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Synthesis and Biology of a 7-Nitro-2,1,3-benzoxadiazol-4-yl Derivative of 2-Phenylindole-3-acetamide: A Fluorescent Probe for the Peripheral-Type Benzodiazepine Receptor

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Benzodiazepines are among the most highly prescribed drugs due to their pharmacological action of relieving anxiety mediated through modulating the activity of γ-aminobutyric acid receptors in the central nervous system.[1] The peripheral-type benzodiazepine receptor (PBR) is another class of binding sites for benzodiazepines distinct from the aforementioned neurotransmitter receptors. The PBR was originally discovered because it binds the benzodiazepine diazepam with relatively high affinity.[2] Further studies demonstrated that in addition to the benzodiazepines, PBR binds with high affinity other classes of organic compounds, such as isoquinolines,[3] imidazopyridines,[4] indole derivatives,[5] and pyrrolobenzoxazepines.[6] In addition to these drug ligands, the polypeptide diazepam binding inhibitor,[7] porphyrins,[8] and benzodiazepine-like molecules[9] have been identified as endogenous PBR ligands. The PBR, although present in all tissues examined, was found to occur in particularly high density in steroid-producing tissues, such as adrenal, testis, ovary, placenta, and brain tissues.[10] In these cells, the PBR was found to be localized primarily in the mitochondria and more specifically in the outer mitochondrial membrane.[11] Nonmitochondrial locations of PBR have also been described in various tissues.[12] An 18 kDa isoquinoline-binding protein was identified as PBR, cloned, and expressed.[13]

On the basis of the pharmacological effects of PBR ligands and the tissue-specific cellular and subcellular localization of the 18 kDa isoquinoline binding protein, the PBR has been shown to be involved in numerous functions including steroid biosynthesis, mitochondrial respiration, heme biosynthesis, calcium channel modulation, cell proliferation and differentiation, and immunomodulation.[10,12] From these functions the role of PBR in steroidogenesis is fairly well established. Using adrenal,[14] testis Leydig,[15] ovarian granulosa,[16] placenta,[17] and brain glia[18] cells, it has been demonstrated that the PBR is a functional component of the steroidogenic machinery mediating cholesterol delivery from the outer to the inner mitochondrial membrane.[19] Further studies demonstrated that pharmacologically induced reduction of adrenal PBR levels *in vivo* resulted in decreased circulating glucocorticoid levels.[20] In addition, targeted disruption of the PBR gene in steroidogenic cells resulted in inhibition of cholesterol transport to the inner mitochondrial membrane and arrest of steroid biosynthesis.[21] Thus, the PBR provides an attractive target molecule for the development of compounds which may be used for the regulation of steroid synthesis in the periphery and the central nervous system and for the regulation of the abovementioned PBR-dependent functions.

The studies described above on the localization and characterization of the structure and function of the PBR in fixed cells or isolated subcellular fractions[3−20] were performed using high-affinity ligands, antisera developed against the entire molecule or fragments of the 18 kDa PBR protein, and cDNA probes. Considering the problems associated with antiserum specificity and sensitivity as well as with subcellular fractionation, one could argue that the results obtained may not represent the actual situation in a living cell. We report herein the synthesis, characterization, and biologic

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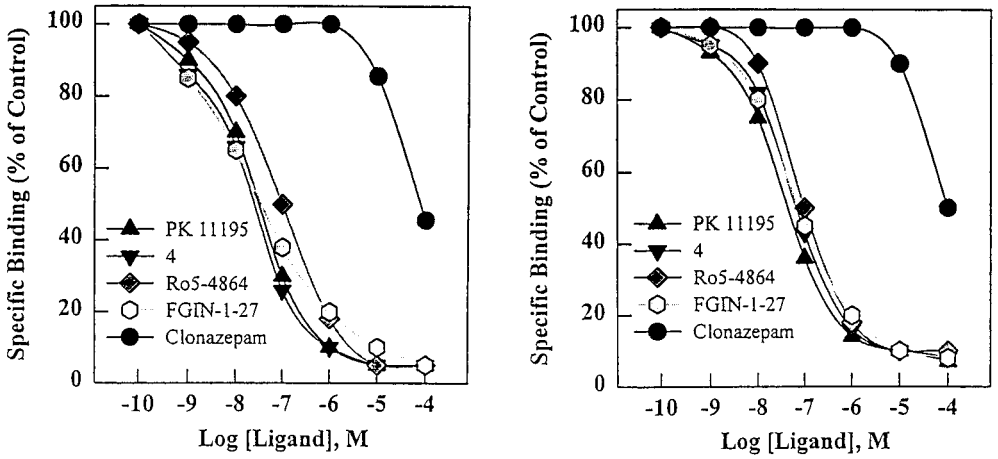
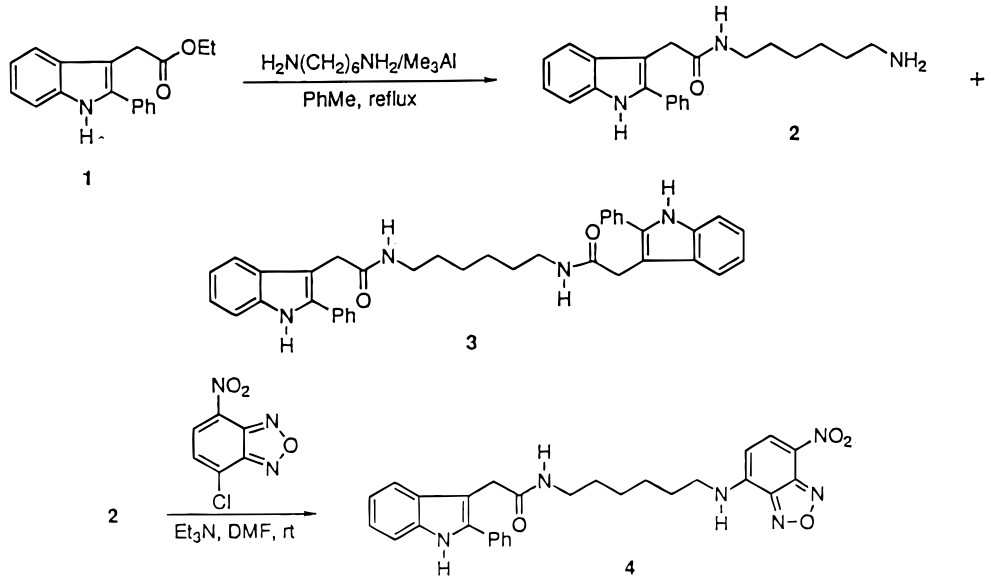


Figure 1. NBD displaces radiolabeled PK 11195. Specific binding of [^{3}H]PK 11195 to MA-10 mouse tumor Leydig (left) and C6-2B rat glioma (right) cells was measured in the presence of 1 nM [^{3}H]PK 11195 with or without the indicated concentrations of **4**, PK 11195, FGIN-1-27, Ro5-4864, and clonazepam.

Scheme 1. Synthesis of Fluorescent Probe **4**



activity of the fluorescent high-affinity PBR ligand (**4**) which provides a tool allowing the direct imaging by fluorescence microscopy of the 18 kDa PBR protein in living cells. Moreover, this probe may be used to access and visualize the PBR in tissues under conditions where antisera cannot penetrate.

Chemistry. The fluorescent derivative **4** was obtained in two simple steps from the previously described[22] ethyl ester **1** (Scheme 1). First, the spacer group was attached by reaction of **1** in boiling toluene with the aluminum amide formed from hexamethylenediamine.[23] A small amount of the 2:1 reaction product **3** is inevitably formed despite the use of an excess of the diamine, but this material is readily separable from the desired product **2** by chromatography on silica gel, and **2** is isolated in 46% yield. In the second step, the remaining amine nitrogen of **2** is alkylated with 4-chloro-7-nitrobenzofurazan (NBD chloride)[24] in DMF to produce the target compound **4** in 52% yield as an orange-colored foam. Using CH_2Cl_2 as the solvent, **4** is also obtained, but only in 24% yield.

Biology. Cells. The MA-10 mouse tumor Leydig and the rat C6-2B rat glioma cell lines were used. We have previously characterized both cell types in regard to their PBR expression, localization, and ability to synthesize steroids.[15,18]

Radioligand Binding Assays. Binding assays were performed as we previously described using [^{3}H]PK 11195 as the radiolabeled ligand.[15,18] The IC_{50} for the compounds tested was determined using the LIGAND program.[25] Figure 1 shows that **4** displaced [^{3}H]PK 11195 with the same efficacy as the isoquinoline PK 11195, the benzodiazepine Ro5-4864, and the 2-aryl-3-indoleacetamide prototype FGIN-1-27. The IC_{50} for all these compounds was approximately 10 nM for both cell types. It should be noted that, at nanomolar and low micromolar concentrations, clonazepam, a benzodiazepine with high affinity for the $GABA_A$ receptor, did not displace [^{3}H]PK 11195. These results demonstrate that the addition of the fluorigenic 7-nitro-2,1,3-benzoxadiazol-4-yl group did not affect the affinity of the indoleacetamide to bind to the PBR. In addition, several other derivatives of 2-phenylindole-3-acetamide were tested for their ability to displace radiolabeled ligands from the $GABA_A$, $GABA_B$, dopamine, 5-HT_1, 5-HT_2, glutamate, opiate, σ, cholecystokinin, β-adrenergic, and cannabinoid receptors.[5] None of the derivatives tested showed such activity. Thus, it is unlikely that the addition of the

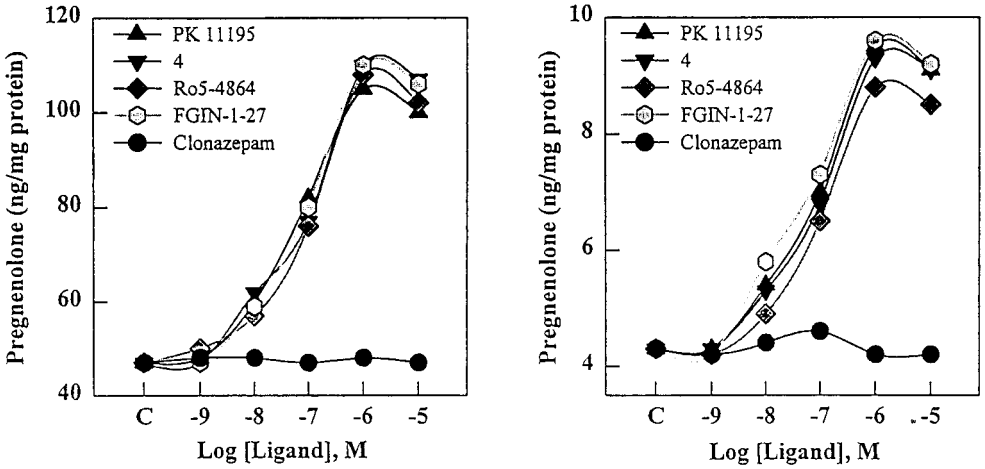


Figure 2. Effect of **4** on mitochondrial steroidogenesis. **4** and the indicated compounds were tested for their ability to stimulate MA-10 Leydig (left) and C6-2B (right) glioma mitochondrial ability to synthesize pregnenolone. Mitochondria were isolated and incubated with the indicated concentrations of different PBR ligands as previously described.[7,15] At the end of the incubation, pregnenolone was extracted and measured by radioimmunoassay.[15] The results shown are the means ($n = 4$) from two independent experiments. Standard deviations are not given for the simplicity of the figure and were <15% of the mean value.

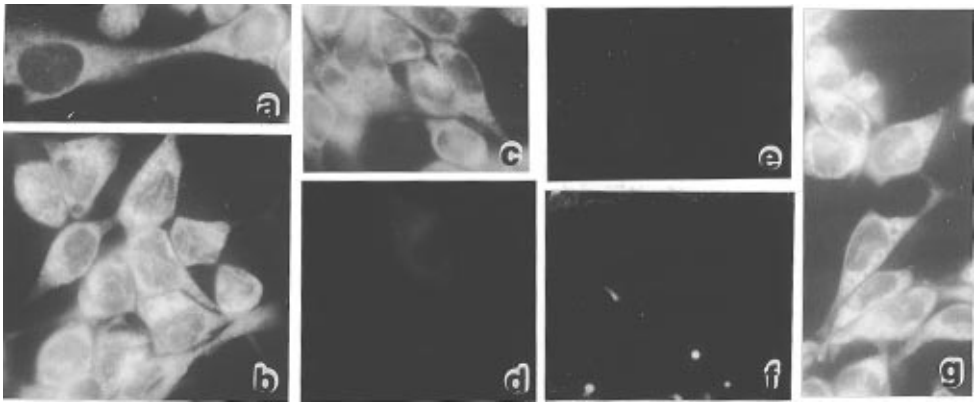


Figure 3. Fluorescent labeling studies of the PBR in MA-10 Leydig cells. MA-10 cells were cultured on coverslips and incubated with **4** (1 μM) for 45 min at 37 °C in the absence (a, b, c) or presence of 100 μM of PK 11195 (d), FGIN-27 (e), Ro5-4864 (f), or clonazepam (g). At the end of the incubation time, the cells were washed, and PBR was localized by fluorescence microscopy. Magnification ×217 (reproduced at 75% of original size).

fluorigenic group will confer to compound **4** novel binding properties for a receptor other than PBR.

Functional Studies. One of the functions of PBR ligands is the activation of cholesterol transport to mitochondria and subsequent steroid formation.[14−20] Most of the PBR ligands have been characterized for their steroidogenesis-inducing activity. Figure 2 shows that compound **4** stimulated mitochondrial pregnenolone formation by both testis Leydig and brain glial cells with the same potency and efficacy as the isoquinoline PK 11195, the benzodiazepine Ro5-4864, and the 2-aryl-3-indoleacetamide prototype FGIN-1-27. As previously shown,[15,18] clonazepam did not affect mitochondrial steroidogenesis.

Fluorescence Microscopy. Glass coverslips were incubated for at least 2 h with fetal bovine serum in order to allow serum components, such as fibronectin, to coat the surface, thus allowing better attachment of the cells. Cells were plated on the coverslips and cultured in media supplemented with serum as previously described for each cell line.[15,18] When cells reached the required confluency, they were washed twice with sterile phosphate-buffered saline (PBS) and incubated for 45 min with 1 μM compound **4** with or without the indicated competing PBR ligands at 100 μM concentrations. At the end of the incubation period, the coverslips were washed with PBS and examined by fluorescence microscopy using an Olympus BH-2 fluorescence microscope. The incubation time of 45 min was established based on preliminary experiments where we observed that intracellular fluorescence was visible after 20 min incubation time and reached the saturation point at around 40 min. The concentration of **4** used was also determined based on preliminary dose−response studies. These experiments showed that intracellular fluorescent labeling could be seen using 10 and 100 nM **4** but that a concentration of 1 μM **4** was required to obtain a consistent and reproducible labeling. The value of 1 μM is about 30 times higher than the IC_{50} obtained from data in Figure 1, suggesting that compound **4** might be labeling a site other than PBR. However, higher amounts of compound **4** may be needed because of the experimental protocol used. It is important to note that the binding studies were performed in cells collected from the dishes, centrifuged, distributed to assay tubes, and incubated at 4 °C whereas fluorescent studies were performed directly on the living cells in culture. Figure 3 shows fluorescent labeling of MA-10 Leydig cell PBR. The labeling was notably cytoplasmic, and when using a low concentration of **4** we could see fluorescent cytoplasmic spots. These data are in agreement with the observations that in MA-10 Leydig cells,

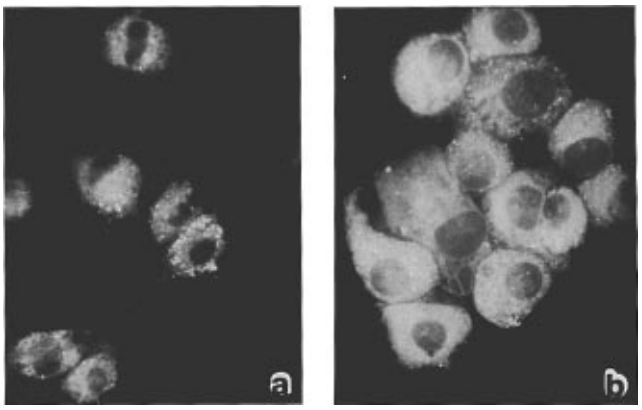


Figure 4. Fluorescent labeling studies of the PBR in C6 rat glioma cell line. C6 cells were cultured on coverslips and incubated with **4** (1 μM) for 45 min at 37 °C (a, b). At the end of the incubation time, the cells were washed, and the PBR was localized by fluorescence microscopy. Magnification ×217 (reproduced at 70% of original size).

which have high numbers of mitochondria, 90% of the PBR is on the mitochondria as shown in our previous studies using anti-PBR antisera and confocal microscopy.[26] The fluorescent staining was completely displaced by a 100-fold excess of the isoquinoline PK 11195, the benzodiazepine Ro5-4864, and the 2-aryl-3-indoleacetamide prototype FGIN-1-27, further demonstrating the specificity of the interaction **4** with the PBR.

The possibility that some of the fluorescent labeling seen may be due to nonspecific trapping of the compound in the lysosomal fraction of the cells should be also considered. Taking into account the subcellular fractionation studies performed with radiolabeled ligands,[10−12] the colocalization of PBR with mitochodrial markers and enzymes,[10−12] the high number of mitochondria in these cells, and the observation that both the fluorescent and the radioactive labeling were displaced by unlabeled ligands, a nonspecific lysosomal labeling is highly unlikely to occur.[27]

Figure 4 shows fluorescent labeling of the PBR of the C6-2B glioma cell line. This cell line was also previously characterized for its PBR content.[18] The fluorescent cytoplasmic spots suggest a mitochondrial localization of the receptor. Because these cells contain smaller amounts of the PBR (25 pmol/mg of protein)[18] than the MA-10 cells (56 pmol/mg of protein),[15] the spots are easier to distinguish.

Conclusions. We report herein the synthesis of a fluorescent derivative related to the 2-aryl-3-indoleacetamide prototype FGIN-1-27. This derivative (i) retained full ability to displace the radiolabeled isoquinoline from the isoquinoline binding site on the 18 kDa PBR protein, (ii) retained full steroidogenesis-activating property when added to mitochondria of both testis Leydig and brain glial cells, and (iii) specifically labeled the intracellular localization of the PBR in a manner consistent with the previously reported localization of the PBR in these cell types.[15,18] We believe that this compound will be a useful tool to probe the localization and function of the PBR in different tissues.[28]

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Supporting Information Available: Preparation and spectroscopic and analytical data for compound **4** (2 pages). Ordering information is given on any current masthead page.

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(28) Compound **4** can be obtained from Alexis Biochemicals, San Diego, CA.

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