# Cancer-related Overexpression of the Peripheral-type Benzodiazepine Receptor and Cytostatic Anticancer Effects of *Ginkgo biloba* Extract (EGb 761)*

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**Abstract.** *The peripheral-type benzodiazepine receptor (PBR) is an 18-kDa high affinity drug- and cholesterol-binding protein that is involved in various cell functions, including cell proliferation and apoptosis. PBR was shown to be overexpressed in certain types of malignant human tumors and cancer cell lines, correlating with enhanced tumorigenicity and cell proliferation rates. The present study was conducted in order to further define the role of PBR in cancer and to extend our recent findings regarding the possible anticancer effects of the standardized Ginkgo biloba extract EGb 761. Treatment with EGb 761 decreased PBR mRNA levels and inhibited the proliferation of breast, glioma and hepatocarcinoma cell lines, further corroborating our previous contention that its mechanism of action is through the modification of PBR expression. In vivo treatment with Ginkgo biloba extract led to dose-dependent decreases in xenograft growth of both MDA-MB-231 breast cancer and U-87 glioma cell lines in nude mice, although the effects were not maintained after 50 days of treatment in the latter. The results obtained in MDA-MB-231 xenografts indicated pronounced inhibition of tumor growth, verified by MRI imaging. These results were obtained using a modified experimental protocol where the animals were treated with the extract before cell inoculation. Although an exact role for PBR in relation to the initiation and progression of various types of cancer remains to be defined, our results indicate that PBR overexpression in certain cancer cells is related to an aggressive phenotype. Since EGb 761 treatment opposes this aggressive phenotype by decreasing PBR overexpression, it could be useful in preventing or treating cancer invasiveness and metastasis.*

The peripheral-type benzodiazepine receptor (PBR) is an 18-kDa, high-affinity drug- and cholesterol-binding protein known to play a crucial role during steroidogenesis and various other cell functions (1-3). The identification of PBR resulted from the discovery of another class of binding sites for benzodiazepines, distinct from the GABAA receptor complex that is involved in inhibitory neurotransmission (4). Convincing evidence indicated that PBR is part of a multimeric receptor complex, whose expression requires both the 18-kDa PBR subunit and a 34-kDa pore-forming, voltage-dependent anion channel (VDAC) (5, 6). Today, this PBR complex may be defined as an entity composed of three subunits [*i.e.*, the 18-kDa PBR, the VDAC and the 30-kDa adenine nucleotide transporter (ADC)] that is localized primarily in the outer mitochondrial membrane (7).

The existence of PBR in non-steroidogenic tissues indicates that it is not only involved in steroidogenesis, but in other processes as well (1-3). As PBR and its putative endogenous ligand, the diazepam-binding inhibitor (DBI), have been detected in many benign and malignant tissues of various species, it has been hypothesized that PBR may be involved in a variety of cancers. One of the earliest studies in this area was published in 1980 and showed that PBR ligands could induce differentiation of murine Friend erythroleukemia (MEL) cells and additionally inhibit the proliferation of Swiss 3T3 cells (8). It has also been shown that PBR expression is increased in many types of tumors and that it is involved in regulating the proliferation rates of several cancer cell lines (9-18).

*This manuscript is dedicated to our co-author, colleague and friend Dr. Francis V. DeFeudis, who died in December 2004.

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Recent studies of human breast cancer cell lines revealed that the levels of PBR expression are higher in more aggressive and invasive estrogen receptor (ER)-negative cell lines (*e.g.*, MDA-MB-231) than in less aggressive cell lines (19), and that the amplified PBR gene may indicate breast cancer progression in more aggressive cell lines (20). PBR-specific ligands also appear to regulate the proliferation rate of the highly aggressive MDA-MB-231 human breast cancer cell line (19). Overexpression of PBR, revealed by a high density of PBR-binding sites, has also been correlated with enhanced tumorigenicity and cell proliferation rates in glioma cell lines (7).

Convincing evidence that supports the involvement of PBR overexpression in various forms of cancer has also been provided in studies of human malignant tumors. In this regard, it was shown that PBR is expressed at higher levels in aggressive metastatic breast carcinoma biopsies of human patients than in normal human breast tissue (19, 21, 22). Overexpression of PBR has also been demonstrated in colorectal carcinoma specimens (21, 23, 24) and in malignant brain tumors (glioblastomas/astrocytomas) (11, 12, 14, 17, 21, 25-27) of human patients. Other studies have shown that PBR is overexpressed in other malignant tumors of human patients, such as hepatocellular carcinoma (28), ovarian carcinoma (21, 29) and prostate carcinoma (21). This analysis indicates that a relationship exists between PBR and cancer.

Our results have shown that treatments with either EGb 761 (used in the form of IPS 200; see Materials and Methods section) or ginkgolide B (GKB) decreased PBR expression and the proliferation of the highly aggressive, human breast cancer cell line MDA-MB-231, but not the proliferation of the non-aggressive human breast cancer cell line MCF-7 (30). EGb 761 and GKB treatments also inhibited tumor growth when MDA-MB-231 cells were used as xenografts in athymic (nude) mice, further substantiating our observation that PBR overexpression is correlated with human breast cancer cell proliferation and the expression of an aggressive phenotype (30).

Considering that these effects were reversible (*i.e.*, cytostatic rather than cytotoxic), it seemed possible that such manipulation of PBR expression might be useful in controlling tumor growth (30) and, therefore, further experiments were warranted. The present study was conducted in an effort to further define the role of PBR in relation to cancer and to extend our recent finding that IPS 200 exerts anticancer effects on human breast cancer cells. Experiments were conducted using several cancer cell lines of different ontogenies (*i.e.*, breast, colon, brain, liver and prostate), as well as xenografts of some of these lines in nude mice. In view of other mounting evidence, which has revealed that PBR expression is increased in aggressive, metastatic cancers in which estrogen receptors (ERs) are either under-expressed or absent (22, 31, 32), we analyzed cancer cells for their levels of both PBR and ER expression in relation to their aggressive/metastatic phenotype. Both ERα and the recently cloned ERβ subtype (33, 34) were studied, since differences in their molecular structures and tissue distributions indicated that they may play different biological roles as ligand-activated transcription factors mediating the effects of 17β-estradiol.

## Materials and Methods

*Materials.* Modified Eagle's Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Mediatech Inc. (cellgro®; Gaithersburg, MD, USA) and the American Type Culture Collection (ATCC; Manassas, VA, USA). Penicillin, streptomycin and crystal violet were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell culture materials were purchased from VWR International (West Chester, PA, USA). N-methyl-[$^3$H]PK 11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline carboxamide; sp. act. 83.50 Ci/mmol] was obtained from DuPont-New England Nuclear (Wilmington, DE, USA), and PK 11195 was obtained from Research Biochemicals Inc. (Natick, MA, USA). The TACS MTT Cell Proliferation and Viability Assay was obtained from Trevigen Inc. (Gaithersburg, MD, USA), the BrdU (5-bromo-2'-deoxyuridine) kit was from Roche Diagnostics (Indianapolis, IN, USA), and the FluorAce Apopain Assay Kit was purchased from Bio-Rad Laboratories (Richmond, CA, USA). IPS 200, the injectable form of the standardized *Ginkgo biloba* extract EGb 761, was provided by the Institut Henri Beaufour-IPSEN (Paris, France) and was used in all *in vitro* experiments presented in this report. EGb 761 (trade mark of IPSEN) was standardized to contain flavonol heterosides (24%), terpenoids such as ginkgolides and bilobalide (6%), proanthocyanidins and organic acids (35) and used for the *in vivo* studies. IPS 200 is devoid of proanthocyanidins or phenolic polymers, known to bind and precipitate proteins *in vitro* (36). In this article, the designations "EGb 761" and "IPS 200" are generally used interchangeably when referring to the standardized extract of *Ginkgo biloba*, except for purposes of detailed discussion of certain results. Antibodies to ERα and the intermediate filament, vimentin, were obtained from Santa Cruz Biotechology, Inc. (Santa Cruz, CA, USA), while the antibody to ERβ was obtained from Research Diagnostics, Inc. (Flanders, NJ, USA). The anti-PBR antibody was produced and used as previously described (21). The Histostain®-Plus Kit was purchased from Zymed Laboratories, Inc. (South San Francisco, CA, USA).

*Cell culture.* The human hepatocellular carcinoma cell lines, Hep 3B and Hep G2, as well as the human prostate carcinoma cell lines, PC-3 and TSU, were obtained from ATCC. The human breast adenocarcinoma cell lines, MDA-MB-231 and MCF-7, as well as the human colon adenocarcinoma cell line HT-29, were obtained from the Lombardi Cancer Center (Georgetown University, Washington, DC, USA). Human U-87 glioma cells were provided by Dr. Philip G. Kasprzyk (Biomeasure Inc., Milford, MA, USA). The Hep3B and HepG2 cells were cultured in MEM, whereas the MDA-MB-231, MCF-7, HT-29, U-87, TSU and PC-3 cells were grown in DMEM. The culture media were supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin, and the

cells were incubated at 37˚C in 6% $CO_2$. The cells were treated for different time-periods with increasing concentrations of IPS 200. For immunocytochemical analyses, the cells were cultured in multi-chambered glass slides. Human hepatocytes were purchased from In Vitro Technologies, Inc. (Halethorpe, MD, USA), kept in culture according to the supplier's recommendations, and treated with increasing concentrations of IPS 200 for 72 h.

*[$^3$H]PK 11195 ligand-binding assay.* Cells were scraped from 150-mm culture dishes into 5 mL phosphate-buffered saline (PBS) and homogenized using a Dounce homogenizer. Radiolabelled [$^3$H]PK 11195 binding assays were performed using aliquots that contained 100 mg protein, as previously described (19, 37). The protein concentrations were determined by the method of Bradford (38) using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. Values for the dissociation constant (*Kd*) and the maximal binding capacity (*Bmax*) were determined by Scatchard analyses using the KELL RadLig program (39).

*Analysis of cell viability.* The cellular reduction of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), to formazan by mitochondrial succinate dehydrogenase was used to quantitate viable cells (40). The TACS MTT cell proliferation and viability assay was performed according to the manufacturer's instructions. The cells were plated in 96-well plates (Corning; Corning, NJ, USA) at a density of approximately 2,000 cells/well and absorbance was measured at 600 nm (reference wavelength: 690 nm) using the Victor$^2$ plate-reader (ECG-Wallac, Inc., Gaithersburg, MD, USA).

*BrdU cell proliferation assay.* The cells were plated on 96-well plates (Corning) at a density of approximately 2,000 cells/well. Differences in cell proliferation were determined by measuring the amount of BrdU incorporation using a BrdU ELISA. Incorporation of BrdU was measured at 450 nm (reference wavelength: 690 nm) using the Victor$^2$ plate-reader (ECG-Wallac, Inc.).

*Crystal violet staining.* After the cells had been plated, cultured and treated as described above, they were washed with PBS, fixed with 100% ethanol and stained with crystal violet (41). They were then washed, dried and resuspended in citrate buffer (0.1 M sodium citrate in 50% methanol), and the absorbance was measured at 570 nm (reference wavelength: 690 nm) using the Victor$^2$ (ECG-Wallac, Inc.).

*Immunocytochemistry.* Eight malignant cell lines of different origins (see above) were screened for the expression of PBR, ERα, ERβ and vimentin. The cells were cultured on eight-chambered, SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA, USA). They were fixed with 10% formalin for 15 min at 4˚C, and then treated with 10% $H_2O_2$ for 10 min. The primary antibodies for PBR (1:400), ERα (1:200), ERβ (1:200) and vimentin (1:200) were applied for 1 h at room temperature. The cells were then washed and incubated with HRP-conjugated secondary antibody for 20 min. Staining was visualized with peroxidase using 3-amino-9-ethyl carbazole as a chromogen. These steps were performed as described in the manufacturer's protocol (Zymed). After counterstaining with hematoxylin, the slides were permanently mounted using Crystal mount medium and analyzed by light microscopy.

*FluorAce apopain assay.* Apopain (also termed caspase-3, CPP32, or YAMA) is a pro-apoptotic member of the family of aspartate-specific, cysteinyl proteases (an "effector" caspase). It is responsible for the cleavage of poly(ADP-ribose) polymerase (PARP) with high efficiency and specificity at the onset of apoptosis ("programmed cell death") (42,43). Since apopain plays a pivotal role in apoptosis, its activity represents an early marker for cells entering this process. After treatment of the Hep G2 and Hep 3B cells with various concentrations of IPS 200 for 72 h, the cells were processed as described by the manufacturer, and the FluorAce apopain assay and fluorometric measurements were performed according to the manufacturer's protocol.

*Isolation of total cellular RNA.* For each cell line, both control and treated cells were cultured in 150-mm plates (Corning). Following treatment with IPS 200, the cells were washed 3 times with 1x PBS (pH 7.4) and subsequently lysed using RLT buffer (QIAGEN, RNeasy Mini kit), according to the manufacturer's specifications. The total RNA was treated with DNase I.

*Reverse transcription for 18S amplicon and PBR.* TaqMan reverse transcription reagents were purchased from Applied Biosystems (Foster City, CA, USA). The reaction mixture for reverse transcription of the 18S amplicon contained 1mg total RNA, 1x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTPs, 2.5 μM random hexamers, 3.125 U/μL MultiScribe™ reverse transcriptase and 0.4 U/μL RNase inhibitor in a total volume of 50 μL. The reaction mixture was sequentially incubated at 25˚C for 10 min, 37˚C for 60 min and 95˚C for 5 min. The reaction mixture for reverse transcription of the PBR gene consisted of 2 μg total RNA, 1x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTPs, 2.5 μM random hexamers, 1.25 U/μL MultiScribe™ reverse transcriptase, and 0.4 U/μL RNase inhibitor in a total volume of 100 mL. The reaction mixture was incubated successively at 25˚C for 10 min, 48˚C for 30 min and 95˚C for 5 min. The resulting cDNAs were then processed for amplification using specific primer sets designed according to the *Primer Express* guidelines.

*Primer design.* In accordance with the *Primer Express* guidelines, primers for the 18S rRNA PBR were as follows: forward primer 18S: 5'TCC CAG TAA GTG CGG GTC AT-3' (nucleotides 1643-1662) and reverse primer 18S: 5'CCA ATC GGT AGT AGC GAC GG 3' (nucleotides 1704-1723). The amplicon for human 18S rRNA was 81 bp. The primers for human PBR were as follows: forward primer; 5'TCT TCT TTG GTG CCC GAC A 3' (nucleotides 380-398) and reverse primer; 5'CCA GCA GGA GAT CCA CCA AG-3' (nucleotides 411-430). The two primers for human PBR were located in different exons, the forward primer being in exon 3, and the reverse primer being in exon 4. The amplicon for PBR was 51 bp and spanned one intron in order to avoid amplification of the target gene in the genomic DNA.

*Quantitative real-time polymerase chain reaction (QRT PCR).* The SYBR Green PCR Master Mix (Applied Biosystems) was used for all components, except for the primers in real-time PCR. Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green Dye to double-stranded (ds) DNA. Using the

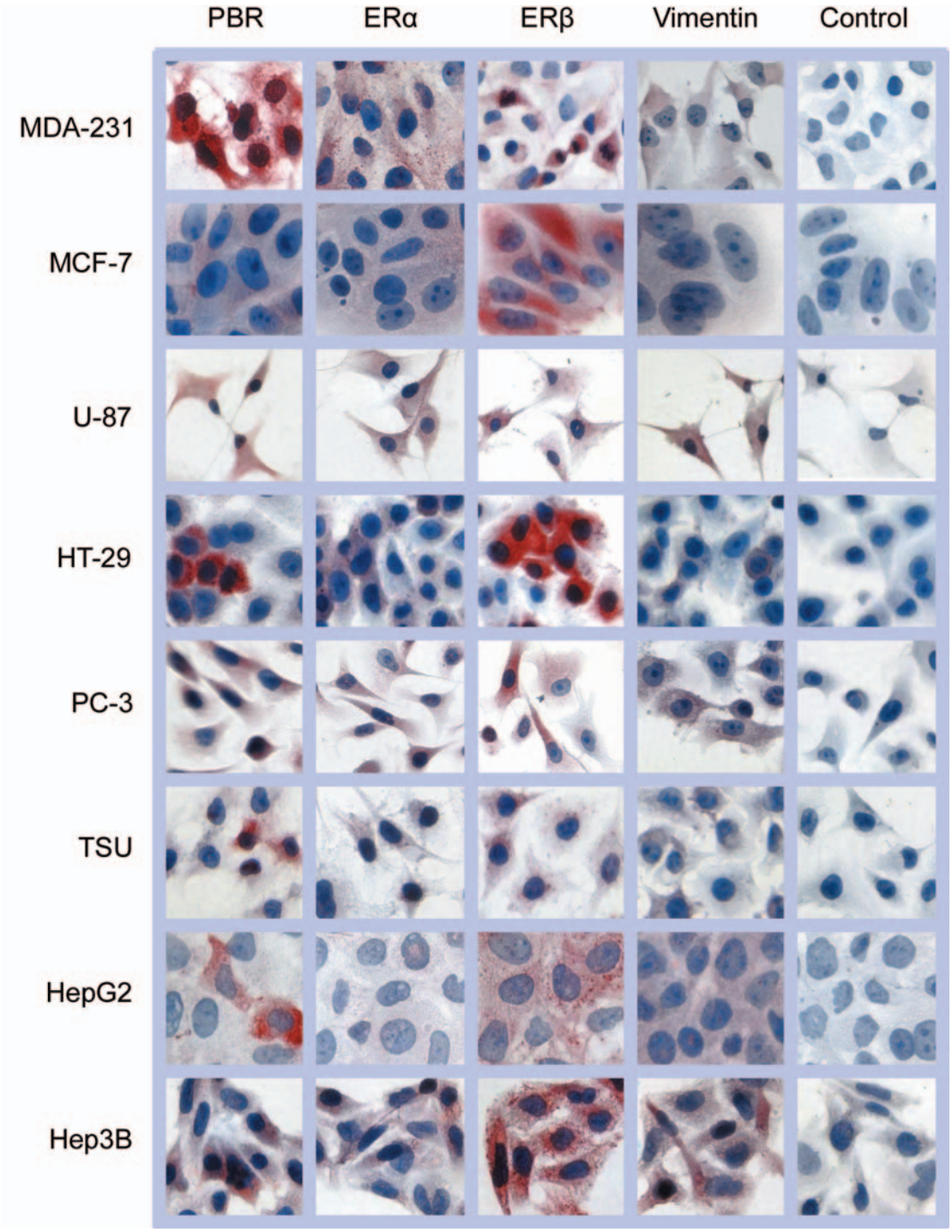


Figure 1. *Immunocytochemical analyses showing the variation in expression of PBR, ERα, ERβ and vimentin that exists between eight human cancer cell lines of different histological origins. A comparison of receptor protein expression among these different cell lines is provided in Table I.*

Comparative $C_T$ method to analyze the data, the amount of human PBR mRNA was normalized to the endogenous reference (18S rRNA). Reactions using PBR and 18S rRNA were performed in separate tubes.

For the PBR and 18S rRNA reactions, the forward/reverse primers were used at equimolar concentrations of 250 nM, in addition to 2 mL cDNA and 10 μL SYBR Green PCR Master Mix 2x in a final volume of 20 μL. Each sample was run in triplicate.

The incubation conditions for the reaction mixture were as follows: 50˚C held 2 min, 95˚C held 10 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, using the ABI PRISM 7700 Sequence Detector.

*In vivo experiments: xenografts.* In order to assess the effects of EGb 761 on tumor cell proliferation and PBR expression in an *in vivo* setting, the mammary fat pad implantation model was used to examine the development of MDA-MB-231 human (estrogen-insensitive) breast cancer cells into tumors in nude mice (30). Considering that the use of EGb 761 in various neurological animal models has been successful, primarily in a preventative mode, we modified the original treatment protocol by including a 7-day oral pretreatment of mice with the indicated daily doses of EGb 761. This pretreatment was followed by inoculation of the MDA-MB-231 cells, allowing the tumors to achieve a volume of 100 to 150 $mm^3$, and then the same indicated daily oral doses of EGb 761 were administered for the indicated time-periods. Female athymic (nude) mice (NCI/Charles River, Frederick, MD, USA) were injected subcutaneously with 0.5-1x$10^6$ MDA-MB-231 cells. The tumor sizes and body weights were recorded twice a week for all animals for the total duration of the experiments (30). To determine whether the effects of EGb 761 were due to a direct action on breast cancer cells or to induced changes in the general physiology (metabolism) of the animal, the control experiment of pretreating MDA-MB-231 cells with IPS 200 (100 μg/mL) for 72 h prior to inoculation was performed. Using this same protocol, the effects of EGb 761 treatment on xenografts of U-87 glioma cells and on both Hep 3B and Hep G2 cells were also examined.

*Magnetic resonance imaging.* Magnetic resonance imaging (MRI) was used as an objective method to evaluate the efficacy of the treatments. This non-invasive method can provide serial data concerning anatomical changes (44, 45). A recent study has revealed that PBR-overexpressing C6 glioblastoma cells can be visualized by administering a cocktail containing a PBR-targeted lanthanide chelate molecular imaging agent (46). MRI was used to measure the size of tumors and identifying the lesions. For such anatomical imaging, segmented T1- and T2-weighted spin-echo pulse sequences were used to acquire high resolution MRI, on both coronal and axial planes, with which the tumor size was measured and tumor texture evaluated. Animals subjected to both control (vehicle) and EGb 761 treatments were examined serially using MRI. All experiments were performed on a Bruker Biospec 7T system, equipped with micro-imaging gradients. A 40-mm diameter surface RF coil was used to transmit RF power and receive MR signals. A respiration gating device was attached to the abdomen of each mouse, and a rodent ventilator was used in anesthetizing the animals. A gas mixture of 30% $N_2O$ and 70% $O_2$ and 4% isoflurane was initially used to anesthetize the mice. A 2% dose of isoflurane was used later to prevent the animals from waking. A constant-temperature water-bath was used to maintain the animal's body temperature. Rectal and skin temperatures were monitored at 35˚C and 33.5˚C, respectively, using biothermal sensors.

*Statistical analysis.* Multiple statistical comparisons were performed with InStat's one- way analysis of variance (ANOVA) (GraphPad Inc., San Diego, CA, USA). Comparisons between individual drug treatments and respective controls were made using an unpaired *t*-test.

Table I. *PBR ligand-binding and expression of ERα and ERβ in various tumor cell lines.*

| Cell lines | Tissue type | Kd (nM) | Bmax (pmol/mg protein) | ERα | ERβ |
|---|---|---|---|---|---|
| MDA-MB-231 | Breast cancer | 7.8 | 8.7 | + | + |
| MCF-7 | Breast cancer | ND | ND | + | ++++ |
| U-87 | Glioblastoma | 1.8 | 7.9 | ++ | ++ |
| HT-29 | Colon carcinoma | 3.7 | 1.56 | + | ++++ |
| PC-3 | Prostate cancer | 2.5 | 0.8 | ++ | ++ |
| TCU | Prostate cancer | 2.9 | 0.3 | - | + |
| Hep3B | Hepatocarcinoma | 4.5 | 0.312 | - | +++ |
| HepG2 | Hepatocarcinoma | 1.5 | 0.045 | - | +++ |

Ligand-binding assays were performed using [$^3$H]PK 11195 and aliquots of tissue suspensions containing 20-100 μg of protein. ND: not detected.

## Results

*Immunocytochemical findings.* As shown in Figure 1, the expressions of PBR, ERα, ERβ and vimentin varied between the eight different human cell types examined. With regard to PBR, the highly aggressive and invasive estrogen-independent MDA-MB-231 breast cancer, U-87 glioma and HT-29 colon adenocarcinoma cell lines uniformly showed high amounts of PBR, as compared to prostate carcinomas, PC-3 and TSU, and hepatocellular carcinomas, HepG2 and Hep3B. The less aggressive, estrogen-responsive breast cancer line MCF-7 expressed the lowest level of PBR protein. The expression of ERα was seen to be highest in U-87 glioma cells and in prostate cancer PC-3 cells (originally isolated from a metastatic bone lesion), whereas the remaining cell lines expressed little or no ERα. All cell lines examined expressed ERβ, with higher levels observed in breast cancer MCF-7, colorectal carcinoma HT-29 and the hepatoma cell lines, HepG2 and Hep3B. Vimentin, a cytoskeletal protein known to be expressed predominantly in cells of mesenchymal origin (47) and in cells adapted to tissue culture conditions (48), was expressed in all cell lines to various degrees, with the highest levels observed in the MDA-MB-231, Hep3B and U-87 cell lines. The relative intensities of protein expression observed in the different cell lines are summarized in Table I.

*Ligand-binding experiments.* Table I presents the results obtained in ligand-binding experiments that were performed using the PBR-specific radioligand, [$^3$H]PK 11195. The dissociation constant (*Kd*; reciprocal of affinity) for binding to this receptor was in the nanomolar range for all cell types examined, indicating the presence of high-affinity binding sites. It is also noteworthy that the binding capacity (*Bmax* value) for PK 11195 was significantly greater for the highly

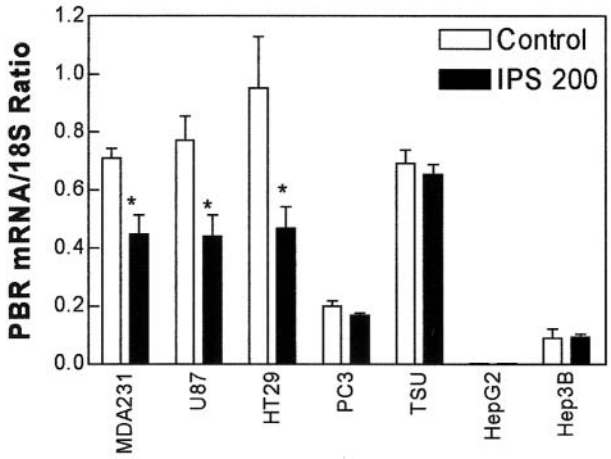


Figure 2. *QRT PCR analyses of the effects of IPS 200 on PBR mRNA expression in cancer cell lines. These data confirm the immunocytochemical results shown in Figure 1, except for the result obtained with TSU prostate cancer cells, which exhibited significant PBR mRNA levels (this figure) in association with low PBR expression (Figure 1). The data are presented as means±SEM of experiments (n=3) that were performed in triplicate. Statistical significance is indicated by the asterisks.*

aggressive/invasive breast cancer cell line, MDA-MB-231, than for the less aggressive MCF-7 breast cancer cell line, in agreement with the immunocytochemical data shown in Figure 1.

*QRT PCR analyses of PBR mRNA expression.* The QRT PCR results shown in Figure 2 indicate high PBR mRNA expression in the MDA-MB-231, HT-29, U-87 and TSU cells. PBR mRNA was expressed to a significantly lesser extent in the PC-3, HepG2 and Hep3B cell lines. These data generally confirm the immunocytochemical results shown in Figure 1, except that TSU prostate cancer cells, which showed low PBR expression by immunocytochemistry (see Table I), contained a significant amount of PBR mRNA. It is also evident that treatment with IPS 200 (200 μg/mL) for 48 h decreased PBR mRNA expression in the aggressive cell lines, MDA-MB-231, U-87 and HT-29.

*MTT cell viability and BrdU cell proliferation assays.* Figure 3 shows that treatment with IPS 200 resulted in concentration-dependent decreases in cell viability (survival) of Hep3B and HepG2 cells (MTT assays) and a decrease in the proliferation of Hep3B cells (BrdU assay). Treatment of U-87 glioma cells with IPS 200 (2-200 μg/mL) for 24 - 72 h also decreased cell proliferation, as indicated by crystal violet and BrdU assays (Figure 4, panels A and B, respectively).

*Apopain/caspase-3 assay.* A representative experiment showing the effects of IPS 200 treatment for 72 h on the apopain

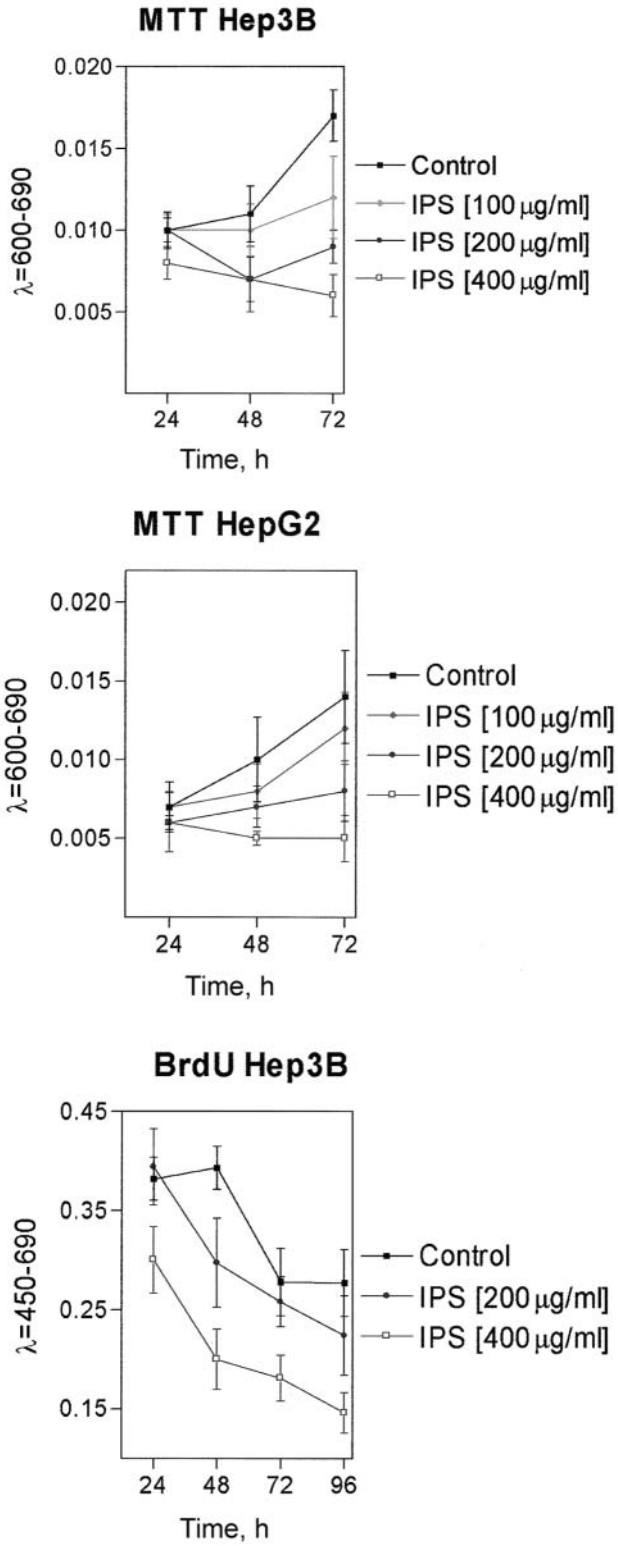


Figure 3. *Dose-dependent decreases in cell viability and proliferation of Hep 3B and Hep G2 cells by IPS 200 (EGb 761), as assessed by MTT and BrdU assays. The data are presented as means±SEM of experiments (n=3) that were performed either in triplicate or quadruplicate.*

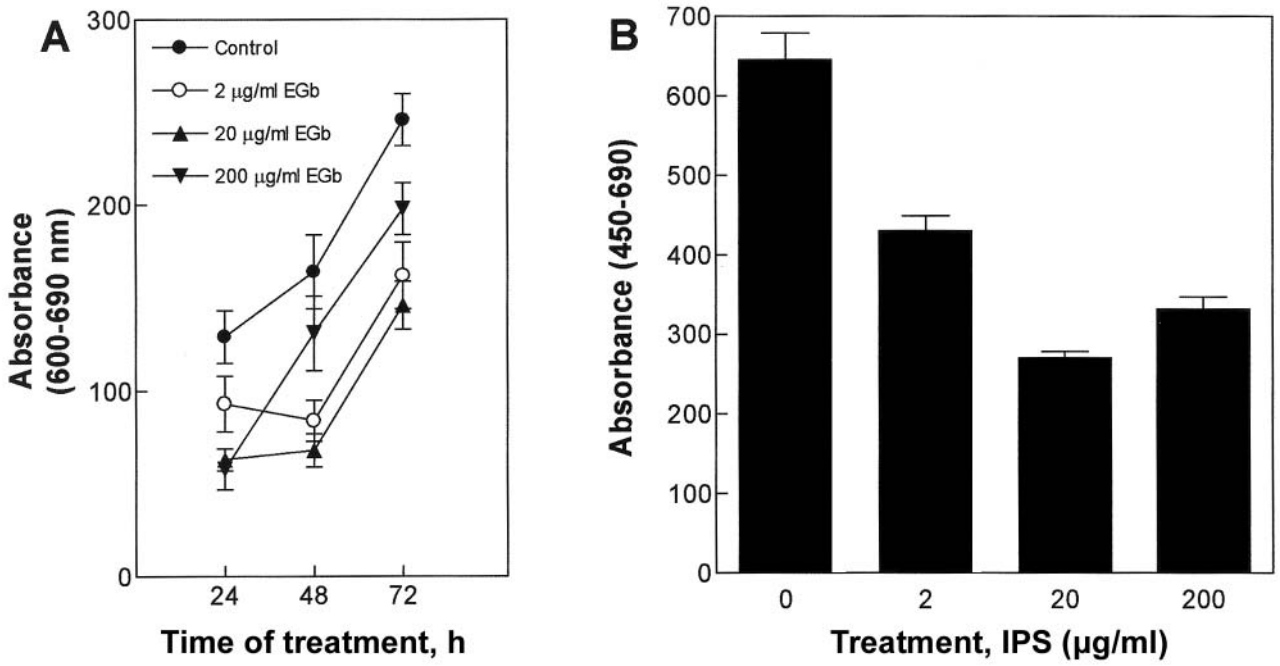


Figure 4. *Effect of IPS 200 on proliferation of U-87 glioma cells. U-87 cells were treated with IPS 200 (2-200 µg/ml) for 24 - 72 h. A: BrdU assay; B: crystal violet assay. The data are presented as means±SEM of experiments (n=3) that were performed either in triplicate or quadruplicate.*

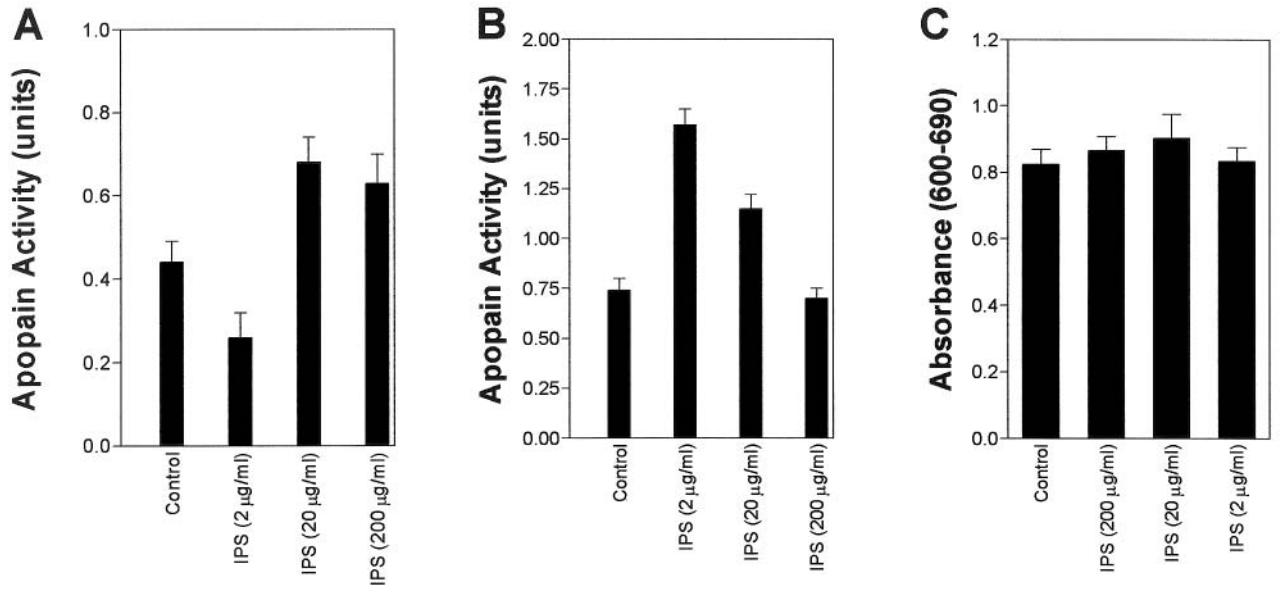


Figure 5. *Representative experiment showing the effects of treatment with increasing concentrations of IPS 200 for 72 h on apoptosis in Hep G2 and Hep 3B cells. (A) Hep G2 cells. (B) Hep 3B cells. Apoptosis was determined using the FluorAce apopain assay and fluorometric measurements were performed according to the manufacturer's protocol. The data are presented as means ± SEM of experiments (n=3) that were performed either in triplicate or quadruplicate. (C) MTT assay of normal human hepatocytes after treatment with IPS 200. The data are presented as means ± SEM (n=3) of experiments that were performed in triplicate.*

(caspase-3) activity of HepG2 and Hep3B cells is illustrated in Figure 5, panels A and B, respectively. It is evident that exposure to a low concentration of IPS 200 (2 µg/mL) inhibited apoptosis (decreased apopain activity) in HepG2 cells, whereas exposure to medium or high concentrations (20 or 200 µg/mL) of the extract significantly increased apoptosis

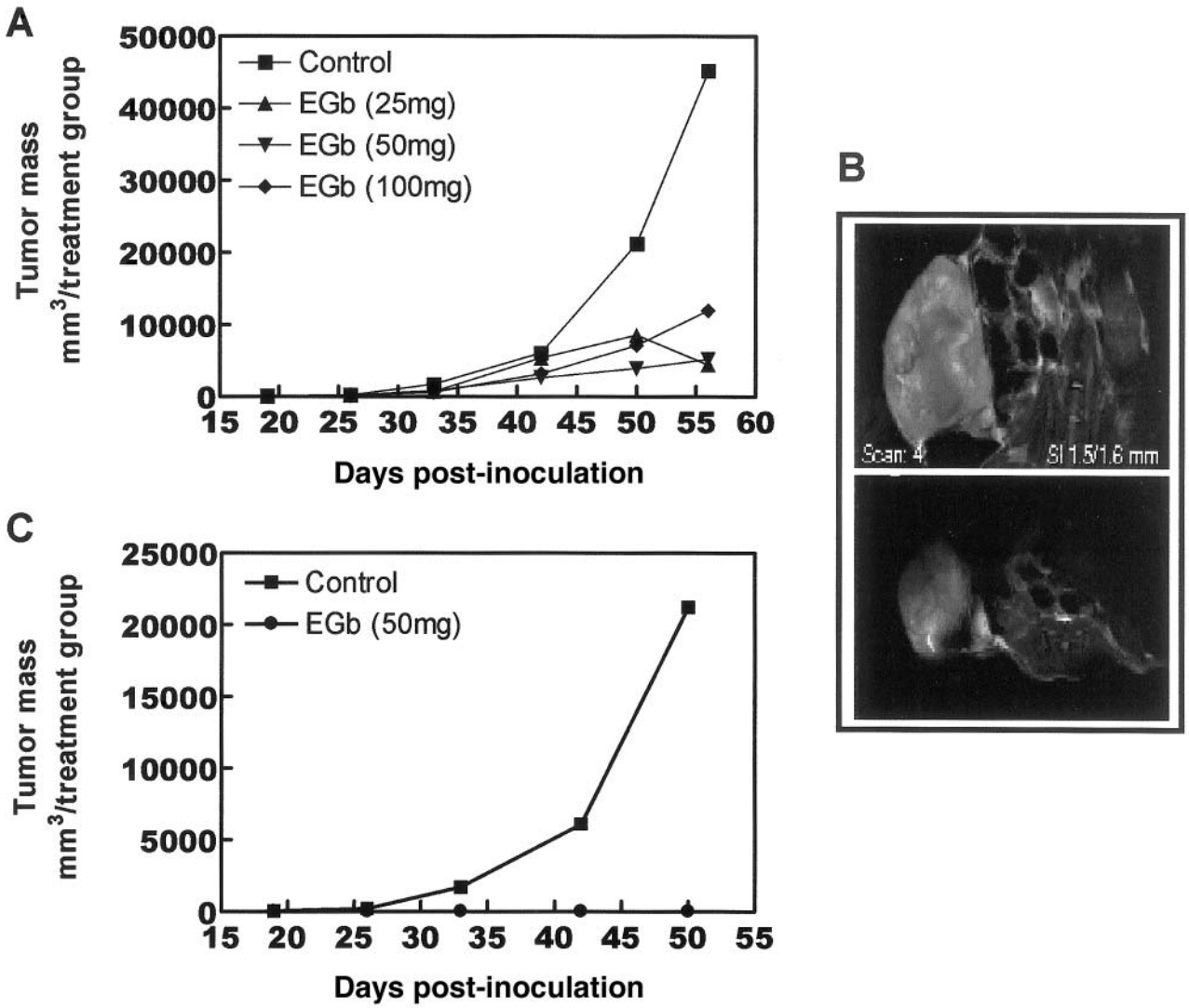


Figure 6. *Effect of EGb 761 on the growth of MDA-MB-231 breast cancer xenografts in nude mice. (A) Preventive protocol: female athymic (nude) mice were pretreated orally with 25, 50, or 100 mg/kg EGb 761 once a day for 7 days, followed by subcutaneous inoculation with 8 x $10^6$ MDA-MB-231 cells, allowing the tumors to form to a volume of about 100 to 150 $mm^3$, and then by continuous treatment with EGb 761 at the same respective daily oral doses for the indicated time-periods. (B) MRI images corresponding to tumor development in a control mouse (upper panel) and in a mouse that was treated with 50 mg/kg/day of EGb 761 for 50 days (lower panel) are also shown. Data shown are mean values of 10 animals for each dose of EGb 761. (C) Effect of orally-administered EGb 761 on the growth of MDA-MB-231 breast cancer xenografts in nude mice. MDA-MB-231 cells were treated with EGb 761 (100 μg/ml) for 72 h before being subcutaneously inoculated into the animals, after which the animals were treated continuously with EGb 761 (50 mg/kg/day). The data shown are the mean values of 10 animals for control animals and for animals treated with EGb 761.*

(Figure 5A). The opposite effect was observed with Hep3B cells (Figure 5B). In this regard, it is noteworthy that treatment of normal human hepatocytes with IPS 200 for 72 h had no effect on cell proliferation or cell viability, as determined by MTT assay (Figure 5C).

*Effects of IPS 200 on the in vivo growth of tumor cell xenografts in nude mice.* Using the modified protocol described in the "Materials and Methods" section, IPS 200 (EGb 761) was found to have a dramatic inhibitory effect on the *in vivo* growth of human MDA-MB-231 breast cancer cells when used as xenografts in nude mice (Figure 6). This inhibitory effect of IPS 200 became more pronounced as a function of time post-inoculation of the tumor cells and reached 80-90% by 55 days post-inoculation (Figure 6A). Changes in tumor volume were confirmed by changes in the MRI images of tumors that were formed in both vehicle- and IPS 200-treated animals (Figure 6B). The results also

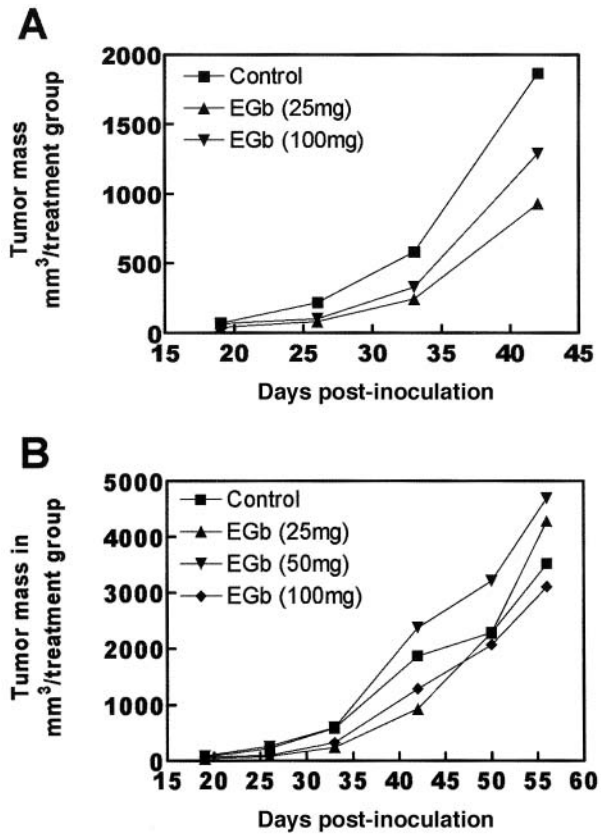


Figure 7. *Effect of EGb 761 on the growth of U-87 glioma xenografts in nude mice. Preventive protocol: animals were treated orally with 25, 50, or 100 mg/kg EGb 761 once a day for one week, followed by subcutaneous inoculation with the tumor cells and by continuous treatment with EGb 761 at the same respective daily doses. Results for the first 42 days ( A) and for the total 55-day period post-inoculation of the tumor cells (B) are shown; mean values of 10 animals for each dose of EGb 761.*

indicated that inoculation of the mice with MDA-MB-231 cells, which had been pretreated *in vitro* with 100 μg/mL of EGb 761 for 48 h, did not form tumors *in vivo* in animals that were treated with EGb 761 (50 mg/kg/day) for 50 days (Figure 6C).

The results obtained using this same protocol showed that treatment with EGb 761 had a less pronounced effect on U-87 glioma growth when used as a xenograft for a time-period up to 45 days (Figure 7A) and that this cytostatic effect was not maintained after 50 days of treatment (Figure 7B). Also, in contrast to the results obtained with xenografts of breast cancer and glioma cells, and to *in vitro* data obtained with Hep3B and HepG2 cells (see above), EGb 761 treatment did not decrease the tumor volume of xenografts of PBR-impoverished Hep3B and HepG2 cells in nude mice (data not shown).

## Discussion

*Overexpression of PBR in cancer cells.* One major aim of the present study was to examine PBR overexpression in relation to cancer. Based on our previous findings, we concluded that PBR expression, perinuclear/nuclear localization and PBR-mediated cholesterol transport into the nucleus are all involved in human breast cancer cell proliferation and expression of an aggressive phenotype, properties that favor disease progression (19). The findings of the present study, using immuno-cytochemistry, QRT-PCR, ligand-binding methods and breast cancer cell xenografts (Figures 1, 2 and 6; Table I), corroborate these previous results, indicating clearly that PRB is overexpressed to a much greater extent in aggressive, ER-negative, MDA-MB-231 breast cancer cells than in the less aggressive, ER-positive, MCF-7 cell line. Our findings are also in agreement with those obtained in a previous study showing that the ER-negative breast cancer cell lines, BT-20, MDA-MB-435-S and SK-BR-3 expressed higher levels of PBR than the ER-positive cell lines, T47-D, MCF-7 and BT-474 (31). Although the precise roles played by PBR in normal human breast tissue and in breast carcinoma remain unclear (49), a study in rats showed that the PBR distribution was limited to functionally specialized, acinar cells in normal breast tissue, but that it was ubiquitously present at higher concentrations in dimethylbenzanthracene-induced breast tumors (50). These findings imply that PBR may be involved in regulating the normal growth and function of rat mammary cells, but that its aberrant expression favors abnormal growth and function associated with the development of breast cancer.

Additionally, the immunocytochemistry and ligand-binding results presented here show, conclusively, that PBR is present at moderate-to-high levels in the human cell lines designated as colon carcinoma, HT-29, glioma, U-87 and prostate carcinoma, PC-3 (see Table I). In contrast, the hepatocarcinoma cell lines, Hep3B and HepG2, and the prostate carcinoma cell line, TSU, showed low PBR levels by immunocytochemistry, although TSU cells contained an appreciable amount of PBR mRNA (Figure 2).

The overexpression of PBR that was observed in colon carcinoma, HT-29, supports early results obtained in ligand-binding experiments with [$^3$H]PK 11195, showing a 3.2-fold increase in PBR density for human colon adenocarcinoma *versus* normal colonic tissue (23). More recent studies, that showed that the cell cycle-interfering and pro-apoptotic effects of PBR-specific ligands were associated with an inhibition of proliferation of colorectal cancer cells, indicated that PBR may play a significant role in colorectal cancer growth *via* its involvement in the regulation of both cell division and cell death (51).

With regard to our results showing the presence of a moderate amount of PBR in U-87 glioma cells, it might be noted that this human glioblastoma/astrocytoma cell line

had not previously been fully characterized with respect to PBR expression. However, high densities of specific PBR-binding sites have been observed on tumors that were derived from the related human glioma cell line ATCC HTB 14, U-87 MG, and grown in either nude mice or nude athymic rats (52). These results indicate that PBR is expressed in this cell line and that its presence might be associated with cancer-related processes. In this regard, studies performed by our group have previously demonstrated that positive correlations exist between increased PBR expression in MGM1 and MGM3 glioblastoma cell lines, their proliferative potential and the aggressiveness of their respective disease processes (41). Such findings signify that the presence of PBR in U-87 glioma cells might be related to the molecular mechanisms underlying the notorious aggressiveness of glioblastomas.

Our finding that the prostate carcinoma cell line, PC-3, contains a moderately elevated level of PBR is consistent with other findings that suggested that PBR might play a role in prostate cancer. At the human level, immunohistochemical analyses performed by members of our group indicated that the PBR levels of prostate carcinoma specimens were higher than those of corresponding non-tumor tissues and benign lesions (21), further substantiating that PBR overexpression is involved in prostate cancer.

Our findings that the hepatocarcinoma cell lines, Hep3B and HepG2, showed low PBR levels, as determined by immunocytochemistry (Figure 1, Table I), ligand-binding and QRT-PCR methods (Figure 2), are not in agreement with previous results of other workers obtained using tissue specimens. Other findings obtained in our laboratory have also not supported this observation of the up-regulation of PBR expression in human hepatocellular carcinomas (21).

*Expression of ERα and/or ERβ and correlation with PBR expression in various cancer cell lines.* The immunocytochemical findings presented here also show that most cancer cell lines studied possess both ERα and/or ERβ to various extents, except for TSU, HepG2 and Hep3B cells that were not positive for ERα. Also, correlations appear to exist between PBR expression and the expression of ERα and/or ERβ in certain cell lines that were studied. The presence of ER and its correlation with PBR expression in cancer cells may be significantly related to carcinogenesis and cancer progression since ER is a conditional transcription factor, belonging to the nuclear receptor superfamily whose interactions with specific ligands activate the transcription of specific genes involved in cell proliferation and/or differentiation.

It has been reported that both the MDA-MB-231 and MCF-7 breast cancer cell lines express low levels of ERβ (53). In contrast, our data revealed a preferential expression of ERβ by these two cell lines; MCF-7 cells contain much more ERβ than MDA-MB-231 cells. This finding is consistent with the observation that ERα and ERβ are not co-expressed in human breast tumors and cancer cell lines (53). In addition, this difference in ERβ expression by these two cell lines showed markedly different levels of aggressiveness and invasive potential, indicating that determination of ERβ levels could be useful as a prognostic, diagnostic and/or therapeutic marker for breast cancer.

Although a functional relationship between ER (or ER subtypes) and PBR has not yet been established, an ER-positive status is considered to be an independent predictor of survival in the clinical management of breast cancer. Studies conducted with flow cytometry have supported this concept by indicating that the more aggressive, ER-negative, breast cancer cell lines show higher PBR expression than the less aggressive, ER-positive, cells (31). In further support of this concept, studies from our laboratory have shown that ER is lacking in the highly aggressive MDA-MB-231 human breast cancer cells, which express high levels of PBR, proliferate at a high rate and are able to form tumors in nude mice (32).

With regard to colon cancer, information concerning the role of ERβ expression in colonic mucosa remains sketchy. However, five human colon cancer cell lines, HT-29, Colo320, LoVo, SW480 and HCT116, have been shown to express ERβ mRNA but not its ERα counterpart, consistent with previous reports that indicate that ERα and ERβ play different biological roles in colon cancer (54). Also, the localization and distribution of ERβ differs significantly between normal colon, adenomas and colorectal carcinoma (55). These observations are in agreement with our findings that showed that ERβ and PBR exhibit a high degree of co-expression in the colon carcinoma cell line, HT-29. With regard to prostate cancer, our data indicated that both PC-3 and TSU prostate carcinoma cells express ERβ, and that the latter cell line shows lower ERα expression. These findings relate to those of Maruyama *et al.* (56), who examined mRNA levels of ERα and ERβ in samples of benign prostatic hyperplasia and prostate carcinoma obtained by radical prostatectomy. Using a competitive PCR technique, they detected both ERα and ERβ mRNAs in all prostate cancer tissues examined, as well as in PC-3 and LNCaP cells, but at variable levels among specimens. Thus, they concluded that, although both ERα and ERβ mRNAs are expressed in prostate cancer, the expression of ER mRNA may not be related to cancer progression but may be negatively correlated with metastasis.

Regarding ER and the other examined cell lines, the results presented here indicate that both ER isoforms are expressed by U-87 glioma cells, with a slight predominance of the α-subtype, and represent the first assessment of ER status of this cell line, to the best of our knowledge. Furthermore, our findings demonstrated that ERβ is expressed at high levels in both HepG2 and Hep3B

hepatoma cells, and that both cell lines are also ERα-negative. The significance of these findings remains to be explained, but we note that estrogen reportedly increases the production of apolipoprotein A-1 in HepG2 cells by modulating gene expression (57).

*Anticancer effects of Ginkgo biloba extract (IPS 200): in vitro experiments (neoplastic cell lines).* The other major aim of the present study was to further define an anticancer effect of *Ginkgo biloba* extract, EGb 761 (used in its injectable form as IPS 200). In our previously published work, we had shown that treatment with IPS 200 inhibited the proliferation of human breast cancer cell lines *in vitro* and decreased tumor development (xenograft growth) initiated by MDA-MB-231 breast cancer cells in nude mice (30). The present report extends these findings in showing that treatment with this extract produces a concentration-dependent inhibition of glioma and hepatocarcinoma cell line proliferation as determined by BrdU cell proliferation and MTT cell viability assays, and a dose-dependent decrease in the growth of U-87 glioma cell line xenografts. The differential expression of PBR protein in the various cell lines examined was confirmed by Q-PCR methodology.

Our finding that the proliferation of normal human hepatocytes was unaltered by exposure for 72 h to increasing concentrations of IPS 200, that influenced hepatoma cell line proliferation, indicates that the extract's molecular mechanism of action might depend upon oncogenic up- or down-regulation of certain key molecules involved in neoplasm-associated pathways. Taken together, our results obtained with hepatoma cell lines and normal hepatocytes imply that, at least in this case, the antiproliferative effect of IPS 200 may be specific to cancer cells.

The biphasic effect obtained with HepG2 cells is of special interest relative to a very recent study that indicated that daidzein (a flavonoid phytoestrogen found in soybean) exerts a biphasic effect on the growth of the human colon cancer cell line, LoVo (58). Considering these results in light of the findings obtained in the present study, it appears possible that a tumor-suppressive effect of IPS 200 (in HepG2 cells) may also be mediated by cell cycle arrest at the G0/G1-phase and apoptosis. Furthermore, as daidzein is a flavonoid and since IPS 200 contains many flavonoid constituents, it is possible that the pro-apoptotic effect of IPS 200 observed in HepG2 cells may be mediated by a flavonoid constituent(s) of the extract. As IPS 200, used in the present study, is devoid of proanthocyanidins and flavonoid aglycones, its effect would probably involve flavonoid glycoside constituents. The opposite findings, obtained with Hep3B cells, may be indicative of an influence on cell proliferation by altering different molecular mechanisms (or additional non-apoptotic mechanisms) in this particular hepatoma cell line and/or that other active constituents of IPS 200 (*e.g.*, other flavonoid glycosides, ginkgolide B, or other terpene trilactones) are involved in mediating its pro-apoptotic effect.

*Anticancer effects of Ginkgo biloba extract (EGb 761): in vivo experiments (xenografts).* Regarding the effects of EGb 761 on the growth of tumor cell xenografts in nude mice, our previous study showed that 30-day treatments of nude mice bearing xenografts of highly aggressive, human MDA-MB-231 breast cancer cells that overexpress PBR with EGb 761 (50 mg/kg) resulted in a 35% decrease in tumor size, measured a month after terminating treatment (30). The results of the present study verified this finding and further showed that a more pronounced inhibition of *in vivo* tumor growth (*i.e.*, up to 80-90%) can be obtained using a modified experimental protocol consisting of treating the animals prior to inoculation. Earlier results were extended by the findings that tumor measurements could be confirmed with MRI images of the tumors, and that cells which had been pretreated with IPS 200 prior to their use as xenografts did not form tumors *in vivo*. As these results agree with our previous finding that MDA-MB-231 subclones that express low PBR levels did not form tumors when injected into SCID mice *in vivo* (32), it seems probable that EGb 761 acts *via* decreasing PBR expression to inhibit tumor growth of MDA-MB-231 cells.

The other results from the experiments with xenografts were not as clear. EGb 761 treatment inhibited U-87 glioma cells growth as xenografts for periods up to 45 days only, and this effect was not maintained after 50 days of treatment. The growth inhibitory effect was much less robust than that found with MDA-MB-231 cells. In contrast to results obtained with xenografts of breast cancer and glioma cells, and with the *in vitro* data obtained with Hep3B and HepG2 cells, EGb 761 treatment did not inhibit the growth of xenografts of these hepatoma cells in nude mice. This lack of a treatment effect could be related to the finding that these hepatoma cells contain very low levels of PBR.

## Conclusion

In summary, overexpression of the 18-kDa PBR represents a valid phenotypic marker of aggressive tumor growth, invasiveness and metastasis, or contributes to these cancer-related processes. Recent studies of human biopsy specimens have provided convincing evidence in support of this concept that PBR overexpression is a biomarker for such aggressive tumorigenesis in human patients afflicted with both breast cancer and colorectal cancer (23, 24). PBR overexpression could also serve as a phenotypic marker and prognostic indicator for certain other forms of cancer that are characterized by aggressive tumor growth and metastasis, such as brain cancer (gliomas) and, perhaps, prostate cancer,

ovarian carcinoma and hepatocellular carcinoma. On this basis, the overexpression of PBR could be useful as a means of identifying specific tissue targets for new anticancer drugs. The modulation of PBR expression that can be produced by treatment with EGb 761 or by specific exogenous compounds, such as the ginkgolide constituents of EGb 761 (30) or their derivatives (59), seems to represent a valid strategy for developing novel anticancer therapies.

Further understanding of PBR-mediated signal transduction mechanisms in malignant cells is required, especially as they relate to apoptosis. It is clear that PBR expression is implicated in the growth control of various tumor models and human cancer specimens, but the signaling pathways leading to cell cycle arrest remain largely unknown. It seems essential to consider that the activation of PBR stimulates multiple signal transduction mechanisms in various malignant cell lines, and that PBR-specific ligands influence cell proliferation by actions that appear to differ among various cell types.

With specific regard to PBR and apoptosis, it seems necessary to consider that PBR might be an element involved in the formation (and opening) of the mitochondrial permeability transition pore (MPTP), which is the major regulatory complex of apoptosis, and that opening of the MPTP, which leads to dissipation of the inner-mitochondrial transmembrane potential [deltapsi(m)], is an early event in apoptosis. On this basis, agents such as IPS 200, by targeting PBR overexpression in human cancer cells, that is, a "knockdown" approach, might not be expected to induce or enhance MPTP-regulated apoptosis.

Today, a standardized extract of *Ginkgo biloba* leaves, EGb 761, is used to treat patients afflicted with cerebrovascular and peripheral vascular insufficiency, disturbances in vigilance, short-term memory and other cognitive functions associated with aging and senility, dementias, neurosensory problems, macular degeneration and non-cognitive functions (mood changes) (35, 60). Further studies may reveal that this extract, acting by eliciting an anti-stress effect that involves a decrease in the overexpression of PBR (61, 62), could be useful in preventing or treating certain later events of the cancer process, such as invasiveness and metastasis, in various forms of cancer, a disease stage that increases an individual's daily stress (60, 63).

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