-4864 induced a decrease of $\Delta\Psi_m$, as evidenced by JC-1 flow cytometry analysis. Our data demonstrate the pro-apoptotic effects of specific PBR ligands on rat C6 glioma cells.


*Keywords:* Peripheral benzodiazepine receptor ligands; Apoptosis; Mitochondrial transmembrane potential; Rat C6 glioma cells; Flow cytometry; Transmission electron microscopy

## 1. Introduction

The peripheral-type benzodiazepine receptor (PBR), originally discovered as an alternative binding site for the benzodiazepine Diazepam (Valium®), forms a unique class of receptors which are pharmacologically and functionally different from the central-type receptor (CBR). [1–3]. Although it shows an ubiquitous distribution in peripheral tissues, in the central nervous system the overall PBR content is low and predominantly localized in glial cells, in which it becomes abundant in pathological conditions including cancer [4–10].

Malignant astrocytic gliomas are the most common primary brain tumors, whose polymorphic de-differentiated cells show resistance to apoptotic cell death induction and rapidly infiltrate adjacent tissue [11]. Despite substantial efforts, no effective therapy has been found and overall-survival of patients is poor [12,13]. At present, in addition to other therapies, medical strategies include the use of agents able to induce apoptotic cell death [14,15].

*Abbreviations:* MPT, mitochondrial permeability transition; PBR, peripheral benzodiazepine receptor; TEM, transmission electron microscopy; $\Delta\Psi_m$, mitochondrial transmembrane potential; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium

* Corresponding author. Tel.: +39-050-2219522/11; fax: +39-050-2219609.
*E-mail address:* cmartini@farm.unipi.it (C. Martini).

[1] These authors contributed equally to this work.

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Several evidences have provided that in the machinery leading to apoptosis the MPT is a critical event of cell death, marking the point of no return of this process. The extensive and prolonged opening of the pore responsible of MPT can cause the dissipation of the transmembrane mitochondrial potential leading to the release of pro-apoptotic inter-membrane proteins [16,17]. PBR has been suggested to be a constituent of this pore [2,18] in association with other mitochondrial proteins (i.e. the voltage-dependent anion channel, the adenine nucleotide translocator and the anti- and pro-apoptotic members of Bcl-2 family) and to take part in the regulation of MPT and in the induction of apoptosis [19]. Specifically, numerous findings have suggested that PBR ligands may act as potential therapeutic agents useful in the management of a large spectrum of diseases, including cancer, through the modulation of the MPT-pore activity [20–26].

In several glioma cell lines, PBR has been shown to be highly expressed, localized at mitochondrial and/or nuclear level and to facilitate the transport of cholesterol. These findings suggested that the receptor might play an important role in regulating cell growth and tumor development/progression [9,10,27]. Moreover, PBR ligands have been demonstrated to inhibit proliferation of rat C6 glioma, mouse neuro-2A neuroblastoma cells and primary astrocytes cultures [28,29]. However, at our knowledge, no data are available in glioma cells regarding the ability of PBR ligands to interfere with MPT-pore functioning and to modulate cell proliferation and survival through apoptosis.

Therefore, the aim of the present paper is to investigate the ability of selective and high affinity PBR ligands to induce mitochondrial transmembrane potential dissipation and apoptosis in rat C6 glioma cells, providing interesting means for overcoming apoptosis resistance of tumoral cells.

## 2. Materials and methods

### 2.1. Drugs and materials

1-(2-chlorophenyl-*N*-methyl-1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864), carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), Diazepam, Clonazepam, Nonidet P-40 (NP-40), soybean trypsin inhibitor, bacitracine, benzamidine and Trypan Blue were obtained from Sigma–Aldrich. Propidium iodide (PI) and fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were obtained from Molecular Probes; DNase-free RNase was purchased from Boehringer/Roche. MTS assay kit (Cell Titer 96® AQueous one Solution Cell Proliferation assay) was from Promega. Caspase-3 Colorimetric Activity Assay Kit was from Chemicon International. Cell culture media and fetal bovine serum (FBS) were from Bio-Whittaker. All other chemicals were supplied by standard commercial sources.

### 2.2. Cell cultures and drug treatments

Rat C6 glioma cells (a kindly gift from Professor Damir Janigro, Cleveland Clinic Foundation, Cleveland, OH) were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL) (complete medium). Cells were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$ and were fed every 3 days.

Cells were seeded in 96-well plates at a density of ∼1000 cells/well for viability assays, or in 24-well plates at a density of 5000 cells/well, at least, for apoptosis measurements. The following day, medium was changed with complete medium (untreated-cells) or with medium supplemented with the tested ligands at several concentrations (1–100 μM) and the cells were incubated for different times (12–96 h).

The percentage of the organic solvent, in which PBR ligands were dissolved, never exceeded 1% (v/v) in the samples. We verified that this amount did not affect cell viability.

### 2.3. Cell viability analysis by MTS conversion and Trypan Blue exclusion assays

Following cell treatments, the number of living cells was measured by quantitative colorimetric MTS assay kit according to manufacturer. After 2 h of incubation with the reagent, the absorbance of individual wells was measured by microplate reader (Wallac Victor 2, 1420 Multilabel counter, Perkin–Elmer).

Each drug concentration was tested in duplicate and the experiments were repeated at least three times.

Cell viability was also measured using Trypan Blue exclusion assay [30].

### 2.4. Flow cytometry analysis of nuclear DNA content

After treatment, detached and adherent trypsinized cells were collected by centrifugation at 200 × *g* for 5 min. Pellets were gently suspended in 500 μL of a hypotonic fluorochrome solution [PI 50 μg/mL in 0.1% (w/v) sodium citrate plus 0.01% (v/v) NP-40 and 10 μg/mL DNase-free RNAse in bi-distilled water]. After incubation in the dark at 37 °C in 5% $CO_2$ for 30 min, cell samples were stored on ice and analyzed for DNA content on logarithmic scale by FACScalibur flow cytometer (Becton Dickinson) with doublet discrimination and Cell Quest research software. $10^4$ events per sample were acquired and the percentage of cells in the sub-$G_0$ fraction (representing apoptotic cells) was determined.

### 2.5. Transmission electron microscope analysis

To evaluate the morphological features of C6 glioma cell death, control cells or cells treated with PBR ligands were processed for transmission electron microscopy (TEM). Briefly, both floating and adherent cells were harvested and pelleted by centrifugation. Pellets were washed three times in phosphate buffered saline (PBS) and fixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4 °C. Cells were then scraped off and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h at room temperature. After rapid dehydration in a graded series of ethanol and propylene oxide, cells were embedded in an ''Epon-Araldite'' mixture. Ultrathin sections, obtained by a diamond knife on an Ultracut Reichert-Jung ultramicrotome, were placed on Formvar-carbon coated nickel grids, stained with uranyl acetate and lead citrate and observed with a Jeol 100 SX transmission electron microscope.

### 2.6. Casapse-3 activity assay

Caspase-3 activity was measured by means of a spectrophotometric assay kit, following manufacturer's instructions. Briefly, after treatment both floating and adherent cells were collected and $2 \times 10^6$ cells were pelleted by centrifugation. The cells were resuspended in 500 μL of cell lysis buffer (as supplied by the manufacturer) and incubated for 20 min on ice. The insoluble fraction was discarded by centrifugation (5 min at 10,000 × *g*) and the protein content in the supernatant was determined according to the method of Bradford [31]. Different protein concentrations were tested, and best results were obtained with a total amount of 50–60 μg. The determination of caspase-3 activity was carried out in a 96-well plate in a total volume of 100 μL of assay buffer in the presence of the caspase-3 substrate (Ac-DEVD-pNA). After incubation of assay mixture for 2 h at 37 °C, the released pNA was measured by spectrophotometer at 405 nm. Caspase-3 activity was expressed as U/mg proteins and the fold-increase can be determined by comparing the value from the treated-cell samples with the value of untreated control cells, at which is attributed the arbitrary value of one. Samples were run in duplicate and three independent experiments were performed.

### 2.7. Flow cytometry analysis of mitochondrial transmembrane potential

After treatments with PBR ligands, the mitochondrial membrane potential was determined by labeling both floating and adherent trypsinized control and treated C6 glioma cells with the fluorescent dye JC-1 for 20 min at room temperature, as previously described by Ceruti et al. [32]. J-aggregate fluorescence was recorded by flow cytometry in the fluorescence channel 2 (FL2) and monomer fluorescence in the fluorescence channel 1 (FL1). Necrotic fragments were electronically gated out, on the basis of morphological characteristics on the forward light scatter versus side light scatter dot plot.

As a control, an aliquot of cells was incubated in the presence of the uncoupler agent CCCP (5 μM) in each experiment.

### 2.8. Data analysis

For data analysis and graphic presentations the non-linear multipurpose curve-fitting program Graph-Pad Prism (GraphPad) was used.

All data are presented as mean ± S.E.M. Statistical analysis was performed by one-way ANOVA (with post hoc Newman–Keuls test). $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Induction of cell death by specific PBR ligands

Specific PBR ligands were investigated for their ability to interfere with cell survival and to induce apoptosis in rat C6 glioma cells.

Cell viability was determined quantitatively by MTS conversion assay. Treatment of rat glioma cells with increasing concentrations of the specific PBR ligands, PK 11195 and Ro5-4864, at different incubation times induce a dose- and time course-dependent inhibition of cell survival as shown in Figs. 1A and 2A. At maximum time of incubation, dose-response curves indicated that, after cell exposure to PK 11195 or Ro5-4864, the $EC_{50}$ values (the concentration inducing 50% cell survival inhibition) were $94.2 \pm 8.50$ μM and $58.4 \pm 4.6$ μM, respectively. At 100 μM ligand concentration percentage of cell survival was reduced to $47.4 \pm 6.75$ and $20.6 \pm 2.30$% versus control (Figs. 1B and 2B). The non selective PBR compound diazepam did not show any significant inhibitory effect on cell survival ($90.1 \pm 1.90$%, $P > 0.05$, versus control, set to 100%) and the central-type site selective benzodiazepine Clonazepam did not elicit any effect on cell viability (Fig. 3).

In addition, the survival of untreated control cells was always greater than 90% after each time of exposure, as assessed by Trypan Blue exclusion test. Moreover, by using this assay we observed that PBR ligands affected cell survival with similar results to those obtained by MTS assay (data not shown).

### 3.2. Induction of apoptosis and activation of caspase-3 by specific PBR ligands

In order to establish the nature of C6 glioma cell death (necrosis or apoptosis) induced by PBR ligands, nuclear

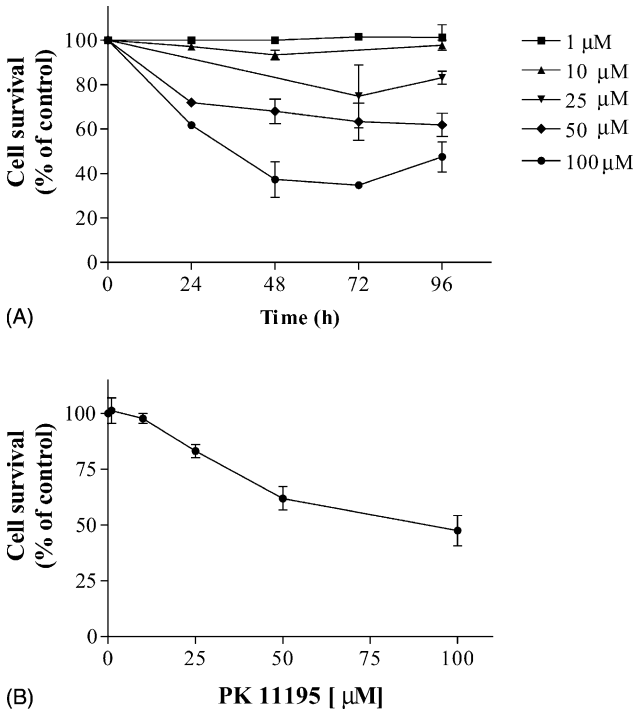


Fig. 1. Time course and dose response of PK 11195-induced C6 glioma cell death: C6 glioma cells were seeded at ∼ 1000 cells/well and incubated with increasing concentrations (range 1–100 μM) of PK 11195 for 24, 48, 72 or 96 h (A) or at 100 μM for 96 h (B). Untreated-cells were used as control. After each incubation time, cell viability was measured by MTS conversion assay as described in Section 2. Results are expressed as percentage of viable cells observed after treatment with PK 11195 vs. untreated control cells (100%) and shown as mean ± S.E.M. derived from at least three separate experiments done in duplicate.

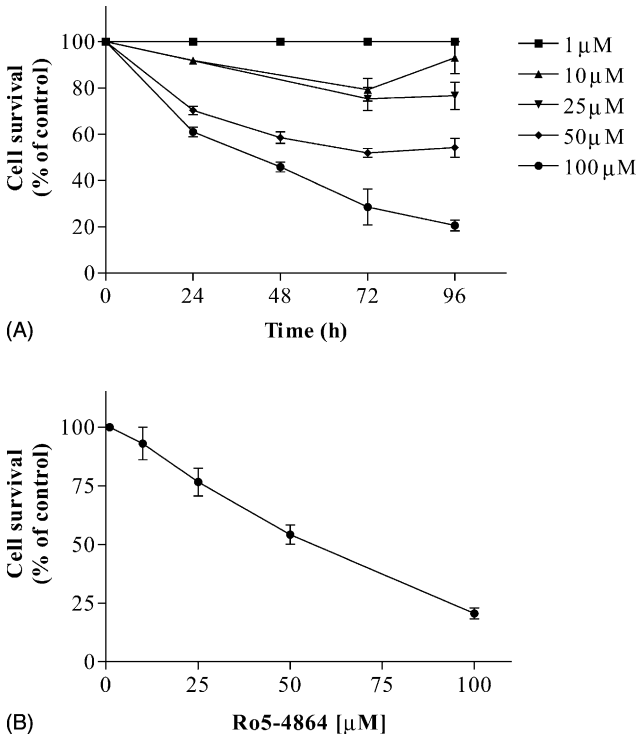


Fig. 2. Time course and dose response of Ro5-4864-induced C6 glioma cell death: C6 glioma cells were seeded at ∼1000 cells/well and incubated with increasing concentrations (range 1–100 μM) of Ro5-4864 for 24, 48, 72 or 96 h (A) or at 100 μM for 96 h (B). Untreated-cells were used as control. After each incubation time, cell viability was measured by MTS conversion assay as described in Section 2. Results are expressed as a percentage of viable cells observed after treatment with Ro5-4864 vs. untreated control cells (100%) and shown as mean ± S.E.M. derived from at least three separate experiments done in duplicate.

DNA fragmentation (an apoptotic marker) was assessed by flow cytometry using the DNA specific PI staining. Since DNA fragments are not easily extractable from apoptotic cells, we employed a hypotonic solution of PI to facilitate the loss of fragmented DNA and to evaluate the effects of PBR ligands in isolated nuclei. DNA fragmentation was evaluated after 12, 24, 48, 72, and 96 h of treatment and in Fig. 4A frequency histograms from a representative experiment of flow cytometric analysis are shown. As evidenced in Fig. 4B, both PK 11195- and Ro5-4864-treated-cells showed a significant increase in the percentage of nuclei with hypodiploid DNA content (sub-$G_0$ cells), a clear sign of apoptotic cells, in comparison with control cells ($12.0 \pm 0.72\%$; $P < 0.001$ and $20.3 \pm 0.90\%$; $P < 0.001$ treated-cells versus $1.41 \pm 0.27\%$ control cells). The amount of apoptotic hypodiploid DNA content of treated-cells did not increase prolonging time exposure (data not shown). By contrast, Diazepam ($3.34 \pm 1.14\%$) or Clonazepam ($2.24 \pm 0.26\%$) did not induce significant nuclear DNA fragmentation ($P > 0.05$), even when treatment was extended to 96 h.

PBR ligand induced apoptosis was confirmed by TEM ultrastructural analysis. Untreated rat C6 glioma cells (control) were irregularly roundish with large euchromatic, sometimes convoluted or segmented nuclei. The cell surface showed numerous microvillar projections and the cytoplasm exhibited a well developed endoplasmic reticulum, free ribosomes and numerous mitochondria with well detectable regular cristae (Fig. 5A). By contrast, the cells

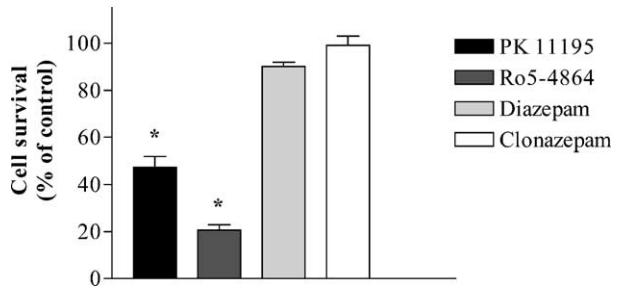


Fig. 3. PBR ligand-induced C6 glioma cell death: C6 glioma cells were seeded at ∼1000 cells/well and incubated with PK 11195, Ro5-4864, Diazepam or Clonazepam at 100 μM for 96 h. Untreated-cells were used as control. Cell viability was measured by MTS conversion assay as described in Section 2. Results are expressed as a percentage of viable cells observed after treatment with ligands vs. untreated control cells (100%) and shown as mean ± S.E.M. derived from least three separate experiments done in duplicate. $^*P < 0.05$ with respect to control, one-way ANOVA (Newman–Keuls test).

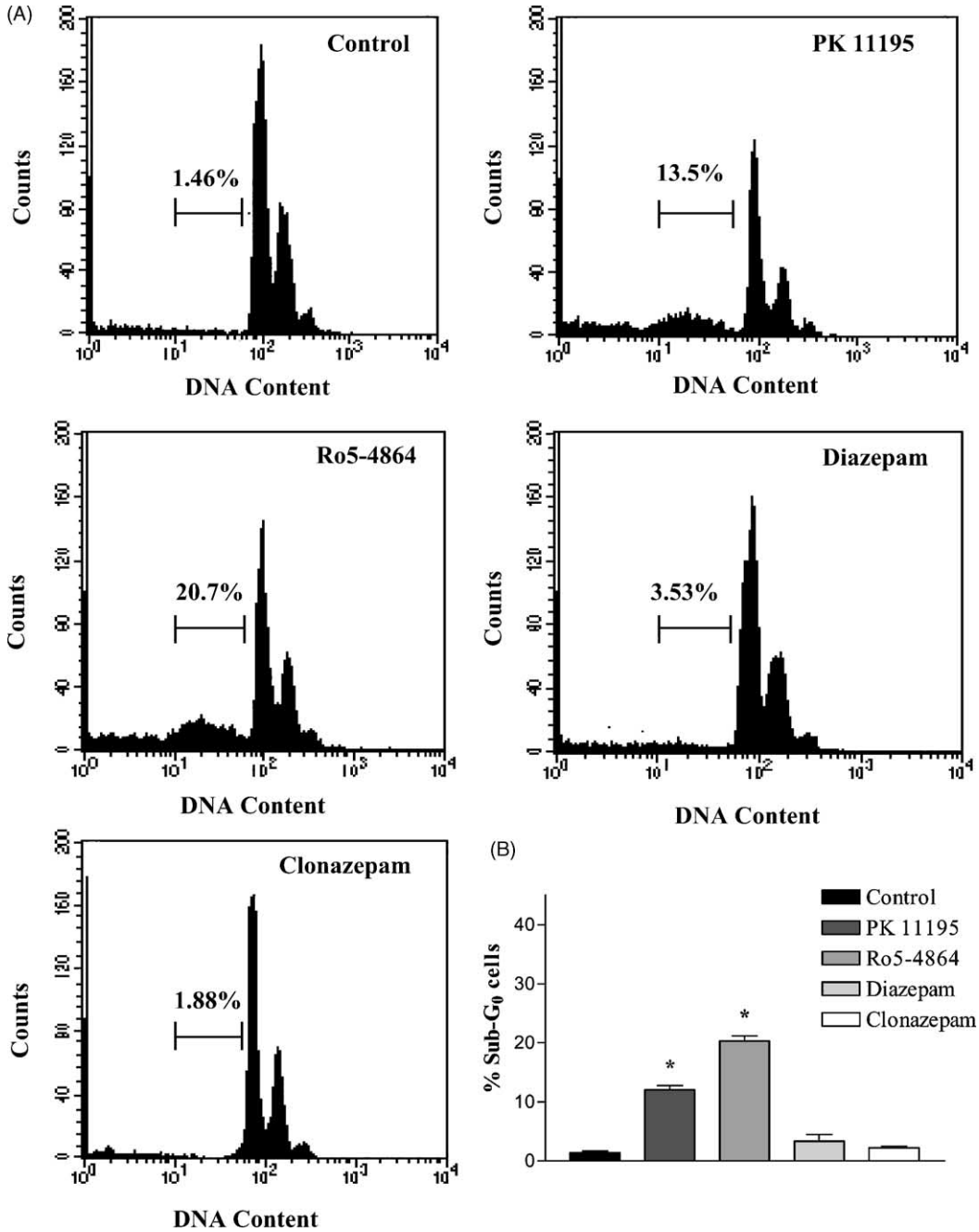


Fig. 4. Flow cytometric analysis of DNA content in PBR ligand-treated C6 glioma cells: C6 glioma cells were seeded at ~5000 cells/well and incubated with PK 11195, Ro5-4864, Diazepam or Clonazepam at 100 μM for 24 h. At the end of incubation, treated-cells and untreated control were stained with PI as described in Section 2 and analyzed for DNA content by FACScalibur flow cytometer on logarithmic scale. DNA content frequency histograms from a representative experiments are shown (A). Results are expressed as percentage of nuclei with hypodiploid DNA content (sub-$G_0$ cells) vs. untreated control cells and shown as means ± S.E.M. derived from at least three separate experiments done in duplicate. $^*P < 0.05$ with respect to control, one-way ANOVA (Newman–Keuls test) (B).

treated with 100 μM PK 11195 or Ro5-4864 for 24, 48, 72, and 96 h showed a cytoplasm with swollen and pale mitochondria only recognizable by their double membranes and displaced, distorted cristae (Fig. 5B). Typical features of apoptosis, such as cytoplasm shrinkage and chromatin condensation and margination were detected in many PK 11195- or Ro5-4864-treated-cells, an example is showed in Fig. 5C. Occasionally, the nuclei of treated-cells appeared to be broken up into small nuclear fragments. (Fig. 5D).

To support the ability of PBR ligands to induce apoptosis in rat C6 glioma cells we measured the activation of caspase-3, the most important "effector" caspase. Caspase-3 analysis revealed that 24 h treatment with PK 11195 or Ro5-4864 (100 μM) caused a significant increase in caspase-3 activity (1.89 ± 0.07; $P < 0.01$ and 1.53 ± 0.05; $P < 0.05$,

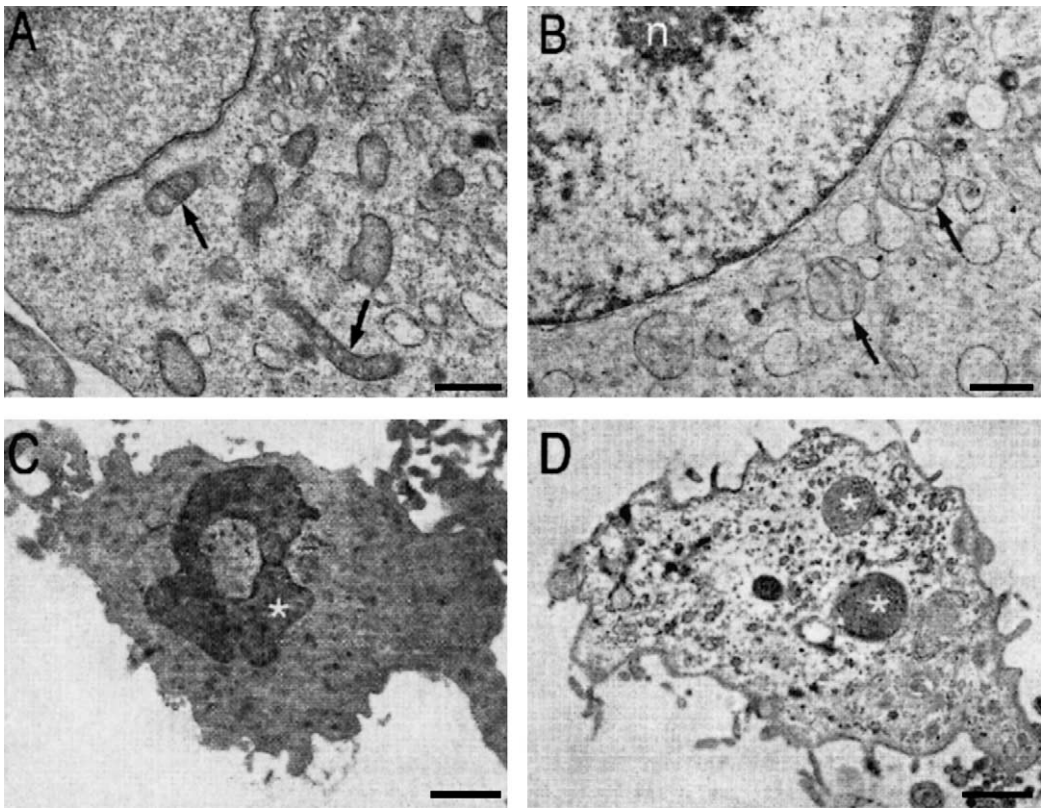


Fig. 5. TEM micrographs of untreated or PBR ligand-treated C6 glioma cells: a representative control cell with a mainly euchromatic nucleus and a well preserved cytoplasm containing several mitochondria (Ø) and vesicles of the endoplasmic reticulum (A). A representative cell treated with 100 μM PK 11195 for 24 h, shows part of the nucleolus (n) and some patches of heterochromatin in the nucleus and swollen and pale mitochondria (Ø) in the perinuclear cytoplasm (B). A representative cell treated with 100 μM PK 11195 for 96 h, showing margination of the chromatin into a large ring-shaped mass (∗) (C). A 100 μM PK 11195-treated-cell showing recognizable nuclear fragments (∗) (D). Scale bars represents 1 μm (A, B) and 3 μm (C, D).

respectively, versus the value of control cells, to which was attributed the arbitrary value of one). Extended 48 h treatment induced further increase of caspase-3 activity ($4.74 \pm 0.24$; $P < 0.001$ for PK 11195 and $4.03 \pm 0.05$; $P < 0.001$ for Ro5-4864) (Fig. 6).

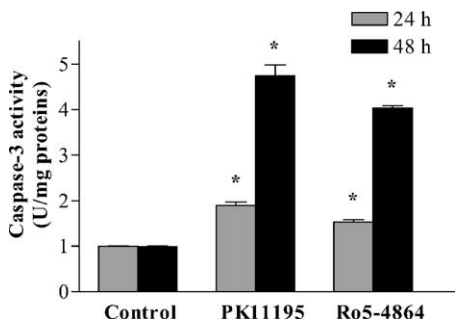


Fig. 6. Caspase-3 activity in PBR ligand-treated C6 glioma cells: after C6 glioma cells exposure to 100 μM PK 11195 or Ro5-4864 for 24 or 48 h, caspase-3 activity was determined by evaluating cleavage of the specific substrate Ac-DEVD-pNA and release of pNA at 405 nm as described in Section 2. Caspase-3 activity is expressed as U/mg proteins. One unit (U) of activity is the amount of enzyme that cleaves 1 nmol of the fluorescent substrate per hour at 37 °C under saturate substrate concentration. Data were normalized to the controls (arbitrary value = 1) and expressed as means ± S.E.M. derived from at least three separate experiments done in duplicate. $^{*}P < 0.05$ with respect to corresponding controls, one-way ANOVA (Newman–Keuls test).

### 3.3. Induction of mitochondrial transmembrane potential dissipation by specific PBR ligands

Since it is well established the pivotal role of mitochondria in triggering apoptosis, we evaluated the mitochondrial transmembrane potential ($\Delta\Psi_m$) in rat C6 glioma cells exposed to PBR ligands. Possible changes in $\Delta\Psi_m$ have been evaluated by means of cytofluorometric analysis. Cells were stained with the specific mitochondrial cationic dye JC-1, that accumulates in the transmembrane space of polarized mitochondria and forms the so-called ‘‘J-aggregates’’, emitting orange fluorescence recorded by fluorescence channel 2. A decrease in $\Delta\Psi_m$ results in disappearance of J-aggregates and formation of JC-1 monomers, which emit in a greenish–yellow fluorescence recorded by fluorescence channel 1. It is therefore possible to monitor changes in $\Delta\Psi_m$ by looking at the number of cells showing fluorescence emission in both channels [32,33]. Representative examples of the cytometric analysis of C6 glioma cells stained with JC-1 are reported in Fig. 7A. In control untreated-cells, due to the equilibrium between J-aggregates and monomers, the majority of cells (96.8%) shows a high emission of fluorescence in both channels and is therefore found in the upper right (UR) quadrant of the plot. Only a very small percentage of

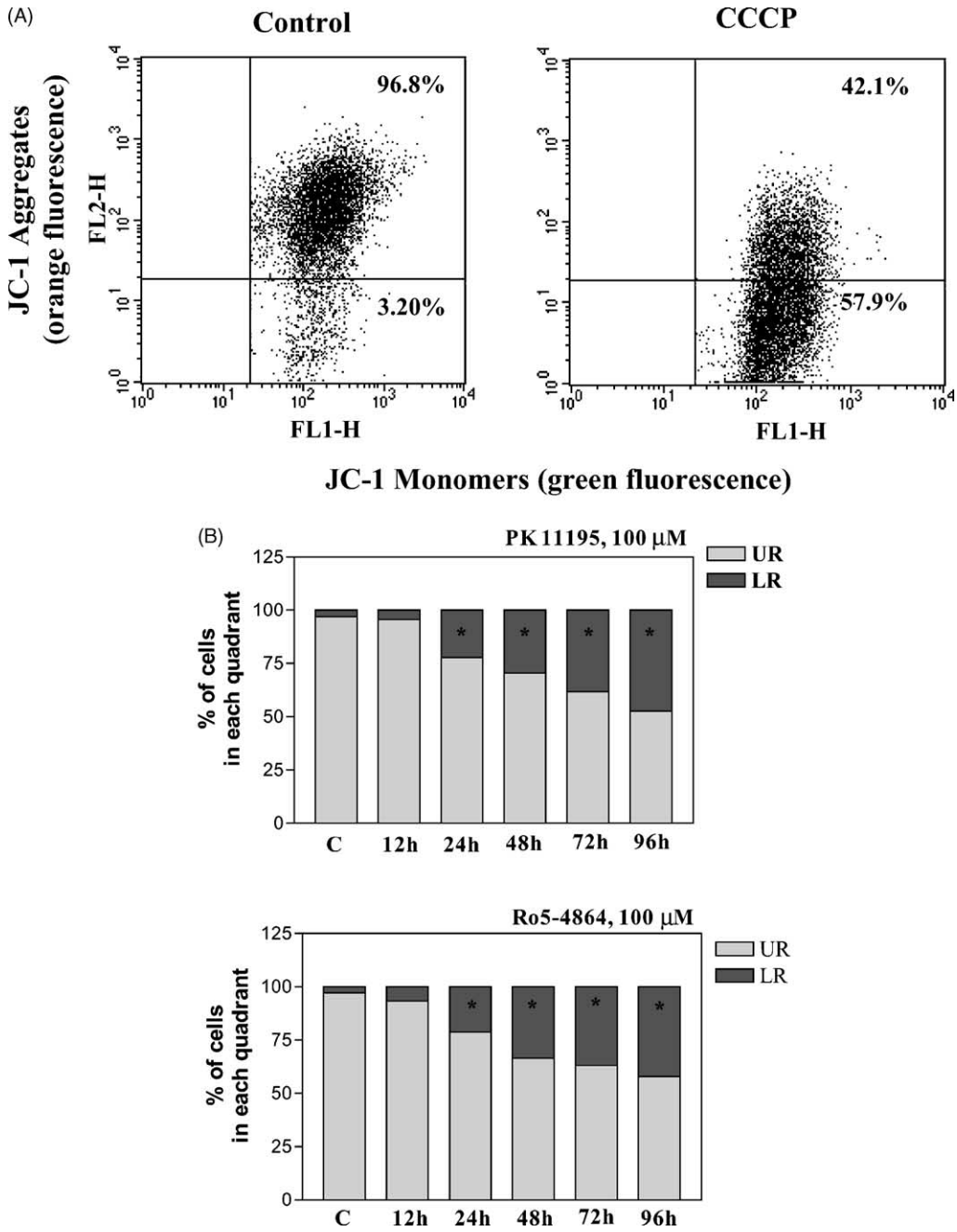


Fig. 7. Flow cytometric analysis of mitochondrial transmembrane potential in PBR ligand-treated C6 glioma cells: representative examples of the fluorescence pattern of C6 glioma cells stained with JC-1 in the presence or absence (control)of the uncoupler agent CCCP (5 μM) are shown. Cells with polarized mitochondria are found in the upper right (UR) quadrant of the plots, corresponding to high emission of fluorescence in both FL1 (green; *x*-axis) and FL2 (orange; *y*-axis) channels. After CCCP treatment, mitochondrial depolarization becomes evident as a decrease in the FL2 and an increase in the FL1 emission, low right (LR) quadrant (A). C6 glioma cells were treated with PK 11195 or Ro5-4864 (100 μM) for graded time periods (12–96 h) and $\Delta\Psi_m$ evaluated by means of cytofluorimetric analysis of JC-1 stained cells (as above). Histograms show the mean values of cell percentages derived from three independent experiments either in the UR (polarized mitochondria) or in the LR (depolarized mitochondria) quadrant of $\Delta\Psi_m$ analysis plots. $^*P < 0.05$ with respect to corresponding control, one-way ANOVA (Newman–Keuls test) (B).

control untreated-cells (3.20%) shows low emission of fluorescence in FL2 and can therefore be found in the lower right (LR) quadrant of the plot. The exposure of glioma cells to the uncoupler agent CCCP, which have been used as positive control, resulted in a dramatic decrease of J-aggregate fluorescence (FL2, *y*-axis) accompanied by a concomitant increase of JC-1 monomer fluorescence (FL1, *x*-axis). As a result, 57.9% of cells where found in the LR quadrant of the plot.

Significant changes of $\Delta\Psi_m$ were detected in C6 glioma cells after 24 h treatment with the specific high affinity PBR ligand, PK 11195 and Ro5-4864 (100 μM), as shown in Fig. 7B, where the percentages of cells in the UR or LR quadrants of the $\Delta\Psi_m$ analysis are reported. By contrast,

the non-site selective PBR ligand Diazepam and the central-type benzodiazepine Clonazepam did not alter $\Delta\Psi_m$ even when used at the highest concentration (100 μM) and for the most extended incubation time (96 h) (data not shown).

## 4. Discussion

The efficacy of chemotherapeutic agents may depend on their effectiveness at inducing cell death in malignant cells resistant to apoptosis induction [34]. In the present study, we evaluated the effects of PBR ligands on rat C6 glioma cell survival. Our results showed that both specific PBR ligands, the isoquinoline carboxamide derivative PK 11195 and the benzodiazepine Ro5-4864, were able to induce inhibition of cell viability in these tumoral cells (over 50% inhibition at 100 μM ligand concentration, for 96 h). By contrast, the non-site selective ligand Diazepam and the central-type benzodiazepine Clonazepam were only slight active or totally ineffective, suggesting that the observed effect was PBR-specific. Consistent with our results, it has been shown an inhibitory effect on cell proliferation by micromolar concentrations of PBR ligands in glioma and neuroblastoma cells [28] as well as in other tumoral cell lines [26,35–39].

By a combination of experimental approaches widely used to investigate the nature of cell death, we evidenced that the specific PBR ligands induced apoptosis in rat C6 glioma cells. PK 11195- and Ro5-4864-treated-cells showed an increase in the percentage of nuclei with hypodiploid DNA content, typical of apoptotic cell death, as assessed by flow cytometry analysis. In line with this, PK 11195 and Ro5-4864 treatment resulted in activation of the ''effector'' caspase-3. Moreover, ultrastructural cell analysis evidenced morphological cell alterations characteristic of apoptosis (i.e. cytoplasm shrinkage and chromatin condensation and margination), suggesting a late stage of this process. Analogous ultrastructural features have been observed in glioma cells after long exposure to cisplatin, a drug commonly used for the treatment of malignant brain tumors [40,41].

A critical event of the apoptotic mechanism is the opening of the MPT-pore, which causes the dissipation of the mitochondrial transmembrane potential and the subsequent release of pro-apototic factors [42,43]. In previous studies, PK 11195 has been shown to induce antioxydant-inhibitable collapse of $\Delta\Psi_m$ and mitochondrial swelling in HL60 human leukemia cells [22], to trigger MPT in isolated cardiac mitochondria inducing nuclear apoptosis [44], to facilitate the MPT-pore opening induced by the tumor necrosis factor-α in L929 cells [45] and to promote the induction of MPT-pore opening by the ANT ligand atractiloside in Bcl-2-overexpressing mitochondria [20]. To better understand the ability of PBR ligands to affect MPT-pore activity, we studied the effects of PBR ligands on mitochondrial potential in glioma cells using a sensitive flow cytometry assay. Our data showed that treatment of rat C6 glioma cells with specific PBR ligands resulted in time-dependent mitochondrial transmembrane potential dissipation, with a significant effect after 24 h of cell exposure. In contrast, Diazepam and Clonazepam did not affect the mitochondrial potential, even after prolonged treatment. The mitochondrial alterations were also evidenced by ultrastructural analysis, which showed pale and swollen mitochondria.

Consistent with these results the opening of the mitochondrial pore could explain how specific PBR ligands induce apoptosis in these brain tumoral cells as well as in several other cell lines from haemopoietical and epithelial malignancies [20,22–24,26,37,46,47]. Indeed, the PBR ligand FGIN-1-27 decreased $\Delta\Psi_m$ and caused apoptosis and cell cycle arrest in several tumor cell lines [26,37] and treatment with PK 11195 was able to overcome apoptosis resistance conferred by Bcl-2 [20,22]. Cell sensitivity to apoptosis induction could be modulated by affecting MPT-pore opening [48]. In fact, it has been shown that rapid and transient pore opening does not impair mitochondrial functions and cell survival, whereas, extensive and prolonged opening triggers the apoptotic pathway culminating in nuclear alterations [17,49]. However, the chronology of apoptotic events are not easy to established due to the complexity of the process. In fact, some apoptotic effectors, like mitochondrial potential and caspase, which are consequences of MPT-pore opening, can themselves induce MPT, triggering a self-amplifying process [16,50]. In our hands, after 24 h exposure to PBR ligands a late stage of apoptosis was evidenced, when both changes of mitochondrial potential and nuclear alterations were already present. However, after 12 h of treatment no significant changes typical of apoptotic process were detectable. Further studies will be necessary to clarify this issue.

Numerous results have suggested that the use of specific PBR ligands modulating PBR activity, might be of significant clinical benefit in the management of a large spectrum of different pathologies, including cancer. In particular, the potential application of PBR-targeted therapies in cancer has been proposed on the base of observations that PBR ligands show antiproliferative and pro-apoptotic properties and that the highest densities of PBR are found in neoplastic tissue and cell lines [19,47]. However, up to now, few data about the selectivity of pro-apoptotic effects of PBR ligand on malignant respect to normal cells are available [45,51,52]. In our experimental conditions, we evidenced that rat primary astrocytes were also sensitive to specific PBR ligands, despite showing higher resistance to cell death induction at low ligand concentrations (data not shown). In order to develop therapeutical compounds able to overcome the resistance to apoptosis induction a clear definition of efficacy dose might be relevant.

Apoptosis induction and antiproliferative effects of PBR ligands have been observed at concentrations significantly higher than expected from their nanomolar affinity to the receptor [26,35–38]. It has been reported that saturating (micromolar) doses of PK 11195 or Ro5-4864 sensitize cancer cells to the induction of apoptosis, both in vitro and in vivo [53]. The discrepancy between the micromolar ligand concentrations, necessary to inhibit cell survival, and the nanomolar-binding affinities was also observed in our results and nanomolar concentration of PK 11195 and Ro5-4864 were not able to induce C6 glioma cell apoptosis. Moreover, no significant differences in the action of the two specific PBR ligands (PK 11195 and Ro5-4864) were observed. Although Ro5-4864 and PK 11195 are thermodynamically classified as agonist and antagonist of the receptor, respectively [54], it has been observed that both can induce similar effects under certain physiological or pathological conditions [2,19].

The identification of the molecular events involved in the pro-apoptotic and antiproliferative effects of the specific PBR ligands still requires clarification. A few hypothesis have been put forward [55]. It could be speculated that binding of PBR ligands to the receptor would trigger a conformational change of the MPT-pore towards a more permeable state, which may sensitize the cell to apoptotic messages.

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## References

[1] Brauestrup C, Squires RF. Specific benzodiazepine receptors in rat brain characterized by high-affinity (3H) diazepam binding. Proc Natl Acad Sci USA 1977;74:3805–9.
[2] Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G, et al. Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev 1999;51:619–40.
[3] Beurdeley-Thomas A, Miccoli L, Oudard S, Dutrillaux B, Poupon MF. The peripheral benzodiazepine receptors: a review. J Neurooncol 2000;46:45–56.
[4] Park CH, Carboni E, Wood PL, Gee KW. Characterization of peripheral benzodiazepine type sites in a cultured murine BV-2 microglial cell line. Glia 1996;16:65–70.
[5] Black KL, Ikezaki K, Toga AW. Imaging of brain tumors using peripheral benzodiazepine receptor ligands. J Neurosurg 1989;71:113–8.
[6] Black KL, Ikezaki K, Santori E, Becker DP, Vinters HV. Specific high-affinity binding of peripheral benzodiazepine receptor ligands to brain tumors in rat and man. Cancer 1990;65:93–7.
[7] Olson JM, Junck L, Young AB, Penney JB, Mancini WR. Isoquinoline and peripheral-type benzodiazepine binding in gliomas: implications for diagnostic imaging. Cancer Res 1988;48:5837–41.
[8] Ikezaki K, Black KL, Toga AW, Santori EM, Becker DP, Smith ML. Imaging peripheral benzodiazepine receptors in brain tumors in rats: in vitro binding characteristics. J Cereb Blood Flow Metab 1990;10:580–7.
[9] Miettinen H, Kononen J, Haapasalo H, Helen P, Sallinen P, Harjuntausta T, et al. Expression of peripheral-type benzodiazepine receptor and diazepam binding inhibitor in human astrocytomas: relationship to cell proliferation. Cancer Res 1995;55:2691–5.
[10] Cornu P, Benavides J, Scatton B, Hauw JJ, Philippon J. Increase in omega 3 (peripheral-type benzodiazepine) binding site densities in different types of human brain tumours. A quantitative autoradiography study. Acta Neurochir (Wien) 1992;119:146–52.
[11] Kleihues P, Burger PC, Scheithauer BW. The new WHO classification of brain tumours. Brain Pathol 1993;3:255–68.
[12] Fine HA, Dear KB, Loeffler JS, Black PM, Canellos GP. Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults. Cancer 1993;71:2585–97.
[13] Hildebrand J, Sahmoud T, Mignolet F, Brucher JM, Afra D. Adjuvant therapy with dibromodulcitol and BCNU increases survival of adults with malignant gliomas. EORTC Brain Tumor Group. Neurology 1994;44:1479–83.
[14] Levin VA. Chemotherapy for brain tumors of astrocytic and oligodendroglial lineage: the past decade and where we are heading. Neurooncology 1999;1:69–80.
[15] Rainov NG. 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 2000;11:2389–401.
[16] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.
[17] Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol 1998;60:619–42.
[18] McEnery MW, Snowman AM, Trifeletti RR, Snyder SH. Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. Proc Natl Acad Sci USA 1992;89:3170–4.
[19] Casellas P, Galiegue S, Basile AS. Peripheral benzodiazepine receptors and mitochondrial function. Neurochim Int 2002;40:475–86.
[20] Hirsch T, Decaudin D, Susin SA, Marchetti P, Larochette N, Resche-Rigon M, et al. PK 11195, a ligand of the mitochondrial benzodiazepine receptor, facilitates the induction of apoptosis and reverses Bcl-2 mediated cytoprotection. Exp Cell Res 1998;241:426–34.
[21] Zisterer DM, Campiani G, Nacci V, Williams DC. Pyrrolo-1,5-benzoxazepines induce apoptosis in HL-60, Jurkat, and Hut-78 cells: a new class of apoptotic agents. J Pharmacol Exp Ther 2000;293:48–59.
[22] Fennell DA, Corbo M, Pallaska, Cotter FE. Bcl-2 resistant mitochondrial toxicity mediated by the isoquinoline carboxamide PK11195 involves de novo generation of reactive oxygen species. Br J Cancer 2001;84:1397–404.
[23] Xia W, Spector S, Hardy L, Zhao S, Saluk A, Alemane L, et al. Tumor selective G2/M cell cycle arrest and apoptosis of epithelial and hematological malignancies by BBL22, a benzazepine. Proc Natl Acad Sci USA 2000;97:7494–9.
[24] Banker DE, Cooper JJ, Fennell DA, Willman CL, Appelbaum FR, Cotter FE. PK11195, a peripheral benzodiazepine receptor ligand, chemosinsitizes acute myeloid leukemia cells to relevant therapeutic agents by more than one mechanism. Leukemia Res 2002;26:91–106.
[25] Larochette N, Decaudin D, Jacotot E, Brenner C, Marzo I, Susin SA, et al. Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. Exp Cell Res 1999;249:413–21.
[26] Maaser K, Hopfner M, Jansen A, Weisinger G, Gavish M, Kozikowski AP, et al. Specific ligands of the peripheral benzodiazepine receptor

induce apoptosis and cell cycle arrest in human colorectal cancer cells. Br J Cancer 2001;85:1771–80.
[27] Brown RC, Degenhardt B, Kotoula M, Papadopoulous V. Location-dependent role of the human glioma cell peripheral-type benzodiazepine receptor in proliferation and steroid biosynthesis. Cancer Lett 2000;156:125–32.
[28] Gorman AM, O'Beirne GB, Regan CM, Williams DC. Antiproliferative action of benzodiazepines in cultured brain cells is not mediated through the peripheral-type benzodiazepine acceptor. J Neurochem 1989;53:849–55.
[29] Bruce JH, Ramirez AM, Lin L, Oracion A, Agarwal RP, Norenberg MD. Peripheral-type benzodiazepines inhibit proliferation of astrocytes in culture. Brain Res 1991;564:167–70.
[30] Ruan S, Okcu MF, Pong RC, Andreeff M, Levin V, Hsieh JT, et al. Attenuation of WAF1/Cip1 expression by an antisense adenovirus expression vector sensitizes glioblastoma cells to apoptosis induced by chemotherapeutic agents 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatin. Clin Cancer Res 1999;5:197–202.
[31] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[32] Ceruti S, Barbieri D, Veronese E, Cattabeni F, Cossarizza A, Giammarioli AM, et al. Different pathways of apoptosis revealed by 2-chloro-adenosine and deoxy-D-ribose in mammalian astroglial cells. J Neurosci Res 1997;47:372–83.
[33] Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess deltapsi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 1997;411:77–82.
[34] Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp Cell Res 2000;256(1):42–9.
[35] Landau M, Weizman A, Zoref-Shani E, Beery E, Wasseman L, Landau O, et al. Antiproliferative and differentiating effects of benzodiazepine receptor ligands on B16 melanoma cells. Biochem Pharmacol 1998; 56:1029–34.
[36] Carmel I, Fares FA, Leschiner S, Scherubl H, Weisinger G, Gavish M. Peripheral-type benzodiazepine receptors in the regulation of proliferation of MCF-7 human breast carcinoma cell line. Biochem Pharmacol 1999;58:273–8.
[37] Sutter AP, Maaser K, Hopfner M, Barthel B, Grabowski P, Faiss S, et al. Specific ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in human esophageal cancer cells. Int J Cancer 2002;102:318–27.
[38] Solowey WE, Pestka S, Spector S, Fryer RI, Fisher PB. Peripheral-acting benzodiazepines inhibit the growth of human melanoma cells and potentiate the antiproliferative activity of recombinant human interferons. J Interferon Res 1990;10:269–80.
[39] Alexander BE, Roller E, Klotz U. Characterization of peripheral-type benzodiazepine binding sites on human lymphocytes and lymphoma cell lines and their role in cell growth. Biochem Pharmacol 1992; 44:269–74.
[40] Krajci D, Mares V, Lisa V, Spanova A, Vorlicek J. Ultrastructure of nuclei of cisplatin-treated C6 glioma cells undergoing apoptosi. Eur J Cell Biol, 2000;79:365–76.
[41] Noda S, Yoshimura S, Sawada M, Naganawa T, Iwama T, Nakashima S, et al. Role of ceramide during cisplatin-induced apoptosis in C6 glioma cells. J Neurooncol 2001;52:11–21.
[42] Decaudin D, Geley S, Hirsch T, Castedo M, Marchetti P, Macho A, et al. Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. Cancer Res 1997;57:62–7.
[43] Marchetti P, Decaudin D, Macho A, Zamzami N, Hirsch T, Susin SA, et al. Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. Eur J Immunol 1997;27:289–96.
[44] Chelli B, Falleni A, Salvetti F, Gremigni V, Lucacchini A, Martini C. Peripheral-type benzodiazepine receptor ligands: mitochondrial permeability transition induction in rat cardiac tissue. Biochem Pharmacol 2001;61:695–705.
[45] Pastorino JG, Simbula G, Yamamoto K, Glascott PA, Rothman RJ, Farber JL. The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. J Biol Chem 1996;271:2992–8.
[46] Sutter AP, Maaser K, Barthel B, Scherubl H. Ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in oesophageal cancer cells: involvement of the p38MAPK signalling pathway. Br J Cancer 2003;89:564–72.
[47] Galiegue S, Tinel N, Casellas P. The peripheral benzodiazepine receptor: a promising therapeutic drug target. Curr Med Chem 2003; 10:1563–72.
[48] Costantini P, Jacotot E, Deucadin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 2000;92: 1042–53.
[49] Ichas F, Mazat JP. From calcium signalling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta 1998;1366(1–2):33–50.
[50] Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta 1998;1366(1–2): 151–65.
[51] Pastorino JG, Simbula G, Gilfor G, Hoek JB, Farber JL. Protoporphyrin IX, an endogenous ligand of the peripheral benzodiazepine receptor potentiates induction of the mitochondrial permeability transition and the killing of cultured hepatocytes by rotenone. J Biol Chem 1994;269:31041–6.
[52] Tanimoto Y, Onishi Y, Sato Y, Kizaki H. Benzodiazepine receptor agonists modulate thymocyte apoptosis through reduction of the mitochondrial transmembrane potential. Jpn J Pharmacol 1999;79:177–83.
[53] Decaudin D, Castedo M, Nemati F, Beurdeley-Thomas A, De Pinieux G, Caron A, et al. Peripheral benzodiazepine receptor ligands reverse apoptosis resistance of cancer cells in vitro and in vivo. Cancer Res 2002;62:1388–93.
[54] Le Fur G, Vaucher N, Perrier ML, Flamier A, Benavides J, Renault C, et al. Differentiation between two ligands for peripheral benzodiazepine binding sites, [$^3$H]RO5-4864 and [$^3$H]PK 11195, by thermodynamic studies. Life Sci 1983;33:449–57.
[55] Castedo M, Perfettini JL, Kroemer G. Mitochondrial apoptosis and the peripheral benzodiazepine receptor: a novel target for viral and pharmacological manipulation. J Exp Med 2002;196:1121–5.