New Fluorescent 2-Phenylindolglyoxylamide Derivatives as Probes Targeting the Peripheral-Type Benzodiazepine Receptor: Design, Synthesis, and Biological Evaluation

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Fluorescent ligands for the peripheral-type benzodiazepine receptor (PBR) featuring the 7-nitrobenz-2-oxa-1,3-diazol-4-yl moiety were synthesized, based on *N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamides, a potent, selective class of PBR ligands previously described by us. All the new ligands are moderately to highly potent at the PBR, with a complete selectivity over the central benzodiazepine receptor. Results from fluorescence microscopy showed that these probes specifically labeled the PBR at the mitochondrial level in C6 glioma cells.

Introduction

The peripheral-type benzodiazepine receptor (PBR) is an 18 KDa protein located on the outer mitochondrial membrane,[1] which is directly or indirectly implicated in numerous cellular functions, including calcium homeostasis, cell proliferation, regulation of mitochondrial permeability transition, apoptosis, steroid biosynthesis, and immunomodulation.[2,3] Although the acronym PBR is widely accepted in the scientific community, Papadopoulos et al. [4] have proposed the revised nomenclature "translocator protein (18 KDa)" or "TSPO" for this protein, which represents more accurately its structure, subcellular role, and putative molecular functions.

Clinical investigations have revealed that PBR basal expression is up-regulated in a number of human pathologies, including a variety of tumors, and PBR expression appears to be related to the tumor malignancy grade. Furthermore, significantly enhanced PBR expression has been observed in neurodegenerative diseases (Huntington's and Alzheimer's diseases and multiple sclerosis), as well as in various forms of brain injury and inflammation.[3] In vivo studies have shown that, following neuronal injury, increased PBR expression is primarily localized on activated microglial cells, whereas undetectable expression has been reported in resting microglia, suggesting that the up-regulation is a consequence of cell activation.[5] All these findings have stimulated the development of new radiolabeled ligands targeting PBR as powerful tools to image and measure the expression level of this protein in both humans and animals.[5−8]

Fluorescently labeled ligands represent a safer, faster, and less-expensive alternative to radioligands in probing the ligand−receptor complex. As a further advantage, fluorescent probes can be displaced from their binding sites of a target protein by nonfluorescent ligands, allowing the identification of the sites recognized by the ligands. Fluorescent agents with high specificity and attractive spectroscopic properties are therefore needed in the field of biomedical research.[9]

In this paper, we report the synthesis, the physical characterization, and the biological application of new PBR fluorescent probes with the general formula **II**, designed on the basis of *N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamides **I**,[10] bearing the fluorescent moiety linked to the *N*-alkyl chain (Figure 1). According to our pharmacophore/topological model (Figure 1),[10] the fluorescent group of derivatives **II** should interact with one of the lipophilic pockets termed L3 and L4 in the receptor binding cleft, thus maintaining the high affinity of the parent ligand **I**. The length of the spacer alkyl chain was modified to introduce some flexibility that would favor self-adaptation of the ligands into the receptor binding site.

We selected the well-known 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group as the fluorophore, because its small size does not generally affect affinity of the parent ligand. Moreover, NBD-containing compounds typically exhibit a low quantum yield in an aqueous solution, but they become highly fluorescent in nonpolar solvents or when bound to membranes or to hydrophobic clefts in proteins.

Chemistry

The procedure for the obtainment of derivatives **1**−**6** represents an improved modification of those used by Kozikowski et al.[11] and Chen et al.[12] for the preparation of analogous PBR fluorescent probes (Scheme 1). The appropriate mono *N*-Boc-protected diamines were condensed with NBD−Cl to obtain compounds **7**−**9**. Deprotection of the Boc group furnished the hydrochloride salts of amino derivatives **10**−**12**, which were directly condensed with 2-phenylindolylglyoxylyl chlorides **13** and **14**,[10] yielding the desired fluorescent compounds **1**−**6** (Supporting Information).

Biological Results

The affinity of fluorescent probes **1**−**6** at the PBR was determined by competition experiments against [^{3}H]PK 11195 performed on rat kidney mitochondrial membranes (Table 1). All the newly synthesized ligands are moderately to highly

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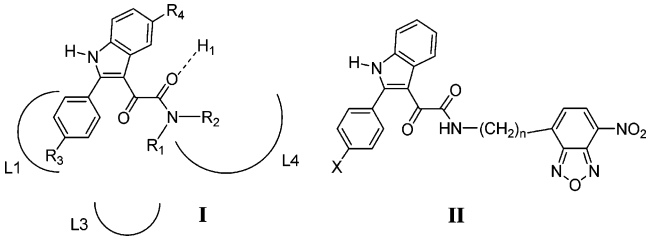
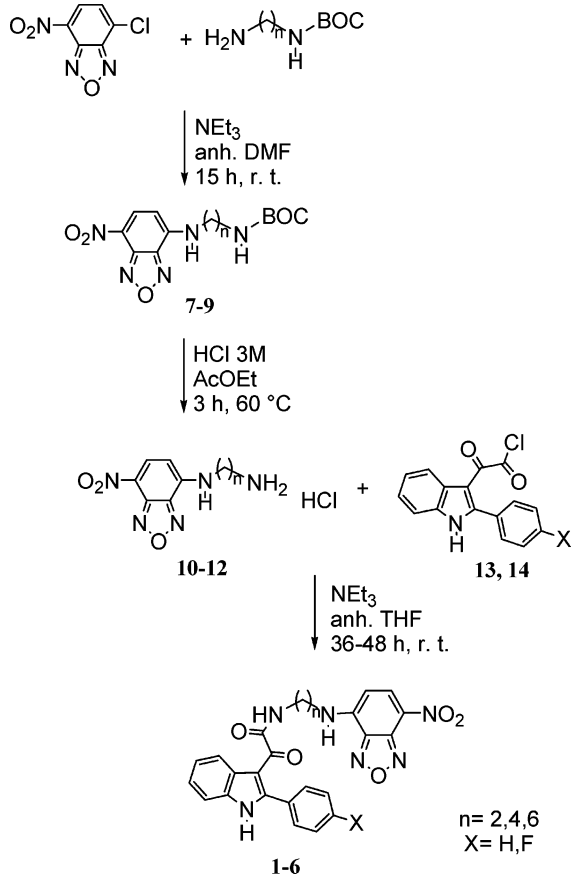


Figure 1. 2-Phenylindolglyoxylamide derivatives **I**[10] in the pharmacophore/topological model and general formula of fluorescent probes **II**.

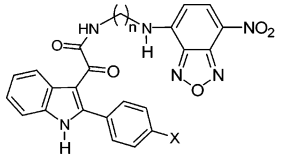
Scheme 1. Synthesis of Fluorescent Probes **1**−**6**

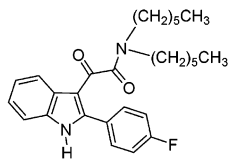


potent at the PBR, with K_i values varying from the submicromolar to the nanomolar range. Compounds **1** and **2**, featuring a short ethylene chain, showed a moderate affinity, whereas probes **3**−**6** with chains of 4 or 6 methylene units displayed nanomolar affinities, probably resulting from a successful accommodation of their side chains into the L3 or L4 lipophilic pocket of the receptor binding site. All compounds **1**−**6** are *N*-monosubstituted glyoxylamides unable to occupy either L3 or L4 subsites. Such a partial occupation could account for the lower affinity of compound **6** with respect to its lead **Ip**.[10] In agreement with the structure−activity relationship data of *N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamides **I**,[10] the presence of an electron-withdrawing fluorine in the 4′-position determines a slight gain in affinity, confirming a possible involvement of the 2-phenyl ring in a π-stacking interaction with an electron-rich aromatic ring within the L1 pocket.

Radioligand binding assays were also performed on C6 glioma cell mitochondrial membranes. In these tests, all the derivatives **1**−**6** demonstrated affinity values similar to those observed in experiments on rat kidney membranes (data not shown).

Table 1. Receptor Binding Affinity of Compounds **1**−**6** for PBR





no.	*n*	X	K_i[a] (nM)
1	2	H	994 ± 95
2	2	F	835 ± 80
3	4	H	42 ± 4.00
4	4	F	23 ± 2.00
5	6	H	17 ± 1.50
6	6	F	12 ± 1.00
Ip[b]			0.37 ± 0.13
PK 11195			9.3 ± 0.50
Ro 5-4864			23 ± 3.10
Alpidem			0.5−7

[a] The concentration of tested compounds that inhibited [^{3}H]PK 11195 binding at rat kidney mitochondrial membranes (IC_{50}) by 50% was determined with six concentrations of the displacers, each performed in triplicate. K_i values are the means ± SEM of three determinations. [b] Data taken from ref 10.

Finally, the PBR/central benzodiazepine receptor (BzR) selectivity of our ligands was evaluated by binding studies carried out on rat brain membranes using [^{3}H]flumazenil as the radioligand. All compounds showed a high selectivity for the PBR over the BzR, as they inhibited the binding of [^{3}H]-flumazenil by less than 10% at the fixed 10 μM concentration (data not reported).

The most potent fluorescent probe, compound **6**, was selected in experiments aimed at labeling PBR in C6 glioma cells. For this purpose, cells were incubated with different concentrations of **6** in cell culture medium and were subsequently processed for microscopy analysis.[11] An intracellular fluorescent labeling was detectable using compound **6** at 1 μM, and a consistent, reproducible staining was obtained by applying the fluorescent ligand at 10 μM. In these conditions, cells appeared to be uniformly stained in the cytoplasm (Figure 2A,B), and no significant differences were observed with higher ligand concentrations. These data are in agreement with our previous observations that in C6 glioma cells the PBR is mainly expressed on mitochondria.[13] To test the specificity of the fluorescent pattern, cells were preincubated with different concentrations of the nonfluorescent lead **Ip**, or PK 11195, and were then stained with 10 μM **6**. The fluorescent staining was significantly displaced by both **Ip** and PK 11195 at 10 μM concentration (Figure 2C,D). The specificity of the staining was further demonstrated by the complete displacement of **6** staining observed when the cells were preincubated with 50 μM PK 11195 (Figure 2E).

Spectroscopic Properties of Fluorescent Ligand 6. The UV (see Supporting Information) and the emission spectra of **6** were measured to investigate the spectroscopic properties of the ligand and how these were affected by the environment. Compound **6**, dissolved in dimethyl sulfoxide (DMSO), was diluted at a final concentration of 10 μM in different assay solutions from aqueous to 60% v/v dioxane−water phosphate-buffered saline (PBS). The percentage of DMSO did not exceed 1% of the final assay volume solution.

Figure 3 shows the emission spectra of compound **6**. As expected, the decrease in polarity of the environment (from aqueous buffer to 60% dioxane in PBS) induced a 9-fold

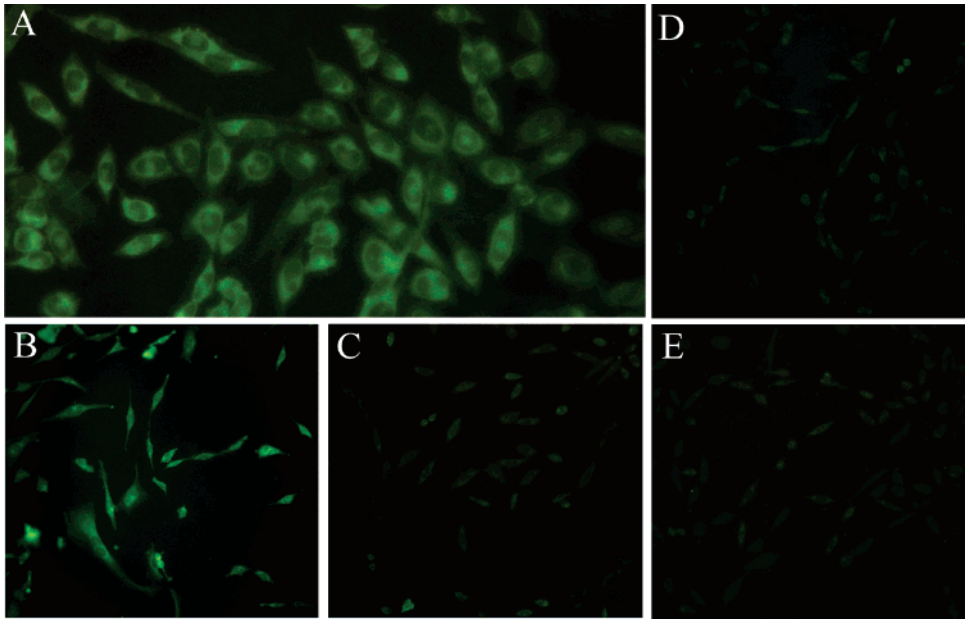


Figure 2. Rat C6 glioma cells stained with 10 μM **6** (A,B); cells stained with 10 μM **6** in the presence of 10 μM PK 11195 (C); cells stained with 10 μM **6** in the presence of 10 μM **Ip** (D); and cells stained with 10 μM **6** in the presence of 50 μM PK 11195 (E).

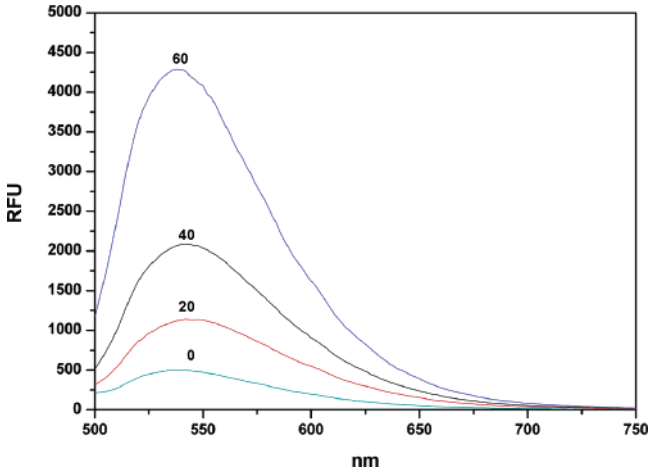


Figure 3. The influence of the polarity of the medium on the fluorescence of compound **6** was investigated by the addition of dioxane (indicated in % v/v) in PBS measured at a λ_{max} of 470 nm.

increase in quantum yield. This phenomenon was accompanied by a blue-shift of the emission maximum from 534 to 540 nm.

Conclusions

Compound **6** can be considered to be a new fluorescent probe eligible for investigating the PBR. In view of its chemical and optical properties, **6** has a great potential as a fluorophore to replace PBR radioligands in studies on the localization, expression level, structure and physiological/pathological roles of this receptor.

Experimental Section

Chemistry. General directions are in the Supporting Information.

General Procedure for the Synthesis of *N*-{[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]alkyl}-2-(4-substituted)phenylindol-3-ylglyoxylamide Derivatives 1−6. A solution of NEt_3 (0.58 mL, 4.2 mmol) in 3.0 mL of anhydrous THF was added dropwise to a stirred suspension, at 0 °C under a nitrogen atmosphere, of the indolylglyoxyl chloride **13** and **14**[10] (2.1 mmol) and the amine hydrochloride **10**−**12** (1.9 mmol) in 25.0 mL of the same solvent. The reaction mixture was left to warm to room temperature, stirred for 36−48 h (TLC analysis) and then filtered. The precipitate was triturated with a saturated $NaHCO_3$ aqueous solution, washed with water, and collected to give a first portion of crude product. The organic solution was evaporated to dryness, and the residue was treated with saturated $NaHCO_3$ aqueous solution, washed with water, and collected to yield an additional amount of crude product. All products **1**−**6** were purified by washing with cold diethyl ether, as the recrystallization process led to partial decomposition of the products (Supporting Information).

Fluorescent Labeling of C6 Glioma Cells. For fluorescence microscopy observation, rat C6 glioma cells were cultured on 96-well plates in Dulbecco's Modified Eagle's medium, as previously described.[13] Cells were grown to subconfluence and incubated with different concentrations (0.5, 1, 5, 10, 20 μM) of the fluorescent PBR ligand **6** in cell culture medium for 30 min under 5% CO_2 at 37 °C. In parallel, some samples were preincubated for 30 min with increasing concentrations (ranging from 10 μM to 100 μM) of PK 11195 or **Ip** and were then stained by adding compound **6** at a final concentration of 10 μM. The cells were then quickly washed with PBS, fixed in 4% paraformaldehyde, and subsequently observed under the fluorescence microscope Axiovert-25 (Zeiss). Excitation was at 458 nm, and fluorescence detection was with a band-pass filter of 516−565 nm. Images of cells were acquired with ACT-2U 1.2 (Nikon).

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Supporting Information Available: Ultraviolet absorption spectra of compound **6**; general chemistry directions; synthesis of compounds **7**−**12**; Tables 1 and 2, including yields, physical, and spectral data of compounds **1**−**12**; analytical data of compounds **1**−**12**; and general biological directions, including [^{3}H]PK 11195 binding to rat kidney and C6 glioma cell mitochondria membranes and [^{3}H]flumazenil binding to rat cerebral cortex membranes. This material is available free of charge via the Internet at http://pubs.acs.org.

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