Antimitotic activity of high affinity ligands for peripheral benzodiazepine receptor (PBR) in some normal and neoplastic cell lines

Miltyk W[1], Pałka M[1], Karna E[1], Jarząbek K[2], Boujrad N[3], Knapp P[4]

[1] Department of Medicinal Chemistry, Medical University of Białystok, Poland
[2] Department of Reproduction and Gynecological Endocrinology, Medical University of Białystok, Poland
[3] Interactions Cellularies et Molecularies, Universite de Rennes, Campus de Beaulieu, Avenue du General Leclerc, France
[4] Department of Gynecology, Medical University of Białystok, Poland

Abstract

Purpose: Overexpression of PBR has been found in several tumor types including ovarian, colon, breast adenocarcinomas, esophageal cancer. There is evidence suggesting that PBR ligands regulate cell proliferation. However, their action is probably cell-type specific. We decided to evaluate mitotic activity of PBR ligands in some normal and neoplastic cell lines.

Material and methods: The cells were maintained according to standard procedures. Ligand binding assay was performed in cell extract using PK-11195 or Ro-54864 and [N-methyl-^{3}H] Ro-54864 or [N-methyl-^{3}H] PK-11195. Cell proliferation was evaluated using 5-[^{3}H]-thymidine assay. Western Immunoblot assay was conducted using polyclonal anti-PBR antibody.

Results: We have found that, macrophages evoked strong binding of both Ro-54864 and PK-11195. This phenomenon was accompanied by drastic decrease in the cell divisions. Similar effect was found only in the case of non-estrogen-dependent breast cancer cells MDA-MB 231. It suggest that PBR-ligand mediated inhibition of mitogenesis may represent a new anticancer strategy in non-estrogen-dependent breast cancer. In respect to macrophages inhibition of the cell division by both PBR ligands may have implication in modulation of inflammatory response. It has been postulated that PBR ligands may have anti-inflammatory activity in rheumatoid arthritis. The presence of peripheral benzodiazepine receptors in chondrocytes, T cells, macrophages and mesenchymal cells suggest that peripheral benzodiazepine receptor ligands may interfere with the cytokine network and thus modulate inflammatory response.

Conclusions: The data suggest that PBR-ligand mediated inhibition of DNA synthesis in non-estrogen dependent breast cancer cells and in macrophages may represent a new therapeutic approach of breast anticancer and anti-inflammatory therapy.

Key words: peripheral benzodiazepine receptors, Ro-54864, PK-11195, macrophages, neoplastic cell lines.

* CORRESPONDING AUTHOR:
Medical University of Białystok
ul. Kilińskiego 1, 15-089 Białystok, Poland
Tel: +48 85 7485702; fax: +48 85 8795703
e-mail: wmiltyk@amb.edu.pl (Wojciech Miltyk)

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Introduction

The peripheral – type benzodiazepine receptor (PBR) is a multimeric complex composed of 18 kDa receptor protein, the 32 kDa voltage anion-dependent channel (VDAC) protein required for benzodiazepine binding [1] and 30 kDa adenine nucleotide carrier [2]. PBR is present in most tissues. It is particularly abundant in steroid producing tissues [3], it is found in the outer mitochondrial membrane [4] and the outer – inner mitochondrial membrane contact sites [5]. Using high affinity PBR drug ligands such as the isoquinoline carboxamide PK-11195, it was shown that PBR is involved in the transport of the substrate cholesterol into mitochondria [6], the rate – determining step in steroid biosynthesis. PBR has been shown to be implicated in mitochondrial respiration [7], apoptosis [8] and cell proliferation [9]. Overexpression of PBR attenuates apoptosis induced by reactive oxygen radicals or ultraviolet light [10]. Overexpression of PBR has been found in several tumor types including ovarian, colon, breast adenocarcinomas [11], esophageal cancer [12].

The benzodiazapine Ro-54864 and isoquinoline carboxamide PK-11195 exhibit nanomolar affinity for PBR. PK-11195 has been classified as an antagonist and Ro-54864 as an agonist of the receptor [13]. Isoquinoline carboxamide bind specifically to the 18 kDa subunit, whereas benzodiazepine ligands such as Ro-54864 bind to a site consisting of porin, as well as adenine nucleotide translocase and 18 kDa subunit [1].

Several lines of evidence suggest that PBR ligands regulate cell proliferation [14]. However, their action is probably cell-

type specific. Therefore, we decided to evaluate mitotic activity of PBR ligands in some normal and neoplastic cell lines.

Materials and methods

Materials

1-(2-chlorophenyl)-N-methyl-n-(1-methylpropyl)-3-isoquinoline carboxamide (PK-11195), 4'-chlorodiazepam (Ro--54864), BCIP/NBT reagent as well as other reagents were from Sigma-Aldrich (St. Louis, MO). [N-methyl-^{3}H]-Ro-54864 (74 Ci/mmol), [N-methyl-^{3}H]-PK-11195 (83,5 Ci/mmol) – PerkinElmer (Boston, MA). [^{3}H]-thymidine (6,7 Ci/mmol) – ICN. D-MEM with Glutamax I, D-MEM, L-15 Leibovitz, Fetal Bovine Serum (FBS), Penicillin, Streptomycin, phosphate buffered saline (PBS) – Gibco (USA). Glas fiber filters were from Whatman (USA). Anti-PBR antibody – Laboratory of Cellular and Molecular Interactions, University of Rennes (France).

Cell culture

Normal human skin fibroblasts, macrophages, estrogen-dependent breast cancer cells MCF-7 and endometrium cancer cells – Ishikawa were maintained in D-MEM with GlutaMax I supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Non estrogen-dependent breast cancer cells MDA-MB 231 cells were maintained in D-MEM with 2% glutamine supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C. Fibroblasts were used in the 8th to 14th passages.

Western immunoblot

Samples of cell extracts (25 μg of protein) were electrophoresed. The proteins were transfered to 0.2 μm pore-sized nitrocellulose at 25 V for 1 h. The nitrocellulose was incubated with polyclonal antibody against PBR at concentration 1:500 overnight. In order to analyze PBR alkaline phosphatase conjugated antibody against rabbit's Fc IgG was added at concentration 1:3000 in TBS-T. Incubation was continued for 1 h. Then nitrocellulose was washed with TBS-T (5 times for 5 minutes) and submitted to Sigma-Fast BCIP/NBT reagent.

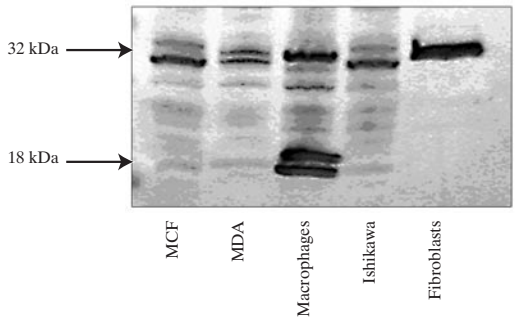
Ligand binding assay

Cell extracts (80 μg) were incubated with [N-methyl-^{3}H]Ro--54864 (30000 cpm) or [N-methyl-^{3}H]PK-11195 (30000 cpm) with or without Ro-54864 or PK-11195 for 3 h at 4°C. Incubation was terminated by transfer of the samples to PBS-prewetted filters and immediate vacuum filtration. The filters were washed three times with PBS and punched into vials and radioactivity was measured. Specific binding was calculated as a difference between total and non-specific binding.

[^{3}H]-thymidine incorporation

To examine the effect of Ro-54864 and PK-11195 on the cells proliferation, the cell were seeded in 24 well plates at 1 x 10^5 cells/well with 1 ml of growth medium. After 48 hours to subconfluent cells various concentrations of the ligands and 0.5 μCi of 5-[^{3}H]-thymidine were added. The incubation was continued for 24 hours at 37°C. Cells were rinsed 3 times with PBS, solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS, then scintillation fluid "Ultima Gold XR" was added and incorporation of the tracer into DNA was measured in scintillation counter.

Figure 1. **Western immunoblot analysis for PBR in different neoplastic (MCF-7, MDA-MB 231, Ishikawa) and normal (human dermal fibroblasts, macrophages) cell lines. The studied cells were cultured in the same conditions to 80% of confluency in D-MEM supplemented with 10% FBS. Samples used for electrophoresis consisted of 25 μg of protein of cell extracts**



Statistical analysis

In all experiments, the mean values from six assays ±standard deviations (SD) were calculated. The results were submitted to the statistical analysis using the Student's t-test, accepting $p<0.05$, as significant.

Results

The expression of peripheral benzodiazepine receptor (PBR) was studied in breast cancer cell lines; MCF-7 (estrogen-dependent) and MDA-MB 231 (non-estrogen-dependent) cells, in cancer cell line from endometrium (Ishikawa) as well in some normal cell lines; human dermal fibroblasts and human macrophages. All studied cells expressed PBR of molecular weight about 32 kDa, while macrophages expressed in addition 18 kDa receptor (*Fig. 1*). The expression of 32 kDa PBR, however, was mostly pronounced in fibroblasts and macrophages.

The differences in expression of PBR among studied cells may suggest their different ligand binding capacity. One of the high affinity ligands for PBR is Ro-54864 [13]. As shown on *Fig. 2* the most binding of Ro-54864 was found in homogenate extracts of macrophages and Ishihawa cells. Much less binding was observed in breast cancer cells and very little binding in human fibroblasts.

Another high affinity ligand for PBR is PK-11195, that may evoke inhibition of biological activity of the receptor [13]. However, in this case binding of PK-11195 to cell homogenate extracts was found to be very weak in both breast cancer cell lines and fibroblasts and much lower in macrophages and Ishikawa cells, compared to the results of Ro-54864 binding in these cells (*Fig. 3*).

The differences in binding of both ligands to PBR suggest, that this phenomenon may affect DNA biosynthesis and subsequently mitotic divisions in studied cells. Therefore, [^{3}H]-thymidine incorporation assay was performed in studied

Figure 2. **Binding of Ro-54864 in cell extracts of various cell lines**

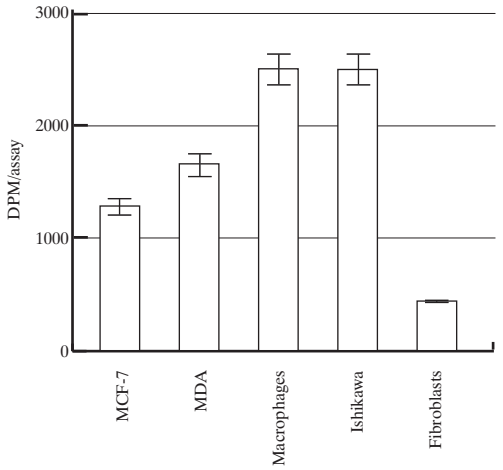


Figure 3. **Binding of PK-11195 in cell extracts of various cell lines**

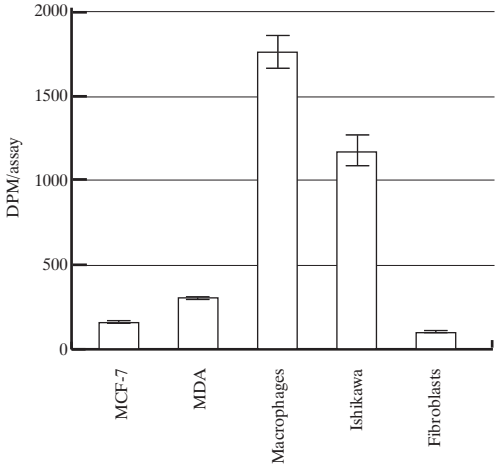


Figure 4. **DNA biosynthesis (measured by 5-[^{3}H]-thymidine incorporation assay) in subconfluent cells treated with different concentrations of Ro-54864 (30, 100 and 300 mM) for 24h in DMEM supplemented with 10% FBS. Mean values from three independent experiments done in duplicates are presented**

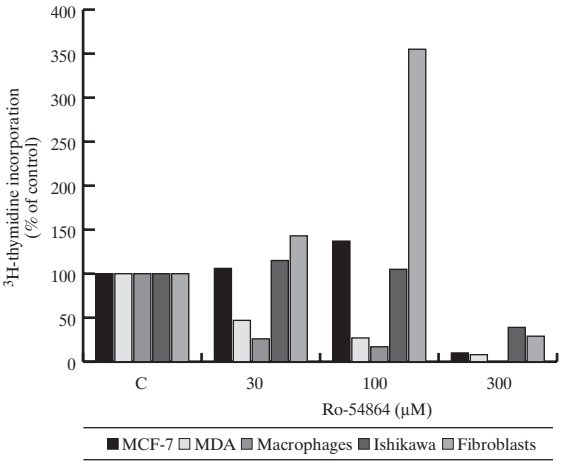
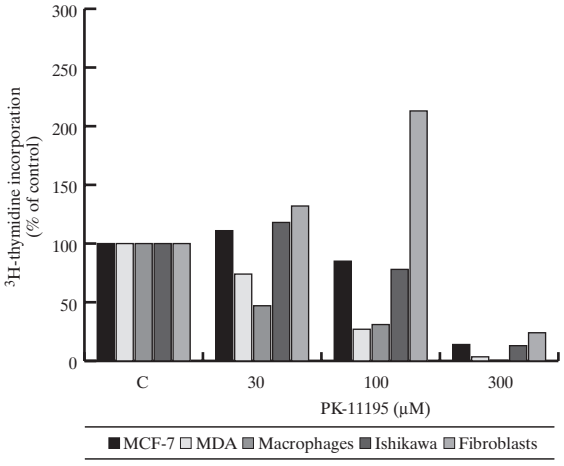


Figure 5. **DNA biosynthesis (measured by 5-[^{3}H]-thymidine incorporation assay) in subconfluent cells treated with different concentrations of PK-11195 (30, 100 and 300 mM) for 24 h in DMEM supplemented with 10% FBS. Mean values from three independent experiments done in duplicates are presented**



cells treated with different concentrations of Ro-54864 or PK-11195, as described in "Materials and methods" section. In the MDA-MB 231 cells and macrophages treated with 30 μmol/l of Ro-54864 the DNA biosynthesis was significantly decreased, while in MCF-7 cells, Ishikawa and fibroblasts it was not much changed. Increase in the concentration of Ro-54864 to 100 μmol/l augmented the inhibition of DNA synthesis in MDA-MB 231 cells and in macrophages, in MCF-7 the ligand slightly stimulated the process, while in fibroblast it was two fold increased. Exposure of the cells to 300 μmol/l of Ro-54864 was found to be toxic for all studied cells (*Fig. 4*).

In case of PK-11195 similar concentration-dependent effects on DNA synthesis in all studied cells was observed (*Fig. 5*).

Discussion

Although several lines of evidence suggest that PBR ligands can regulate cell proliferation [11,12] their action is controversial. In this report we presented that all studied normal and neoplastic cells express PBR. However, functional significance of PBR expression is studied by using high affinity PBR ligands. In this study we used PBR agonist Ro-54864 and PBR antagonist PK-11195. All studied cells, except fibroblasts evoked intense binding of Ro-54864. Similar level of PK-11195 was found only in Ishikawa cells and macrophages. It suggests cell-type specific PBR binding. In fact it is known that PBR function is particularly important in steroid producing cells [3]. However, we provide evidence that other cells that are not involved in steroidogenesis strongly bind PBR.

We have found that, macrophages evoked strong binding of both Ro-54864 and PK11195. This phenomenon was accompanied by drastic decrease in the cell divisions. Similar effect was found only in the case of MDA-MB 231. It suggest that PBR--ligand-mediated inhibition of mitogenesis may represent a new anticancer strategy in non-estrogen dependent breast cancer. In respect to macrophages inhibition of the cell division by both PBR ligands may have implication in modulation of inflamma-

tory response. It has been postulated that PBR ligands may have anti-inflammatory activity in rheumatoid arthritis.

The peripheral benzodiazepine receptor anti-inflammatory effect may also be explained by modifications of cytokines level. Cartilage destruction is a major characteristic of rheumatoid arthritis and is also linked to abberant cytokine and growth factor expression in affected tissues [15]. Interleukin-1, tumor necrosis factor-α and interferon-gamma are known to affect chondrocytes function [16-18], and interleukin-6 has been shown to boost progression from initial inflammation to a chronic state [19]. Peripheral benzodiazepine receptor ligands are known to reduce macrophage secretion of interleukin-1, interleukin-6 and TNF-α [20]. Furthmore, Ro-54864 and PK-11195 dramatically reduce both IL-6 and IL-13 expression in pleural exudation of mice injected with carrageenan [21]. The presence of peripheral benzodiazepine receptors in chondrocytes, T cells, macrophages and mesenchymal cells suggest that peripheral benzodiazepine receptor ligands may interfere with the cytokine network and thus modulate inflammatory response.

Those data suggest that PBR-ligand mediated inhibition of DNA synthesis in non-estrogen dependent breast cancer cells and in macrophages may represent a new therapeutic approach of anti-inflammatory and breast anticancer therapy.

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