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# Relation of Cell Proliferation to Expression of Peripheral Benzodiazepine Receptors in Human Breast Cancer Cell Lines

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**ABSTRACT.** Peripheral benzodiazepine receptor (PBR) agonist [$^3$H]Ro5-4864 has been shown to bind with high affinity to the human breast cancer cell line BT-20. Therefore, we investigated different human breast cancer cell lines with regard to binding to [$^3$H]Ro5-4864 and staining with the PBR-specific monoclonal antibody 8D7. Results were correlated with cell proliferation characteristics. In flow cytometric analysis, the estrogen receptor (ER)-negative breast cancer cell lines BT-20, MDA-MB-435-S, and SK-BR-3 showed significantly higher PBR expression (relative fluorescence intensity) than the ER-positive cells T47-D, MCF-7 and BT-474 ($P < 0.05$). Accordingly, BT-20 and MDA-MB-435-S had the highest capacity for binding [$^3$H]-Ro5-4864, while the ER-positive cells exhibited only low binding of the benzodiazepine. PBR expression correlated inversely with cell doubling time ($r = 0.78$) and positively with Ki-67 expression ($r = 0.77$). The amount of mitochondria was significantly higher in cells with high PBR expression. As PBR could be demonstrated only after permeabilization of cells, PBR is suggested to be localized within the cytoplasm. Moreover, colocalization of PBR and mitochondria was shown by confocal microscopy analysis. The highest amounts of both PBR and mitochondria were found in cell lines with high mitotic activity. Therefore, it is concluded that the level of PBR is dependent on the number of mitochondria. PBR and its putative endogenous ligand diazepam-binding inhibitor are possibly involved in the regulation of cell proliferation of human breast cancer cell lines. BIOCHEM PHARMACOL **60**;3:397–402, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** peripheral benzodiazepine receptor; breast cancer cell lines; cell proliferation

Due to their anxiolytic and sedative effects, BZ† are widely prescribed drugs. Their action is mediated by central-type $GABA_A$ (γ-aminobutyric acid) receptors located in the brain. Moreover, BZ have been observed to bind to another, pharmacologically different type of receptor which is predominantly found in peripheral organs, the so-called PBR [1, 2]. Though it is present throughout the body, its density may vary, showing high amounts in steroid-producing tissues such as ovary [3], testes [4], adrenal, and placenta [5]. While the molecular structure of PBR has been analyzed [6], their function and effector mechanisms have not been definitively elucidated. They are involved in multiple physiological functions such as steroidogenesis [7], immune responses [8], insulin secretion [9], porphyrin transport [10], mitochondrial oxidative phosphorylation [11], and regulation of cell proliferation [12]. In human astrocytomas, PBR expression correlated with tumor malignancy grade, proliferation index, and patient survival [13]. In brain tumors, increased PBR concentrations have been used for diagnostic imaging and as targets for antineoplastic agents [14]. As compared to normal tissues, elevated PBR density has been demonstrated in ovarian carcinomas [15] as well as in chemically induced rat mammary tumors [16]. Recent studies indicate a role for PBR in breast cancer cell proliferation [17], where PBR expression has been linked to growing aggressive potential of breast cancer cells *in vitro* and *in vivo* [18]. The supposed endogenous ligand of PBR, a polypeptide named DBI, is capable of binding acyl-CoA and may therefore play an important role in the regulation of mitosis. In cells with a fast turnover that are able to metabolize fatty acids, acyl-CoA may be the primary energy supplier.

Our previous experiments have shown that the human breast cancer cell line BT-20 binds the PBR agonist [$^3$H]Ro5-4864 with high affinity [19]. Therefore, we investigated in different human breast cancer cell lines whether binding of [$^3$H]-labeled BZ corresponds to binding of the PBR-specific Mab 8D7 [20] and whether there is a correlation with cell proliferation characteristics.

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† *Abbreviations:* BZ, benzodiazepine; PBR, peripheral benzodiazepine receptor; DBI, diazepam-binding inhibitor; Mab, monoclonal antibody; FITC, fluorescein isothiocyanate; NAO, nonyl-acridine orange; ER, estrogen receptor; and RFI, relative fluorescence intensity.

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## MATERIALS AND METHODS

### *Cell Culture*

The human breast cancer cell lines BT-20, MCF-7, BT-474, T47-D, MDA-MB-435-S, and SK-BR-3 were purchased from cell line service (CLS). All cell lines were grown at 37° with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) (pH 7.4) supplemented with 10% fetal bovine serum, 1% glutamine, and 180 IU/mL penicillin–streptomycin. Cell culture plasticware was obtained from Greiner and Nunc. Cell passages ranged from 10 to 30. Experiments were started with $10^6$ cells/9-cm-diameter Petri dishes. For all experiments except laser microscopy, cells in the log phase of cell growth were used. Log phase was reached after 2 (SK-BR-3, MDA-MB-435-S, BT-20) to 3 days (T47-D, MCF-7, BT-474) and represents that phase of cell growth during which cell doubling time is constant.

### *Flow Cytometry*

For flow cytometry, cells were harvested by trypsinization, adjusted to $10^6$ cells/sample, and washed in PBS. For PBR staining, cells were fixed in 0.4% paraformaldehyde in PBS for 5 min. After permeabilization with saponin solution (0.1% saponin in PBS containing 0.1% BSA), cells were labeled for 30 min at 4° with 1 μg/mL of the anti-PBR 8D7 antibody, a gift from P. Casellas, Sanofi Recherche. The Mab 8D7 was produced from mice immunized with the human PBR C-terminal peptide (last eleven amino acid residues) and thus represents a sequence-specific Mab to PBR. Its specificity has been shown by Western blot and ELISA [20]. Thereafter, cells were washed twice in PBS and resuspended in FITC-conjugated goat $F(ab')_2$ antibody to mouse IgG (immunoglobulin G) (Sigma-Aldrich) diluted 100-fold in PBS. Control samples were prepared as described without permeabilization.

For Ki-67 (Ki-67 Antigen R-phycoerythrin, DAKO) and NAO (Molecular Probes) labeling, cells were fixed in 70% ice-cold ethanol for 10 min and rehydrated in PBS. Thereafter, 0.2 mL of Ki-67 solution (diluted 1/100 in PBS) was added, and after incubation for 30 min, cells were washed twice with PBS. Mitochondria were stained with 10 mM NAO for 15 min at room temperature and washed twice with PBS. Samples were measured on a FACScan (fluorescence-activated cell sorting, Becton Dickinson) flow cytometer and analyzed using Lysis II software. Forward scatters (FSC) and side angle scatters (SSC) were used to gate and exclude cellular debris. Fluorescence of cells was excited using the 488-nm wavelength of an argon laser. Green and red fluorescence were collected on FL 1 and FL 2 channels at 530 nm and 585 nm, respectively. The level of background fluorescence, due to the nonspecific binding of the FITC- and PE (phycoerythrin)-conjugated antibodies, was established using control specimens and processed in the same manner as the samples, but without exposure to the primary antibodies. Ten thousand events were collected in a list-mode file for each sample. The RFI is expressed as multiple of fluorescence intensity of control samples.

### *Laser Scanning Confocal Microscopy*

Cells were grown on glass cover slips and fixed in 70% ice-cold ethanol. Samples were simultaneously incubated with biotin-conjugated anti-PBR Mab and antimitochondria M117 Mab for 30 min at room temperature. After washing in PBS containing 0.1% BSA, cells were incubated with FITC-conjugated streptavidin (1 μg/$10^6$ cells) and PE (phycoerythrin)-conjugated goat anti-mouse IgG (immunoglobulin G) for 30 min. Specimens were washed twice in PBS and embedded in glycerol containing 5% of the antibleaching reagent DABCO (1,4-diazabicyclo[2.2.2] octane) (Sigma). A laser scanning confocal microscope (Leica TCS 4D) based on the inverted microscope Leica DMB and equipped with a PlanApo oil (100x) immersion lens was used for analysis of colocalization of mitochondria and PBR.

### *Cell Doubling Time*

Cells were seeded at $5 \times 10^5$/100-mm plastic dish, and triplicates were harvested each day until the stationary growth phase was reached. For determination of cell growth, cells were washed three times with PBS and the total protein concentration of each culture was measured according to Lowry [21], using BSA as standard. Protein concentrations were plotted logarithmically against time, and cell doubling time was determined from the exponential phase of cell growth.

### *Binding*

The [$^3$H]Ro5-4864 binding assay was performed as described previously [19]. Cells were incubated for 40 min at 0° in a final concentration of $1 \times 10^6$ cells/mL in Hanks' buffer containing 1.25 to 40 nM [$^3$H]Ro5-4864 (4′-chlorodiazepam) (70–90 Ci/mM; DuPont NEN). Non-specific binding was determined by adding 10 μM unlabeled Ro5-4864. Specific binding was calculated from the difference between total and non-specific binding. Incubation was terminated by transfer of aliquots of radiolabeled cells ($1 \times 10^5$ cells/0.1 mL) to PBS-prewetted wells of microfiber plate (0.65-μm Durapore glass fiber, MultiScreen system, Millipore) and immediate vacuum filtration. The filters were washed three times with PBS and dried for 5 min at 37°. Filter discs were punched into vials and radioactivity was counted by liquid scintillation spectrometry (Rackbeta, LKB Wallac) after brief shaking and incubation for 12 hrs in scintillation cocktail (Rotiszint, Roth).

### *Statistics*

Results represent the means of 3 to 8 independent experiments. Standard deviation (SD) was ≤5% within and

**TABLE 1. Expression of PBR in human breast cancer cell lines after permeabilization with saponin solution**

| | | PBR Expression | | [$^3$H]Ro5-4864 binding | |
|---|---|---|---|---|---|
| Human breast cancer cell line | ER | RFI ± SD | % Pos Cells ± SD | $K_d$ (nM) ± SD | $B_{max}$ (fM/$10^6$ cells) ± SD |
| BT-20 | − | 28.9 ± 1.5* | 90.4 ± 4.7* | 8.5 ± 1.8 | 339 ± 2.1 |
| MDA-MB-435-S | − | 25.0 ± 2.0* | 78.9 ± 4.0 NS† | 17.3 ± 2.0 | 216 ± 3.3 |
| SK-BR-3 | − | 26.5 ± 1.1* | 87.1 ± 3.9* | 26.3 ± 4.1 | 228 ± 4.3 |
| T47-D | + | 13.2 ± 0.3 NS† | 60.3 ± 3.9 NS† | below detection limit | |
| MCF-7 | + | 11.2 ± 1.4 NS† | 63.0 ± 2.1 NS† | below detection limit | |
| BT-474 | + | 10.9 ± 0.9 | 64.2 ± 3.8 | below detection limit | |

*, $P < 0.05$ as compared to BT-474.
†, not significantly different from BT-474.

between experiments for FACS analysis of PBR, for cell doubling time, for FACS of Ki-67, and mitochondrial mass. Statistical analysis including the regression line of proliferation data and Student's *t*-test was carried out using the Statistical Program for the Social Sciences (SPSS).

## RESULTS

### *PBR Expression in Different Human Breast Cancer Cell Lines*

The ER-negative breast cancer cell lines BT-20, MDA-MB-435-S, and SK-BR-3 showed the highest PBR expression with regard to RFI and the percentage of positive cells (Table 1). The difference between the RFI of the ER-negative cells and that of ER-positive cell lines was significant ($P < 0.05$). Accordingly, the ER-negative BT-20, MDA-MB-435-S, and SK-BR-3 cells bound [$^3$H]Ro5-4864 with nanomolar affinities, while the ER-positive cell lines exhibited only low binding of the BZ. Due to minimal specific binding (25 to 50 fM/$10^6$ cells at 40 nM [$^3$H]Ro5-4864) and high non-specific binding (>80%), calculation of $K_d$ (dissociation constant) and $B_{max}$ (maximal binding capacity) of the ER-positive cells was not possible. PBR expression as determined by flow cytometric analysis (RFI) was present in these cell lines, but relatively low (Table 1).

### *PBR Expression and Cell Proliferation Characteristics*

PBR expression (RFI) showed a close, inverse correlation with the cell doubling time ($r = 0.78$) (Fig. 1): the faster the cell growth, the higher the expression of PBR. ER-negative cells proliferated faster than ER-positive cells. To evaluate the growth fraction of the different cell cultures in log phase of cell growth, the nuclear matrix-associated proliferation-related antigen Ki-67 was determined. We found that the percentage of Ki-67 positive cells was high in cultures with high amounts of PBR (Fig. 2), indicating a correlation ($r = 0.77$) between PBR expression and the number of proliferating cells in culture.

### *PBR Localization*

Flow cytometric analysis of the various cell lines showed no labeling with 8D7 Mab when cells were not permeabilized prior to staining. The same results were obtained using fluorescence microscopy (data not shown). In contrast, all permeabilized samples were specifically labeled (Fig. 3, a and b), suggesting that the epitope recognized by the antibody was located within the cytoplasm, but not on the plasma membrane. Analysis of double-stained specimen (anti-PBR and antimitochondria Mab) by confocal laser microscopy clearly showed an identical subcellular distribution of mitochondria and PBR in BT-20 cells (Fig. 3, a and b). MCF-7 cells showed a similar distribution of PBR in the cytoplasm. No nuclear staining was found in either cell line.

### *PBR Expression and Amount of Mitochondria*

Since PBR have been shown to be expressed on mitochondria, we evaluated the cellular mitochondrial mass by flow cytometry by means of NAO staining. NAO binds specif-

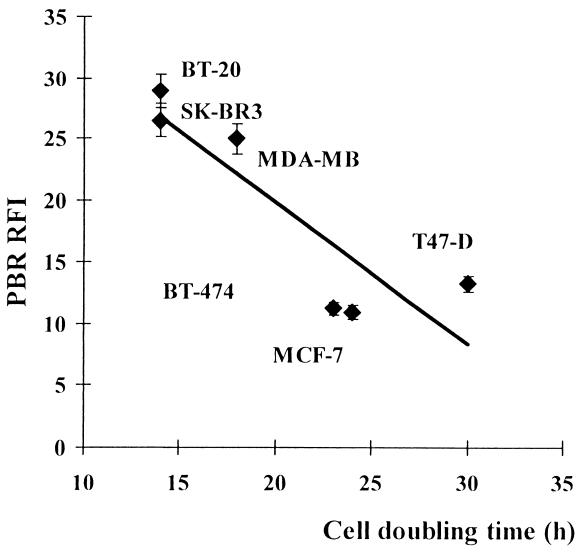


**FIG. 1. PBR expression as measured by RFI in relation to cell doubling time (hr), $r = 0.78$.**

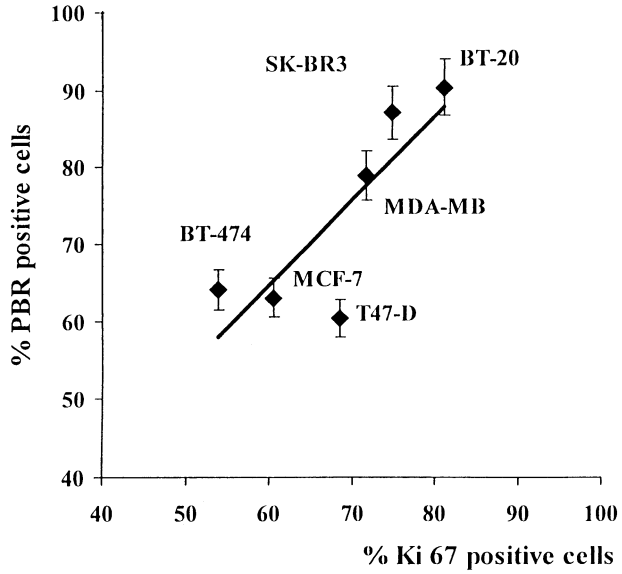


**FIG. 2. PBR expression (% positive cells) in relation to Ki-67 labeling (% positive cells), $r = 0.77$.**

ically to cardiolipin at the inner mitochondrial membrane and can therefore be used to measure the mitochondrial mass [22]. The highest relative NAO fluorescence (29.4) was found in exponentially growing cultures of MDA-MB-435-S cells, which have a high number of PBR (Fig. 4). Relatively high RFI was also observed in BT-20 cells. All ER-positive cell lines with low PBR expression also had lower values (<12) of relative mitochondrial fluorescence, which differed significantly from the ER-negative cell lines ($P < 0.05$ for MDA-MB-435-S and BT-20). In this experiment, SK-BR-3 cells appeared as outliers, showing high PBR expression but low numbers of mitochondria.

## DISCUSSION

PBR have been demonstrated in many tissues of different species. Binding properties of the PBR vary, however, with regard to species, tissues, and ligands. In general, PK 11195, an isoquinoline carboxamide, showed high-affinity binding in tissues from many species [23]. In contrast to rodent PBR ($K_d$ 1–10 nM), binding of Ro5-4864 to PBR of most human tissues occurred only with low affinity ($K_d$ about 1 μM) [23, 24], except for human lymphocytes and platelets [25] and the human breast cancer cell line BT-20 ($K_d$ 8.5 nM) [19].

The present study clearly demonstrates that the ER-positive breast cancer cell lines MCF-7, BT-474, and T47-D possess PBR, but exhibit little specific binding capacity. After FACS analysis with PBR-specific antibody 8D7, the proportion of PBR-positive cells was in the range of 60 to 70% in these cell lines. Nevertheless, in terms of PBR quantity (RFI), the low binding of the benzodiazepine agonist correlated with a low binding of specific PBR antibodies, as represented by RFI. The only cell lines devoid of PBR reported so far are the human Jurkat

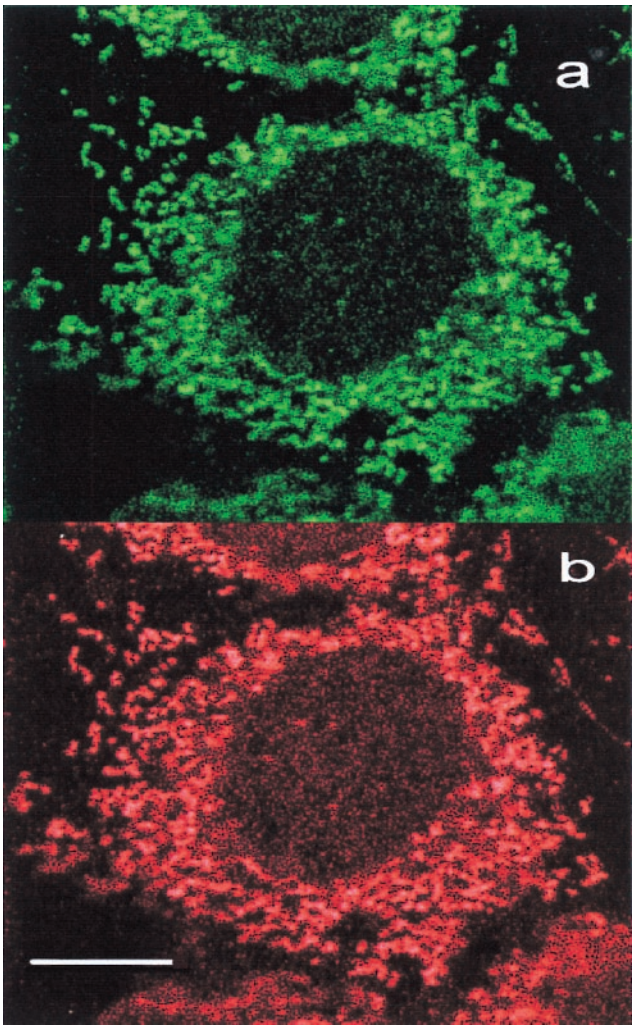


**FIG. 3. (a) PBR and (b) mitochondrial localization of a BT-20 cell by confocal microscopy. Cells were simultaneously labeled with anti-PBR Mabs (8D7) and antimitochondria Mabs as described in Materials and Methods. The green color (a) corresponds to the staining of PBR, the red color (b) to the labeling of mitochondria. This image shows an addition of six optical sections. Bar: 10 μm.**

T-lymphoid cell lines [20]. Out of the six breast cancer cell lines examined, at least 4 follow a pattern, which shows that cells expressing a high level of PBR are ER-negative and have relatively short doubling times and a high amount of mitochondria. In general, the more aggressive breast tumors are characterized by a high growth rate and the absence of ER. These results agree with recent data from the literature [18], showing a more aggressive phenotype for breast cancer cell lines as well as for breast cancer biopsies in the case of high PBR expression.

Autoradiographic and radioligand binding studies showed that PBR are located mainly on the outer mitochondrial membrane, facilitating cholesterol transport as a rate-limiting step in steroidogenesis [26]. This was corroborated by FACS analysis and confocal microscopy [20]. Accordingly, in the present study, FACS analysis revealed that labeling was observed only after permeabilization of

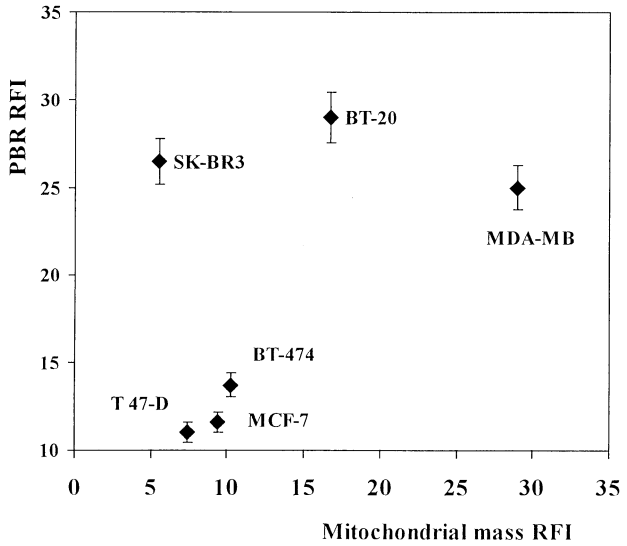


**FIG. 4. PBR expression as measured by RFI in relation to mitochondrial mass (RFI). SK-BR-3 cells represent outliers, showing high PBR expression, but low mitochondrial mass.**

the plasma membrane. Thus, we conclude that in these breast cancer cell lines PBR are exclusively associated with mitochondria. Despite this, the location of PBR remains the subject of ongoing discussions. Previously, PBR were also demonstrated in erythrocytes [27] and, by means of confocal microscopy, on the plasma membrane [28]. Moreover, in liver tissue, PBR binding to two distinct subcellular locations, hepatocyte mitochondria and a non-mitochondrial fraction of binary epithelial cells, has been observed [29]. The role of these PBR might differ from that of mitochondrial PBR.

In a recent study, not only was cytoplasmatic PBR staining observed (MCF-7 and ADR cells), but nuclear PBR staining was also found in another cell line (MDA-231) [18]. While we used a Mab originating from mice immunized with the human PBR C-terminal peptide [20], Hardwick *et al.* applied a fluorescent PBR ligand and an anti-PBR antibody directed against an internal PBR peptide [18]. The reason for this discrepancy might be a slight alteration in sequence in the C-terminal fragment of the receptor [18], so that the sequence-specific Mab 8D7 might not recognize PBR with distinct subcellular localizations. Furthermore, it is possible that changes in PBR localizations are associated with differences in signal peptides or chaperone proteins, which influence PBR subcellular localization and functioning.

In contrast to Carayon *et al.* [11], we observed that cells with higher numbers of mitochondria showed a higher PBR expression (except for SK-BR-3 cells). While we examined fast-growing tumor cell lines of epithelial origin, these authors [11] investigated cells from the hematopoietic system, which have no proliferative activity in peripheral blood. As cells replicate the mitochondria during the cell cycle [30], the amount of mitochondria should be high if there is a high percentage of mitotic cells. Accordingly, as PBR are located on the mitochondrial membrane, a high amount of mitochondria is associated with a high amount of PBR. Further investigations to analyze the relation between mitochondrial content and phase of the cell cycle are needed.

In addition to the subcellular distribution of PBR, its physiological role is still under discussion. In brain tumors, increased levels of DBI have been found compared to normal brain tissue [31], and a clinical correlation between PBR expression and patient survival has been established [13]. PBR/DBI expression was also elevated in liver tumors, while it was low in normal liver tissue and liver hyperplasia [32]. Furthermore, the present results demonstrate that the proliferation rate of breast cancer cell lines is associated with a higher amount of PBR.

The presumably endogenous ligand of PBR, DBI, which is, according to molecular analysis, a typical house-keeping gene expressed in almost all tissues, appears to be involved in energy metabolism by forming an intracellular pool of acyl-CoA [33]. DBI is able to activate mitochondrial acyl-CoA synthetase and to transport acyl-CoA esters for mitochondrial β-oxidation. In conclusion, PBR and DBI might be involved in the regulation of cell proliferation, especially in tissues that use fatty acids as the primary source of energy. Accordingly, a higher level of DBI was found in *Drosophila* in cells that metabolize fatty acids than in those using carbohydrates as a source of energy production [34]. After addition of nanomolar concentrations of PBR agonists, a PBR-specific growth stimulation of cells has been observed [18, 19, 28]. An involvement of PBR in the proliferation of the MDA-231 breast cancer cell line has been shown in terms of an increased cholesterol transport into the nuclei [18]. No such effect could be observed in MCF-7 cells. While the precise function of PBR and its mechanism of action in cell growth remain to be elucidated, the presence of PBR in human breast cancer cell lines, particularly in fast-growing cells, might indicate an involvement in the energy supply of these cells.

Sensitivity of cells to estrogen represents a histomorphologic feature of a more differentiated tissue that proliferates more slowly and expresses less PBR than estrogen-independent tumors. A functional relation between ER and PBR has not yet been established. For the clinical management of breast cancer, the presence of ER is an independent predictor of survival [35].

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