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PERIPHERAL BENZODIAZEPINE RECEPTOR: CHARACTERIZATION IN HUMAN T-LYMPHOMA JURKAT CELLS

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running title: Jurkat cells express Peripheral Benzodiazepine Receptor.

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Abbrevations: PBR, peripheral benzodiazepine receptor; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinolinecarboxamide; Ro5-4864, [7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2-*H*-1,4benzodiazepin-2-one].

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ABSTRACT

Peripheral Benzodiazepine Receptor (PBR) has been considered a promising drug target for cancer therapy and several ligands have been developed for this purpose. Human T-lymphoma Jurkat cells have been considered as lacking PBR and often utilized as negative control to prove the specificity of PBR ligands effects. Surprisingly, we evidenced PBR protein expression in this cell line by the means of Western blotting and immunocytochemistry assays using specific anti-PBR antibodies. PBR intracellular localization was evidenced in mitochondria and nuclei as demonstrated by confocal and elctron microscopy. The binding of both the [^{3}H]4'-chloro derivative of diazepam [^{3}H]Ro5-4864 and the isoquinoline carboxamide derivative [^{3}H]PK11195 evidenced a single class of binding sites with an unusual affinity constant (Kd) of 1.77 ± 0.30 μM and 2.20 ± 0.20 μM, respectively. The pharmacological profile of the classical ligands showed that PK11195 was the most potent inhibitor in the radioligand binding assays followed by Ro5-4864 and diazepam, whereas clonazepam, a specific ligand for the central-type receptor, showed a Ki > 1.0 X 10^{-4} M. By a combined strategy of RT-PCR and Southern blot experiments we succeeded in the isolation and cloning of the full-length Jurkat PBR cDNA, called *JuPBR*. *JuPBR* gene showed two single nucleotide polymorphisms resulting in the two substitutions Ala147→Thr and His162 →Arg of PBR aminoacidic sequence. In conclusion, for the first time we demonstrated PBR expression in Jurkat cells: the protein bound classical PBR ligands with micromolar affinity constants and presented a modified aminoacidic sequence consequently to detection of two gene polymorphisms.

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INTRODUCTION

The Peripheral Benzodiazepine Receptor (PBR), originally discovered as an alternative binding site for the benzodiazepine Diazepam (Valium), forms a unique class of receptors which are pharmacologically and functionally different from the central-type receptor (CBR) (Gavish et al., 1999). At subcellular level, it has been found to be primarily localized in mitochondria at the contact sites between the outer and the inner membrane, where it seems to take part in formation of a heteromeric receptor complex with other proteins including the 30 KDa Adenine Nucleotide Transporter (ANT) and the 34 KDa Voltage-Dependent Anion Channel (VDAC) (McEnery et al., 1992). This mitochondrial multiproteic complex has been suggested to constitute the Mitochondrial Permeability Transition (MPT) pore, whose extensive and prolonged opening can cause the dissipation of the transmembrane mitochondrial potential leading to the release of pro-apoptotic inter-membrane proteins. So in the machinery leading to apoptosis, MPT pore modulation has been suggested as a critical event in the regulation of processes underlying cellular survival/death (Casellas et al., 2002). Specifically, numerous findings have suggested that PBR ligands, including the benzodiazepine Ro5-4864 and the isoquinoline carboxamide derivative PK11195 (Braestrup and Squires, 1977; Le Fur et al., 1983), may act as potential therapeutic agents useful in the management of a large spectrum of diseases, including cancer, through the modulation of the MPT-pore activity (Chelli et al., 2005; Chelli et al., 2004; Decaudin et al., 2002; Hirsch et al., 1998).

The PBR full-length cDNA has been originally cloned from the rat adrenal (781 pb). Since then, human, bovine and mouse (Gavish et al., 1999) PBR cDNAs have been also cloned and their nucleotide sequences have been found to be significantly conserved. The human PBR gene promoter is known to contain Sp1-binding sites (Lin et al., 1993), which is

consistent with housekeeping gene family. Indeed, ubiquitous localization of the gene product has been observed in mammalian tissues (Gavish et al., 1999), including cells from the haematopoietic system (Canat et al., 1993; Carayon et al., 1996). Moreover, it has been demonstrated that PBR is abundantly expressed in a wide variety of malignant cells, even being the hallmark of cancerogenesis in some tissues, e.g. gliomas (Miettinen et al., 1995). PBR expression level has been also suggested to be a clinically relevant prognosis factor in cancer (Hardwick et al., 1999; Hardwick et al., 2001). Among lymphoma cell lines (Canat et al., 1993; Alexander et al., 1992) and among myeloid/lymphoid cells from leukaemia patients (Carayon et al., 1996) a wide range of PBR expression levels have been evidenced. A peculiar condition is represented by the cell line Jurkat, deriving from a patient affected by acute lymphoblastic leukemia, in which the presence of PBR mRNA and protein is still debated. Indeed, several authors have failed to detect PBR expression (Canat et al., 1993; Carayon et al., 1996; Hans et al., 2005). Nevertheless, in the Jurkat cell line PBR selective ligands have been demonstrated to induce the same effects observed in other PBR expressing cells (Zisterer et al., 2000). In addition, Decaudin and coworkers have succeeded in demonstrating detectable PBR mRNA levels in the Fas-resistant Jurkat T-cell line (Decaudin et al., 2002).

Within a general project in which we demonstrated the pro-apoptotic effects of classical and new synthesized PBR ligands in the C6 glioma cell line (Chelli et al., 2004; Chelli et al., 2005), we found that PBR ligands induced cell death also in Jurkat cells, utilized as control (unpublished data). These findings prompted us to investigate the expression and the cellular localization of PBR in this cell line. Here we report that Jurkat cells expressed PBR with unusual affinity constants for PBR ligands. Moreover, we demonstrated that the Jurkat PBR gene contains two point mutations previously reported in other human tumoral cell lines (Hardwick et al. 1999) and normal cells (Kurumaji et al. , 2001) .

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MATERIALS AND METHODS

Materials. The human T-lymphoma cell line Jurkat was obtained from Interlab Cell Line Collection (ICLC) (http://www.biotech.ist.unige.it/interlab/cldb.html). Rat C6 glioma cells were kindly gifted by Prof Damir Janigro, Cleveland Clinic Foundation, Cleveland, OH. Cell culture media and fetal bovine serum were from Bio-Whittaker. PBR polyclonal primary antibodies were obtained from Santa Cruz Biotechnology and Trevigen. Cytochrome c primary antibody and rhodamine-conjugate anti rabbit secondary antibody were purchased by Santa Cruz Biotechnology. HRP-conjugated secondary antibodies and non-fat dry milk were from Bio-Rad. FITC-conjugate anti mouse secondary antibody was from Molecular probes. [^{3}H]1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3 isoquinolinecarboxamide ([^{3}H]PK11195) (specific activity 83.5 Ci/mmol), [^{3}H]7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one ([^{3}H]Ro5-4864) (specific activity 83.5 Ci/mmol) were obtained from Perkin-Elmer, Life Science; PK11195, Ro5-4864, Diazepam, Clonazepam and DAPI were from RBI/Sigma. All other reagents were from standard commercial sources.

Cell culture and Membrane Preparations. The human T-lymphoma cell line Jurkat (T cell derived from a patient with acute lymphoblastic leukemia) was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 100U/ml penicillin, 200 μg/ml streptomycin in a humidified atmosphere of 95% air / 5% CO_2 at 37 °C. Nuclear membranes from Jurkat cells were prepared as previously described (Zamzami et al., 1996). Mitochondrial membranes from Jurkat cells were prepared as previously described with minor modifications (Miccoli et al., 1999). In brief, cells were collected by centrifugation at 1000 g for 5 min. The cell pellet was homogenized in 20 vol. of ice-cold buffer (0.32 M

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sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4, containing 160 μg/ml benzamidine, 200 μg /ml bacitracin, and 20 μg/ml trypsin inhibitor) by seventy strokes of a tight-fitting glass pestle in a Potter Elvehjem glass homogenizer and centrifuged at 1500 *g* for 5 min at 4°C. Supernatant was centrifuged at 12000 *g* for 10 min at 4°C. The resulting mitochondria pellet, suspended in 20 volume of 50 mM Tris/HCl, pH 7.4 buffer and homogenized by Ultraturrax, was centrifuged at 48000 *g* for 20 min at 4°C. Protein concentration was estimated by the method of Lowry et al. (Lowry et al., 1951), using BSA as standard.

Cloning and sequencing of PBR gene. The full length cDNA for the PBR gene was obtained from Jurkat cells RNA by RT-PCR. Total RNA was isolated using NucleoSpin Kit reagents, according to the manufacturer's instructions. The first strand cDNA was obtained from 1 μg of total RNA using SuperscriptTM II RNAse H-reverse transcriptase (Invitrogen) and oligo(dT)15-18. Amplification of the full-length PBR cDNA was obtained with the sequence-specific primers PBR forward, 5'-ACAGCAGCTGCAGCAGCC-3' and PBR reverse, 5'-ACGGCCACCACATCACAAG-3' designed on the basis of human PBR sequence (GenBank Accession number: BC001110) by using Advantage 2 PCR enzyme system (BD Biosciences). After initial denaturation of cDNA at 95°C for 2 min, 35 cycles of PCR reaction were performed as follow: at 95°C for 50 sec, 54°C for 45 sec, and 72°C for 1 min. The last cycle was extended to 10 min. at 72°C. Water instead of cDNA was amplified as a negative control. Some of the amplification reaction products were resolved by agarose gel electrophoresis, transferred to a nylon membrane (Hybond N+, Amersham Biosciences), and probed with an internal PBR specific DIG-labelled oligonucleotide (5'-CGGCTCCTACCTGGTCTG-3') according to the manufacturer's instructions (Roche Applied Science). Chemiluminescent detection was performed with a DIG luminescent detection kit (Roche Applied Science). The remaining PBR amplification reaction product

was resolved by electrophoresis, and the PCR product corresponding to the band hybridized by the internal DIG-labeled oligonucleotide was gel purified, and TA cloned in pGEMT-Easy vector (Promega). Several clones were sequenced by automated fluorescent cycle sequencing (ABI).

Western-blot analysis. Jurkat and lymphocyte cell protein samples (30 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for several hours in 5% blotting grade blocker non-fat dry milk and incubated over-night with a 1:1000 dilution of anti-PBR (FL-169, Santa Cruz Biotechnology: developed against the full length PBR of human origin) or 1:1000 of anti-PBR (Trevigen: developed against an internal sequence of PBR protein consisting of amino acids 71 to 88) in 1% blotting grade blocker non-fat dry milk. After several washes in TBS (10mM Tris-HCl, pH8, 150 mM NaCl) containing 0.05% Tween 20, antibodies binding was detected using anti-rabbit HRP-conjugated secondary antibodies (1:100000). Cross reactivity was detected using the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology Inc., USA). Protein concentration was assayed using a Bio-Rad assay system. In some cases, the specificity of the bands recognized by the anti-PBR antibody was demonstrated using preabsorbed antibody, prepared incubating overnight at 4°C the anti-PBR antibody (Trevigen) with a 5-fold (by weight) excess of recombinant full length PBR protein in a small volume of phosphate buffered saline (PBS). In parallel, the same amount of anti-PBR antibody was incubated overnight at 4°C in PBS alone.

Immunocytochemistry and fluorescence microscopy. Jurkat cells were collected by centrifugation, washed in PBS and fixed with 4% paraformaldehyde at room temperature for 30 minutes. A thin-layer cell preparation was obtained on poly-D-lysin coated glass slides by

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using a cytospin cytocentrifuge. Cells were blocked in PBS supplemented with 0.1% Triton X-100 and 0.5% BSA, and incubated for 1 h at room temperature in blocking solution containing 1:50 anti-PBR antibody (Santa Cruz Biotecnologies) and 1:50 anti-cytochrome c antibody (Santa Cruz Biotecnologies) for fluorescence microscopy, or with 1:200 anti-PBR antibody (Trevigen) and 20 μg/ml DAPI for confocal microscopy. After extensive washes in blocking solution, cells were incubated for 1 hour at room temperature with a 1:200 dilution of FITC-conjugate anti-mouse secondary antibody and 1:200 dilution of rhodamine-conjugate anti-rabbit secondary antibody in blocking solution for fluorescence microscopy, or with 1:200 dilution of rhodamine-conjugate anti-rabbit secondary antibody in blocking solution for confocal microscopy. Cells were washed in PBS and mounted for microscope analysis. The analysis were performed using an Axioplan (Zeiss) epifluorescence microscope and a TCS-SP laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany). A confocal series was collected every 0.15 μm. Each confocal image shown here corresponded to the middle image from a 66-section series**.** Negative controls were performed omitting the primary antibodies.

Immunogold Electron Microscopy. Jurkat cells were processed for EM immunocytochemistry as previously described (Trincavelli et al., 2002) using a rabbit polyclonal anti-PBR antibody (Trevigen) diluted 1:10 in PBS containing 0.1% gelatin and 0.5% bovine serum albumin. Negative controls were performed in the absence of anti-PBR antibody. Ultrathin sections were examined using a 100 SX electron microscope (Joel, Tokyo).

Radioligand Binding assays. For equilibrium binding parameters determination [^{3}H]PK 11195 and [^{3}H]Ro5-4864 were used. [^{3}H]PK 11195 (specific activity 0.44 Ci/mmol) binding

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assays were conducted in a final volume of 500 µl of 50 mM Tris/HCl, pH 7.4 buffer containing membranes (50 µg of protein) and 100 nM-10 µM [^{3}H]PK 11195. Non-specific binding was determined in the presence of 100 µM unlabelled PK11195. Samples were incubated in triplicate for 90 min at 0°C. In parallel, [^{3}H]PK1195 Scatchard analysis were carried out on human lymphocyte and C6 glioma cell membranes, prepared as described for Jurkat cells. C6 glioma cells were cultured as described by Chelli et al. (Chelli et al., 2005).

[^{3}H]Ro5-4864 (specific activity 0.42 Ci/mmol) binding assays were performed in a final volume of 500 µl of 118 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 15 mM Tris/HCl, pH 7.4 buffer containing membranes (50 µg of protein) and 100 nM-10 µM [^{3}H]Ro5-4864. Non-specific binding was determined in the presence of 100 µM unlabelled Ro5-4864. Samples were incubated in triplicate for 60 min at 4°C. Incubations were stopped by centrifugation of the samples for 2 min at 13,000 x *g*. Pellets were quickly washed three times with 1 ml of cold binding assay buffer. Radioactivity in the pellets was counted after the addition of 1 ml of 0.2 N NaOH and 0.19 ml of 1N Acetic acid. Bound radioactivity was measured in a Packard TopCount (Packard Instruments Company, Meriden, CT, U.S.A.) using scintillation liquid (65% counting efficiency). Incubation of samples was performed in safe-lock eppendorf (Eppendorf). In competition experiments, membranes (50 µg of protein) were incubated with 1.5 µM [^{3}H]PK11195 or 1.5 µM [^{3}H]Ro5-4864 in binding assay buffer and increasing concentrations of Ro5-4864, PK11195, diazepam, and clonazepam.

The equilibrium binding of [^{3}H]PK11195 was also measured using homologous binding displacement, whose theoretical validity has been discussed elsewhere (Rovati, 1998; Bylund and Murrin, 2000). Jurkat membranes (150 µg) were incubated with a fixed concentration of labeled ligand (0.5 µM; specific activity 1.33 Ci/mmol) and increasing concentrations of unlabeled ligand up to 100 µM. Binding equilibrium was reached following a 90 min incubation at 0°C. Samples were filtered rapidly under vacuum through GF/C glass fiber

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filters to separate bound and unbound ligand. After being washed four times with 3 ml of assay buffer, radioactivity trapped on the filter was measured by liquid scintillation counting. Non specific binding was measured in the presence of excess PK11195 (100 μM).

Data Analysis. Saturation experiments of [^{3}H]PK11195 and [^{3}H]Ro5-4864 were analyzed with the EBDA and the LIGAND computer programs (Biosoft-Elsevier, Cambridge, England) (Munson and Rodbard, 1980; Mc-Pherson, 1985). Single- and multiple-site models were statistically compared to determine the best fit, and differences between models were tested by comparing the residual variance using a partial F test and a significance level of $P< 0.05$. Displacement curves were analyzed and fitted using the Graph-Pad Prism (Version 3.0) computer program (GraphPad Software). Derived IC_{50} values obtained from displacement curves were converted to Ki values by the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Values represent the means ± SEM of at least three independent experiments. Saturation data were also fitted with the GraphPad Prism program.

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RESULTS

Detection of PBR transcript in Jurkat cells. By using a combined strategy of RT-PCR and Southern blot experiments, we succeeded for the first time in the isolation of a full-length cDNA clone, called *JuPBR*, encoding for PBR in Jurkat cells (GenBank accession number: AY998017; Fig. 1A). *JuPBR* is 593 base pair long and contains an open reading frame (ORF) encoding for a 169 amino acid putative PBR protein, with an estimated molecular weight of 18 KDa. Sequence comparison indicates that *JuPBR* shares high nucleotide identity with the other previously isolated human PBR genes. However, *JuPBR* shows a point mutation at base 526, which changes Ala (ACG) to Thr (GCG), and a point mutation at base 572, which changes Arg (CGT) to His (CAT (Fig. 1B). The same variations were reported in the PBR gene isolated from other human tumoral cell lines (Hardwick et al., 1999) and, with high frequency, in genomic DNA of normal cells (Kurumaji et al., 2001).

Detection and subcellular localization of PBR protein in Jurkat cells. Using commercially available PBR polyclonal antibodies, PBR protein was identified in Jurkat cells.

Western blot analysis on total proteins obtained from Jurkat and lymphocytes cells, as control, were performed. Two different commercial polyclonal anti-PBR antibodies recognised the typical 18 kDa PBR protein in both Jurkat and lymphocyte cells. In addition, a 36 kDa molecular mass protein was also detected, suggesting the presence of polymers of PBR in these cells (Fig.2A). Immunoreactivities were specific as demonstrated by preabsorbing the anti-PBR antibody with the recombinant full length PBR protein (Fig.2 B).

Immunocytochemistry analysis revealed that PBR protein appeared distributed all over the cytoplasm and at nuclear level (Fig. 3). The use of the specific mitochondrial marker anti-

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cytochrome c antibody (Fig. 3B) evidenced that the pattern of the sub-cellular distribution of mitochondria partially overlapped the pattern of PBR staining (Fig. 3C). No signal was detected in negative controls carried out in the same experimental conditions except that no primary antibody was added. By performing immunohistochemistry using anti-PBR antibody in combination with the specific nuclear dye DAPI and subsequent confocal microscopy, nuclear localization of PBR was demonstrated (Fig. 3D-F). Mitochondrial and nuclear PBR localization was also confirmed by immunogold electron microscopy (Fig. 3G).

[^{3}H]PK11195 binding characterization. As first step we employed Jurkat cell mitochondrial membranes for radioligand binding assays using nanomolar concentrations of [^{3}H]PK11195 as described in several cellular systems (Alexander et al., 1992; Canat et al., 1993, Hardwick et al., 1999). In these conditions no [^{3}H]PK11195 specific binding was detected. The presence of PBR protein evidenced using the specific anti-PBR antibody, as described above, prompted us to study a potential lower [^{3}H]PK11195 binding affinity constant in this cell line. Binding at higher ligand concentrations (>100 nM) was investigated in Jurkat cell membranes using radioligand diluted with non radioactive ligand for lowering its specific activity (Bylund and Murrin, 2000). Moreover, bound and unbound radiolabeled ligands were separated by centrifugation procedure according to low affinity receptor binding assays (Kd about 10^{-7} M or 10^{-6} M) (Bennett and Yamamura, 1985). The conditions necessary for the [^{3}H]PK11195 binding were initially studied to optimise yield. In mitochondrial membrane preparations the specific binding of [^{3}H]PK11195 increased linearly with increasing protein concentrations in the range 20-150 μg protein (data not shown). In order to study the saturability of specific [^{3}H]PK11195 binding to Jurkat mitochondrial membranes aliquots of membrane preparations were incubated with increasing concentrations of [^{3}H]PK11195 (100 nM-10 μM). Specific binding of [^{3}H]PK11195 was saturable, whereas non specific [^{3}H]PK11195 binding increased linearly (data not shown). Scatchard analysis of the saturation data yielded a straight line plot,

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compatible with the existence of a single population of binding sites (F-test, $P < 0.05$) (Fig. 4). In mitochondrial membranes the dissociation constant (Kd) for [^{3}H]PK11195 was 2.20 ± 0.20 μM and the maximum amount of specifically bound ligand (Bmax) was 706 ± 75 pmol/mg of protein.

The low affinity [^{3}H]PK11195 binding site was investigated using 'cold' saturation experiments too. Scatchard analysis of these data indicated the existence of one class of binding sites as the one-site model yielded a significantly better fit (F-test, $P < 0.05$) than fitting experimental data to a two-site model. The best fit estimated of the Kd value (9.90 ± 1 μM) for this low affinity [^{3}H]PK11195 binding site was comparable to those obtained by 'hot' saturation experiments (Fig. 5).

In nuclear membranes Scatchard analysis of [^{3}H]PK11195 saturation data evidenced a single population of binding sites with a Kd value of 1.10 ± 0.10 μM, comparable to that obtained in mitochondrial membranes. The Bmax value (27 ± 3 pmol/mg of protein) resulted about 26 times lower than that found in mitochondria extracts, suggesting a significant lesser expression level of PBR protein in this cell compartment. Competition experiments with specific and selective PBR ligands were performed in order to test the pharmacological properties of binding sites for [^{3}H]PK11195 in Jurkat cell membranes. Displacement studies of [^{3}H]PK11195 specifically bound to Jurkat cell membranes were performed by incubating aliquots of membranes with [^{3}H]PK11195 and 9-10 different concentrations of a ligand, as described in experimental procedures. Analysis of displacement data fitted using the Graph-Pad Prism computer program revealed that both the selective PBR ligands PK11195 and Ro5-4864 and the specific PBR ligand diazepam displaced specific [^{3}H]PK11195 binding in a concentration-dependent manner. Ki values of ligands are shown in Table 1. The binding of radioligand was effectively displaced by PBR ligands PK11195, Ro5-4864 and diazepam at

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micromolar concentrations, in contrast clonazepam, a selective ligand for the central benzodiazepine receptor, was not effective up to 10^{-4} M.

In order to clarify whether the unusual [^{3}H]PK11195 low affinity binding site evidenced in Jurkat cells was also present in cells showing [^{3}H]PK11195 high affinity, we performed on human lymphocyte and C6 glioma mitochondrial membranes [^{3}H]PK11195 saturation analysis. For this purpose we used the same experimental conditions described for Jurkat cells. Data reported in Table 2 showed [^{3}H]PK11195 binding parameters were similar in all analyzed cells.

[^{3}H]Ro5-4864 binding characterization. Radioligand binding assays, performed as previously described (Mak and Barnes, 1989), with nanomolar concentration of [^{3}H]Ro5-4864 did not evidence specific binding of radioligands in Jurkat cell membranes. Thus we used the above mentioned experimental approach for extending the [^{3}H]Ro5-4864 concentrations range. Binding assays demonstrated specific, saturable binding of [^{3}H]Ro5-4864 to human Jurkat cell membranes. Scatchard analysis of [^{3}H]Ro5-4864 saturation curve revealed a single class of binding sites with a dissociation constant (Kd) of 1.77 ± 0.3 μM and a maximal binding capacity (Bmax) of 200 ± 21 pmol/mg of protein. Scatchard plots of the binding data were linear, consistent with the interpretation that a homogeneous population of binding sites was present in membranes of Jurkat cells (Fig. 6).

The pharmacological specificity of the binding of [^{3}H[Ro5-4864 was studied in the Jurkat cell membranes, using various displacing drugs. Displacement curves were obtained in the presence of 1.5 μM [^{3}H]Ro5-4864: the estimated Ki for each competing ligand were calculated from these data and are presented in Table 1. In Jurkat cell membrane preparations, the tritiated ligand was effectively displaced by PK11195 and Ro5-4864. Diazepam was less

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effective, while clonazepam, at the concentration of 100 μM**,** was able to displace 65% of [^{3}H]Ro5-4864 binding from its binding site.

DISCUSSION

In the present report we demonstrate that PBR is expressed in the human T-lymphoma Jurkat cell line and that Jurkat PBR protein presents benzodiazepine and isoquinoline carboxamide ligand low affinity binding sites.

In Jurkat cells PBR expression is still debated. By using RT-PCR no PBR transcript has been evidenced by Canat et al. (1993) and Hans et al. (2005). On the contrary, some evidences have demonstrated that PBR mRNA is expressed in human Fas-resistant T-cell line Jurkat (Decaudin et al., 2002). By using a combined strategy of RT-PCR followed by Southern blot hybridization, we succeeded in the isolation and cloning of the Jurkat PBR full-length cDNA (*JuPBR*). PBR mRNA detection required highly sensitive experimental strategy, suggesting that Jurkat cells expressed PBR mRNA at lower level than other tumoral cell lines. Compared to the human Gene Bank PBR sequence, *JuPBR* showed two point mutations resulting in the change of alanine to threonine (Ala147Thr) and of histidine to arginine (His162Arg) localized in the fifth putative transmembrane region and in the carboxyl terminal region of the PBR, respectively. The missense variants (Ala147Thr; His162Arg) have been found in other tumoral cell lines, such as MDA-231 (Hardwick et al., 1999) and, with high frequency, in genomic DNA of normal cells (Kurumaji et al., 2001), suggesting that they can be the consequence of single nucleotide polymorphisms of human PBR gene. Presently, it is not known whether these mutations have any significant effect on PBR structure and function. Molecular modeling of the receptor indicates that the first residue (Ala147Thr) lies within the colesterol entry region of the receptor. However, this mutation does not seem to alter the

ability of cholesterol to move through PBR because cholesterol is incorporated into MDA-231 nuclei (Hardwick et al., 1999). The second mutation (His162Arg) is a neutral mutation given that both histidine and arginine are basic aminoacids, and is unlikely to have any significant effect on PBR function.

The PBR amino acidic substitution His162Arg may explain why some authors have not detected protein in Jurkat cells by using the monoclonal anti-PBR 8D7 antibody (Carayon et al., 1996; Hans et al., 2005), which recognized the human PBR C-terminal region that contains His 162 instead of Arg.

In our conditions, PBR expression was evidenced in Jurkat cells by using two different polyclonal anti-PBR antibodies that specifically recognized the 18 KDa reactive band. Interestingly, the data obtained by Western blot showed two different bands at 18 and 36 KDa, accordingly as previously reported in other cell lines (Delavoie et al., 2003; Corsi et al., 2005). Immunolocalization experiments provide a further demonstration of the presence of PBR protein in Jurkat cells. We evidenced PBR in mitochondria in line with other studies (Gavish et al., 1999) and in nuclei as previously demonstrated in other tumoral cell lines (Hardwick et al., 1999; Brown, 2000; Delavoie, 2003; Corsi et al., 2005).

Emerging data have indicated that different PBR sub-cellular localization is frequently associated with cell malignancy. In particular, in the human aggressive phenotype of breast tumor the PBR protein is primarily expressed in and around the nucleus (Hardwick et al., 1999). A peculiar association between PBR nuclear localization and cell malignance has also been evidenced in human gliomas (Brown et al., 2000). Further studies are necessary to understand the association of Jurkat PBR subcellular localization and cell malignancy.

Jurkat cell line membranes were employed in quantitative radioligand binding assays using the specific and selective PBR radioligands [^{3}H]PK11195 and [^{3}H]Ro5-4864. According to literature data (Canat et al., 1993) no specific [^{3}H]PK11195 binding was

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detected using nanomolar radioligand concentrations. However, the presence of receptor protein revealed by immunoblotting, TEM and confocal microscopy analysis prompted us to investigate the possibility of more low radioligand equilibrium binding parameters.

For this aim, we span in a higher radioligand concentration range (100 nM-10 μM) according to the general guidelines for receptor saturation experiments (Bylund and Murrin, 2000). Scatchard analysis of the saturation [^{3}H]PK11195 binding data yielded a Kd value about 200 times lower than affinity constants derived from saturation studies performed on membranes of human lymphocytes and other leukemia cells (Canat et al., 1993).

A comparable low affinity [^{3}H]PK11195 Kd value was also evidenced by using 'cold' saturation experiments.

PBR nanomolar affinity constants have been detected in leukemia cell lines as CCRF-CEM, Raji and K562, whereas no binding has been evidenced using until to 20 nM PBR radioligand concentration in Molt-4B, suggesting that the absence of the high-affinity PK11195 binding parameter may not be exclusive of the Jurkat cell line (Alexander et al., 1992). Using micromolar radioligand concentration in this paper we demonstrated the presence of a low affinity PK11195 binding site in normal immune system cells like lymphocytes and in a cell line of solid tumours like C6 glioma cells, in which a high affinity PK11195 binding site has been reported (Alexander et al., 1992; Zisterer et al., 1998). These data suggested that the low affinity of PK11195 binding sites evidenced in Jurkat cells corresponded to a second population of binding sites, usually present in other cells.

In the Jurkat cell line competition curve data with specific and selective PBR ligands PK11195, or Ro5-4864, or diazepam or the CBR specific ligand clonazepam allowed us to derive Ki values for specific PBR ligands (listed in Table 1) while clonazepam was not effective up to 10^{-4} M. Although the binding affinity observed for each of these ligands ranged over several orders of magnitude compared to that estimated in human lymphocytes

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and in other human cell types (Parola et al., 1993), the characteristic rank order of ligand binding potency (PK11195> Ro5-4864 > diazepam >>> clonazepam) was typical of PBR.

In the Jurkat cell line [^{3}H]Ro5-4864 did not show specific binding at radioligand nanomolar concentrations. Scatchard analysis of [^{3}H]Ro5-4864 saturation curve, performed with experimental approches as described above for [^{3}H]PK11195 binding assay, showed the presence of a single population of low-affinity binding sites with a dissociation constant of 1.77 ± 0.3 μM. Unlike PK11195 binding properties, that usually demonstrated a high-affinity binding in tissues from many species, Ro5-4864 binding varied depending on species and tissue. In contrast to rodent PBR (Kd 1-10 nM), binding of Ro5-4864 to PBR of most human tissues occurred only with low affinity (Kd about 1 μM) (Awad and Gavish, 1991), except for human lymphocytes and platelets and the human breast cancer cell line BT-20 (Kd 8.5 nM) (Beinlich et al., 1999). In accordance with our data, in HL-60 acute promyelocytic leukemia cell membranes a second class of benzodiazepine binding sites with lower binding affinity has been detected (Kd of the benzodiazepine drug [^{3}H]diazepam 28.6 μM, Bmax 199 pmol/mg protein) (Ishiguro et al., 1987). In Jurkat cell membranes competition experiments of [^{3}H]Ro5-4864 binding with various ligands displayed the typical PBR order of potency: PK11195>Ro5-4864> diazepam>>>clonazepam.

The first conclusion we can draw is that Jurkat cells contain PBR which presents a micromolar affinity constant for classical ligands and these cells cannot be used as control in an assay in which high ligand concentrations are used. The hypothesis that PBR classic high affinity binding site could be changed in Jurkat cell as result of the two aminoacidic substitutions has been discarded since human breast cancer cell lines MCF-7 and MD-231, with the same missense variants (Hardwich et al., 1999), bind the isoquinoline carboxamide derivative with high affinity ([^{3}H]PK11195 Kd respectively, of 2.9 nM (Carmel et al., 1999) and of 7.8 nM (Hardwich et al., 1999)). Since PBR is associated with other proteins to

constitute the mitochondrial pore, it can be hypothesized that in these tumoral cell lines a different PBR-proteins stechiometric ratio might exist, resulting in a conformational receptor change for high affinity binding sites. The presence of the PBR low affinity binding site can justify micromolar PBR ligand concentration required to obtain a functional effect mediated by PBR, as apoptosis induction or facilitation.

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Footnotes

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LEGENDS FOR FIGURES

Fig. 1. PBR cDNA isolation in Jurkat cells. (A) Southern blot of PBR amplification products. Lane 1: Jurkat cDNA amplified with PBR specific primers. An autoradiographic band of about 600 bp was visible. Lane 2: Negative RT-PCR control. (B) Comparison of the deduced amino acid sequence of Jurkat PBR (*JuPBR*) with those of other human PBR protein isoforms: MCF-7, Human breast cancer cell line accession No. AAC31173.1; MDA-231, Human breast cancer cell line (Harwick et al., 1999); *JuPBR*,; wtPBR, Mammalian Gene Collection (MGC) Program accession No. AAH01110. Asterisks indicate the amino acid substitutions.

Fig. 2. Western blot analysis of PBR protein. (A) PBR polyclonal antibody (anti-PBR) (Trevigen) recognize two proteins of about 18 and 36 KDa in both Jurkat and lymphocyte cells. (B) Specificity of the immunoreactivities seen in (A) examined by preabsorbing anti-PBR with the recombinant full length PBR protein.

Fig. 3. Subcellular distribution of PBR protein in Jurkat cells. (A) Distribution of PBR as visualized by means of an anti-PBR antibody by using fluorescent microscopy. (B) Sub-cellular distribution of mitochondria as revealed by means of an anti-cytochrome c antibody and fluorescent microscopy. (C) Merged panels A and B demonstrate that although PBR protein is accumulated in mitochondria, a widespread signal is also observable in other subcellular compartments. Scale bar (A-C): 10 μm. (D-F) Confocal fluorescence images obtained from immunocytochemistry preparations. (D) Distribution of PBR as revealed by use of anti-PBR antibody; (E) The section depicted in (D) stained with the nuclear dye DAPI; (F) Merged panels D and E demonstrate the nuclear localization of JuPBR. Scale bar (D-F):

5 μm. (G) Electron microscope immunocytochemistry. Large clusters of gold particles (arrows) are visible on the nucleus and cytoplasm of Jurkat cells. m, mitochondrion; N: nucleus. Scale bar: 1,5 μm.

Fig. 4. Saturation curve and Scatchard plot (inset) of specific [^{3}H]PK11195 binding to mitochondrial Jurkat cell membranes. Mitochondrial membranes were incubated with increasing concentrations of [^{3}H]PK11195 ranging from 100 nM to 10 μM. Non specific binding was measured in the presence of 100 μM PK11195. Data are from a single experiment carried out in triplicate. The Kd and Bmax values were respectively of 2.05 μM and 662 pmol/mg of protein. Three such experiments yielded similar results.

Fig. 5. Homologous displacement of [^{3}H]PK11195 in Jurkat cell mitochondria membranes. Bound radioactivity is expressed as a percentage of specific binding in the absence of competitor. EBDA/LIGAND analysis indicated a single class of binding sites with an estimated Kd= 9.90 ± 1 μM. Data points represent the means (± SEM) of triplicate determinations pooled from three independent experiments.

Fig. 6. Saturation curve and Scatchard plot (inset) of specific [^{3}H]Ro5-4864 binding to mitochondrial Jurkat cell membranes. Mitochondrial membranes were incubated with increasing concentrations of [^{3}H]Ro5-4864 ranging from 100 nM to 10 μM. Non specific binding was measured in the presence of 100 μM R05-4864. Data are from a single experiment carried out in triplicate. The Kd and Bmax values were respectively of 1.05 μM and 210 pmol/mg of protein. Three such experiments yielded similar results.

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TABLE 1

Pharmacological characterization of [^{3}H]PK11195 and [^{3}H]Ro5-4864 binding to Jurkat cell line membranes.

	Ki(μM) or % of inhibition	
Compound	vs [^{3}H]PK11195	vs [^{3}H]Ro5-4864
PK11195	1.90 ± 0.20	3.68 ± 0.35
Ro5-4864	32.3 ± 4.00	8.74 ± 0.79
Diazepam	79.0 ± 8.00	18.4 ± 1.90
Clonazepam	no dispacement up to 10^{-4} M	35 ± 4 % at 10^{-4} M

Jurkat cells membranes were incubated with 1.5 μM [^{3}H]PK11195 or [^{3}H]Ro5-4864 and increasing drugs concentrations. The IC_{50} values were calculated and the Ki inhibitory constant derived (Cheng and Prusoff, 1973). Each value represents the mean ± SEM of three experiments, each performed in triplicate.

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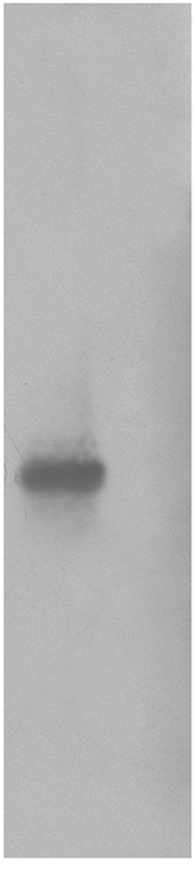
TABLE 2

[^{3}H]PK11195 binding parameters in Jurkat cells, lymphocytes and C6 glioma cells.

cell type	Kd (μM)	Bmax (pmol/mg protein)
Jurkat	2.20 ± 0.20	706 ± 75
Lymphocytes	1.80 ± 0.20	726 ± 83
C6	1.98 ± 0.20	391 ± 42

Lymphocytes, Jurkat and C6 cell membranes were incubated with increasing concentrations of [^{3}H]PK11195 (100 nM-10 μM, specific activity 0.44 Ci/mmol), as described in methods section. Each value represents the mean ± SEM of three experiments, each performed in triplicate.

A



B

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                    10        20        30        40        50        60
                     |         |         |         |         |         |
MCF7        -------------------------HGEGLRWYAGLQKPSWHPPHWVLGPVWGTLYSAM
MDA-231     -------------------------HGEGLRWYAGLQKPSWHPPHWVLGPVWGTLYSAM
JuPBR       MAPPWVPAMGFTLAPSLGCFVGSRFVHGEGLRWYAGLQKPSWHPPHWVLGPVWGTLYSAM
wtPBR       MAPPWVPAMGFTLAPSLGCFVGSRFVHGEGLRWYAGLQKPSWHPPHWVLGPVWGTLYSAM

                    70        80        90       100       110       120
                     |         |         |         |         |         |
MCF7        GYGSYLVWKELGGFTEKAVVPLGLYTGQLALNWAWPPIFFGARQMGWALVDLLLVSGAAA
MDA-231     GYGSYLVWKELGGFTEKAVVPLGLYTGQLALNWAWPPIFFGARQMGWALVDLLLVSGAAA
JuPBR       GYGSYLVWKELGGFTEKAVVPLGLYTGQLALNWAWPPIFFGARQMGWALVDLLLVSGAAA
wtPBR       GYGSYLVWKELGGFTEKAVVPLGLYTGQLALNWAWPPIFFGARQMGWALVDLLLVSGAAA

                   130       140       150       160
                     |         |         |         |
MCF7        ATTVAWYQVSPLAARLLYPYLAWLAFTTTLNYCVWRDNHGWRGGRRLPE
MDA-231     ATTVAWYQVSPLAARLLYPYLAWLAFTTTLNYCVWRDNHGWRGGRRLPE
JuPBR       ATTVAWYQVSPLAARLLYPYLAWLAFTTTLNYCVWRDNHGWRGGRRLPE
wtPBR       ATTVAWYQVSPLAARLLYPYLAWLAFATTLNYCVWRDNHGWHGGRRLPE
                                      *                   *
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