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Peripheral-type benzodiazepine receptor (PBR) and PBR drug ligands in fibroblast and fibrosarcoma cell proliferation: role of ERK, c-Jun and ligand-activated PBR-independent pathways

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Abstract

Peripheral-type benzodiazepine receptor (PBR) is a 18-kDa high-affinity drug and cholesterol binding protein, that has been implicated in several physiological processes, such as cholesterol transport and mitochondrial respiration. Specific PBR ligands regulate cell proliferation, although their action is controversial and probably cell-type specific. The aim of the present study was to examine the expression of PBR in cells of mesenchymal origin, i.e. human fibroblasts and fibrosarcoma cells, as well as its role in the regulation of their proliferation. Both mesenchymal cell types express high levels of PBR, localized exclusively in mitochondria. PBR-specific drug ligands, the isoquinoline carboxamide PK 11195 and the benzodiazepine Ro5-4864, at relative high concentrations (10^{-4} M), exert a strong inhibitory effect on cell proliferation by arresting the cells at the G0/G1 phase of the cell cycle, while no apoptotic cell death was observed. In normal fibroblasts, this inhibition was correlated with a decrease in the activation of the cell cycle markers ERK and c-Jun. PBR knockdown by RNA inhibition did not affect the proliferation of either cell type and did not influence the inhibitory effect of PK 11195 and Ro5-4864 on cell growth. These data suggest that in fibroblasts and fibrosarcoma cells PBR drug ligands inhibit cell proliferation in a PBR-independent manner. These results are in contrast to data reported on cells of epithelial origin, suggesting that the origin of the cells is crucial in defining the role of PBR in their proliferation, and raise caution in the commonly made assumption that PBR mediates cell functions affected by PBR drug ligands.

Keywords: PBR; PK 11195; Proliferation; Fibroblasts; Fibrosarcoma; siRNA

1. Introduction

Benzodiazepines exert their anxiolytic, hypnotic and anticonvulsant action by binding to the central-type benzodiazepine receptor, located on the neuronal plasma membrane, where they interact with the $GABA_A$ receptor. A second receptor for benzodiazepines, with distinct pharmacological and molecular properties, is the peripheral-type benzodiazepine receptor (PBR), a 18 kDa hydrophobic protein, primarily localized in the outer mitochondrial membrane [1]. PBR binds, in addition to some benzodiazepines, drug ligands of diverse chemical nature, it is abundantly expressed in steroid-synthesizing tissues, but it is also widely expressed throughout the body [1]. This protein seems to be involved in numerous physiological processes, such as steroid production, mitochondrial respiration, immunomodulation, porphyrin transport, heme biosynthesis, apoptosis, as well as cell proliferation [1,2]. The latter was mainly investigated using PBR-specific high-affinity drug ligands, such as the benzodiazepine Ro5-4864 and the isoquinoline carboxamide PK 11195 [3]. However, the data on the effect of these compounds on cell proliferation seems to be contradictory, ranging from stimulatory to inhibitory, probably reflecting the tissue-specificity of PBR function [4,5]. Moreover, it has been suggested that the effect of these drug ligands on cell proliferation could be independent of their ability to bind to PBR [6]. Recently, using gene silencing approaches, we observed that the presence of PBR is required for human epithelial breast cancer cell proliferation (Li W, Hardwick MJ, Papadopoulos V, unpublished data). Although the effect of PBR drug ligands, and par extension PBR, in

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the proliferation of several cancer cells has been extensively investigated, less attention had been devoted on stroma cells, such as fibroblasts. Accordingly, the aim of the present study was to examine in normal and cancer cells of mesenchymal origin, i.e. human fibroblasts and fibrosarcoma cells, the presence of PBR and its role in their proliferation.

2. Materials and methods

2.1. Cells and culture conditions

Normal human fibroblasts (HFFF2, ECACC, Salisbury, UK), human fibrosarcoma (HT-1080) and breast cancer epithelial cells (MDA-MB-231) (both from ATCC, Manassas, VA) were cultured in MEM (HFFF2) or DMEM (HT-1080 and MDA-MB-231) supplemented with 10% FCS, in an environment of 5% CO_2, at 37 °C.

2.2. Laser scanning confocal microscopy

Cells were incubated for 1 h with 2 μM of compound 4—a high affinity fluorescent PBR drug ligand [7]—and for 5 min with MitoTracker Red (Molecular Probes, Eugene, OR) (1:5000). Then, they were fixed with 4% formaldehyde and analyzed in an Olympus laser scanning confocal microscope, equipped with a Fluoview software.

2.3. Immunoblot analysis

Cells were washed with Tris-buffered saline (TBS) and scraped in hot SDS–PAGE sample buffer containing β-mercaptoethanol, protease- and phosphatase-inhibitors (Sigma Chemical Co., St. Louis, MO). Following sonication (10 s) the samples were separated on 4–20% acrylamide gels and the proteins were transferred onto nitrocellulose membranes. Membranes were blocked with 1% BSA in TBS containing 0.1% Tween-20 (T-TBS) for 1 h and subsequently incubated with the primary and HRP-conjugated secondary antibodies. Immunoreactive bands were visualized on Kodak-X-OMAT AR films by chemiluminescence (ECL kit), according to the manufacturer's (Amersham, Staffordshire, UK) instructions.

2.4. Cell proliferation and DNA synthesis assay

Cells were plated on 96-well plates at a cell density of 10,000 cells/well, in MEM supplemented with 10% FCS. Twenty-four hours later the compounds to be tested were added and after another 24-h incubation period DNA synthesis was measured by BrdU incorporation by using the BrdU ELISA kit (Boehringer Mannheim, Indianapolis, IN), as previously described [4]. Cell proliferation assay was performed in 6-well dishes and measured by direct cell counting after trypsinization.

2.5. Cell cycle analysis

Cultures were treated with 10^{-4} M PK 11195 and Ro5-4864 for 24 h, fixed in 50% ethanol, stained with a solution containing propidium iodide (50 μg/ml) and RNase (10 μg/ml) and subjected to flow cytometric analysis in a FACS Scan flow cytometer (Becton Dickinson, Menlo Park, CA) equipped with a Modfit software program.

2.6. RNA analysis, siRNA preparation and transfections

PBR mRNA expression was estimated with quantitative real-time-PCR (Q-PCR) as described in [8]. Twenty-one-nucleotide RNAs were chemically synthesized by Dharmacon Research (Lafayette, Colorado) using 2′-ACE protection chemistry.[1] The small interfering (siRNA) strands were deprotected according to manufacturer's instructions, mixed in equimolar ratios and annealed at 60 °C for 45 min, and at ambient temperature 30 min. The GeneBank accession number for human PBR is gI21536444. siRNAs with the following sense and antisense sequences were used: PBR 361siRNA targeting human PBR exon 3, 5′-AACTGGGCATGGCCCCCCATCCCTGTCTC-3′ (sense), 5′-AAGATGGGGGGCCATGCCCAGCCTGTCTC-3′ (antisense); PBR 537siRNA targeting human PBR exon 4, 5′-AACTACTGCGTATGGCGGGACCCTGTCTC-3′ (sense), 5′-AAGTCCCGCCATACGCAGTAGCCTGTCTC-3′ (antisense); PBR 548siRNA targeting human PBR exon 4,5′-AACCATGGCTGGCATGGGGGACCTGTCTC-3′ (sense), 5′-AATCCCCCATGCCAGCCATGGCCTGTCTC-3′ (antisense); scrambled siRNA, 5′-AAGGCTACTATGCGGCGACTGCCTGTCTC-3′ (sense), 5′-AACAGTCGCCGCATAGTAGCCCCTGTCTC-3′ (antisense). PBR 361, 537, 548 siRNAs corresponded to the PBR coding regions, scramble siRNA was used as control. Single transfections of siRNA duplexes were performed using Oligofectamine Reagent (Invitrogen/Life Technologies) and assayed for silencing 3 days after transfection.

2.7. Radioligand binding assay

[^{3}H]PK 11195 binding studies and Scatchard plots on cell lysates from HFFF2 and HT-1080 cells were performed as previously described [4].

3. Results and discussion

3.1. PBR expression and localization

PBR expression in normal human fibroblasts and fibrosarcoma cells was studied at the mRNA level by real-time Q-PCR. Cycling and arrested fibroblasts express comparable PBR mRNA levels, interestingly at a near equal amount to that of the human breast cancer MDA-MB-231 cell line, known to express high PBR mRNA levels among

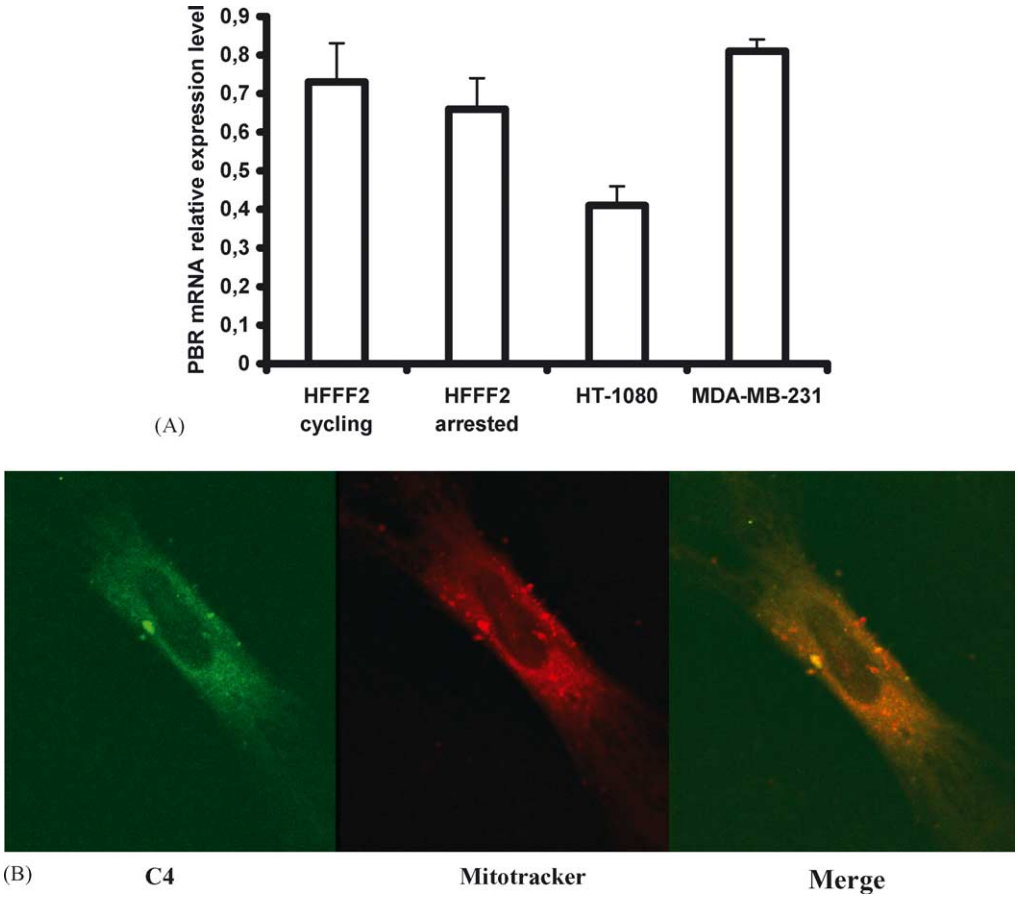


Fig. 1. Expression and localization of PBR in human fibroblasts and fibrosarcoma cells. (A) Total RNA was extracted from growing HT-1080, HFFF2 cells (cycling and arrested) and from MDA-MB-231 human breast cancer epithelial cells, and was subjected to Q-PCR analysis. PBR mRNA levels were expressed in relative level to endogenous reference 18S rRNA. Values represent the means ± S.D. of three individual experiments, each one performed in quadruplicates. (B) Human fibroblasts were incubated with the fluorescent high-affinity PBR ligand compound 4 and with MitoTracker Red and were observed under a laser scanning confocal microscope. The green color indicates the presence of PBR and the red color that of mitochondria. Co-localization of both fluorescent images after superposition results in a yellow color.

non-steroidogenic cells [4]. HT-1080 fibrosarcoma cells contain less PBR mRNA, in comparison to normal fibroblasts (Fig. 1A), in agreement with our recent findings that while in breast, colon-rectum and prostate tissues elevated PBR expression is associated with tumor progression, malignant skin tumors express lower PBR levels than normal skin [8]. These data, suggest that the relation between enhanced PBR levels and increased proliferation is tissue-specific. Confocal laser microscopy indicated that PBR is localized to the cytoplasmic/perinuclear region, in both normal and fibrosarcoma cells (Fig. 1B). An identical pattern was seen when the specific mitochondrial probe MitoTracker was used and superposition of these images resulted in a yellow color, indicating the co-localization of mitochondria and PBR in these cells (Fig. 1B).

3.2. PBR ligands inhibit the proliferation of human fibroblasts and fibrosarcoma cells

The effect of PBR ligands PK 11195 and Ro5-4864 on the proliferation of normal human fibroblasts and fibrosarcoma cells was studied by measuring bromodeoxyuridine incorporation into newly synthesized DNA. Drug ligand concentrations ranging from 10^{-5} to 10^{-11} M had no effect on DNA synthesis (not shown). However, at the concentration of 10^{-4} M a strong inhibition of BrdU incorporation by PBR drug ligands was observed in both cell types; PK 11195 exerted a more pronounced effect than Ro5-4864 (Fig. 2A). This action of PBR ligands can be considered as cytostatic, because no signs of cytotoxicity were observed. This was further reinforced by experiments performed in quiescent normal fibroblasts, where the PBR-ligand-mediated inhibition is only modest at the same ligand concentration (not shown). Interestingly, clonazepam and flunitrazepam that bind preferentially to central-type benzodiazepine receptors did not exert any inhibitory effect, while diazepam that can bind with a mixed affinity to both peripheral- and central-type receptors provoked a significant growth arrest, suggesting the involvement of PBR in the regulation of the proliferation of fibroblasts and fibrosarcoma cells (Fig. 2A).

Cell cycle analysis by flow cytometry indicated that both PK 11195 and Ro5-4864 induce an accumulation of the cells in the G0/G1 phase of the cell cycle, with a

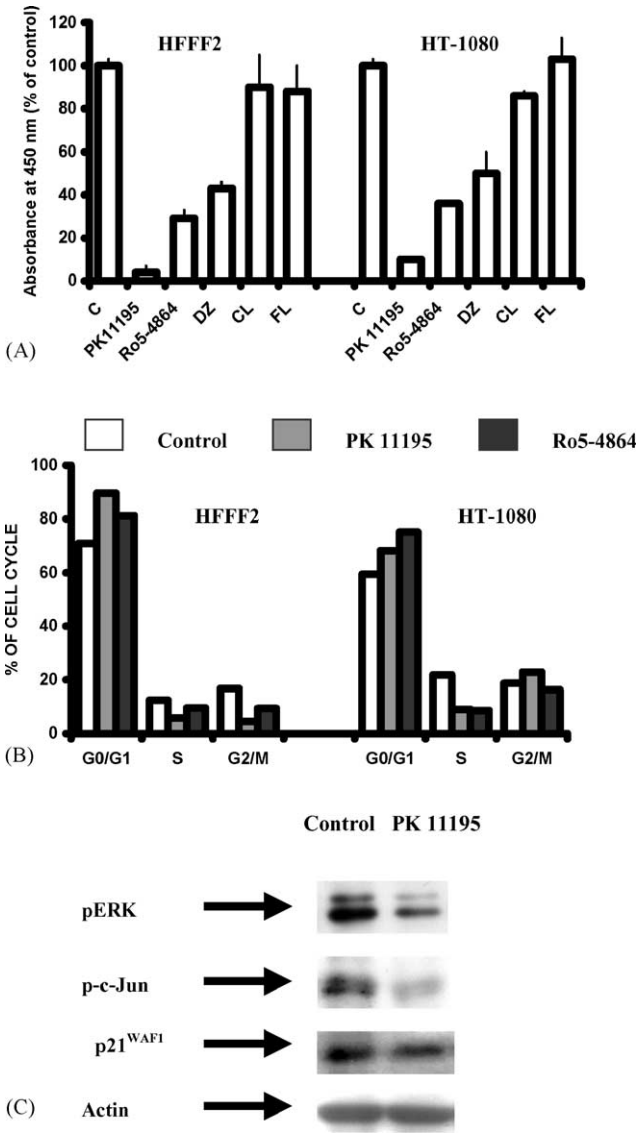


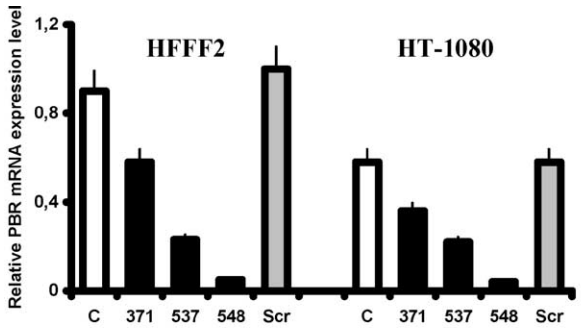
Fig. 2. The effect of peripheral- and central-type benzodiazepines on the proliferation of HFFF2 and HT-1080 cells. (A) Cells were grown in 96-well plates and treated with 10^{-4} M of PK 11195, Ro5-4864, diazepam (DZ), clonazepam (CL) and flunitrazepam (FL). Twenty-four hours later BrdU incorporation was measured. (B) Effect of 10^{-4} M PK 11195 and Ro5-4864 on cell cycle regulation, as estimated by FACS analysis. (C) Expression of the cell-cycle markers ERK, c-Jun and $p21^{WAF1}$ after treatment with 10^{-4} M PK 11195 for 24 h, by immunoblot analysis. For parts (A) and (B), values represent the means ± S.D. of three individual experiments, each one performed in quadruplicates. For part (C), data shown is from a representative experiment. Similar results were observed in three independent experiments.

concomitant reduction in the proportion of cells being in S phase. However, only in HFFF2 cells an additional reduction of the G2/M phases was also observed (Fig. 2B). Interestingly, no sub-G1 peak was observed in any case, indicating that the inhibition in DNA synthesis is not due to apoptotic cell death.

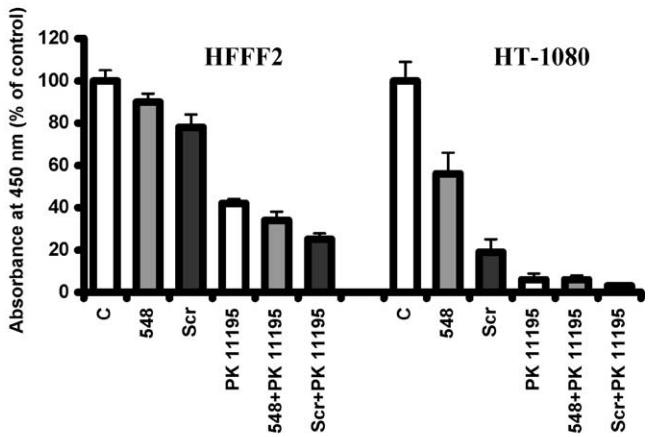
The ability of PK 11195 to inhibit cell proliferation was further tested at the level of cell cycle-markers' expression. Fig. 2C shows that in normal human fibroblasts 24-h incubation with 10^{-4} M PK 11195 resulted in a significant reduction of the activated (phosphorylated) forms of ERK (member of the MAPK family) and c-Jun (one of the components of the AP-1 transcription factor), both being involved in the regulation of cell proliferation [9,10]. On the other hand, the levels of cyclin-dependent kinase inhibitor $p21^{WAF1}$ were found to be unaffected. Interestingly, the changes in the activation of ERK and c-Jun were not observed in fibrosarcoma cells (data not shown), implying the presence of an alternative mechanism for the regulation of their proliferation.

3.3. PBR does not mediate the inhibitory effect of PBR drug ligands on cell proliferation

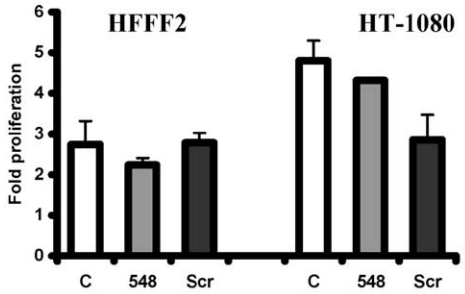
To further access the involvement of PBR on the proliferation of human fibroblasts and fibrosarcoma cells, we inhibited its expression using dsRNAs. To this end, three 21-nucleotide RNAs were constructed and their effect on PBR mRNA expression levels was tested. As can be seen in Fig. 3A, all three siRNAs significantly inhibited the production of PBR mRNA in both cell types, as shown by Q-PCR. PBR 548siRNA was found to be the more potent, as it decreased mRNA expression by more than 90% and consequently it was used in all subsequent experiments. A scrambled siRNA had no effect on PBR mRNA expression. In addition, radioligand binding studies followed by Scatchard analysis demonstrated a strong inhibition of PK 11195 binding (B_{max}) to PBR, compared to control and scrambled siRNA-treated cells (2.3 pmol/mg protein in 548siRNA-treated cells versus 9.6 and 12.9 pmol/mg protein in control and scrambled siRNA-treated cells). The effect of PBR silencing on the inhibition of DNA synthesis induced by benzodiazepines was then investigated. HFFF2 and HT-1080 cells were transfected with the 548 and scrambled siRNAs and 2 days later cells were treated with 10^{-4} M PK 11195. Fig. 3B shows that both siRNAs had a negligible effect on DNA synthesis. Furthermore, they did not alter the inhibitory effect of PK 11195 on DNA synthesis, suggesting that the effect of PBR drug ligands on cell proliferation could be independent from their interaction with the 18 kDa PBR protein. The role of PBR on proliferation was further tested by direct cell counting. To this end, siRNA transfected human fibroblasts and fibrosarcoma cells were allowed to proliferate and we measured the increase in cell numbers between days 2 and 6 after transfection. Fig. 3C shows that transfected cells proliferate at the same ratio as untransfected cells, suggesting that in these cells PBR levels may be unrelated with the regulation of proliferation. These results are in agreement with data reported in mouse MA-10 Leydig cells where PBR levels were decreased using a PBR antisense knockout approach [11]. It should be noted that like fibroblast and fibrosarcoma cells, Leydig cells are also of mesenchymal origin [12]. These findings suggest that the 18-kDa PBR protein may not be crucial for the regulation of



(A)



(B)



(C)

Fig. 3. The effect of PBR siRNA on human fibroblasts and fibrosarcoma cell proliferation. Cells were transfected with the indicated siRNAs, while scrambled siRNA (Scr) was used as control. (A) Three days later total RNA was collected and analyzed by Q-PCR for measuring relative PBR mRNA expression. (B) Two days after transfection with the 548 and the Scr siRNAs, cells were treated with 10^{-4} M PK 11195 and DNA synthesis was measured 24 h later by BrdU incorporation, as described in Fig. 2. (C) One day after plating, HFFF2 and HT-1080 cells were transfected with the 548 and the Scr siRNAs and cell proliferation between days 2 and 6 after transfection was estimated by direct cell counting. Values represent the means ± S.D. of three individual experiments, each one performed in quadruplicates.

proliferation of human fibroblast and fibrosarcoma cells, and cells of mesenchymal origin in general. Nevertheless, the effect of PBR ligands is specific, as shown by the action of central- and peripheral-type type benzodiazepine receptor ligands (Fig. 2A). Thus, it can be hypothesized that specific PBR drug ligands may act through a yet unidentified, PBR-independent, pathway.

It has been proposed that PBR ligands can inhibit cell proliferation by blocking Ca^{2+}-influx through voltage activated channels and this effect can be reversed by increased calcium concentrations [13]. However, in the case of fibroblast and fibrosarcoma cells addition of high calcium concentrations had no effect on the inhibitory effect of PBR ligands PK 11195 and Ro5-4864 (data not shown), suggesting an alternative intracellular target for these compounds.

In conclusion, the data presented herein demonstrate that PBR is present in the mitochondria of cells of mesenchymal origin, such as human fibroblast and fibrosarcoma cells and that specific PBR drug ligands, at relatively high concentrations, exert a strong antiproliferative effect by arresting these cells at the G0/G1 phase of the cell cycle. In normal fibroblasts, this inhibition correlates with a decrease in the activation of the signaling molecules ERK and c-Jun. However, data is also presented indicating that PBR-devoid fibroblasts and fiborsarcoma cells maintain their ability to proliferate and to respond to PBR drug ligands, suggesting that in these cells of mesenchymal origin this event may be PBR-independent. Although these results further support the future use of PBR drug ligands in cancer therapy either alone or in combination with other treatment regimes they also indicate that the commonly made assumption that PBR mediates cell functions affected by PBR drug ligands is not always true.

Acknowledgments

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