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Novel Irreversible Fluorescent Probes Targeting the 18 kDa Translocator Protein: Synthesis and Biological Characterization

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The 18 kDa translocator protein (TSPO) is a mitochondrial protein whose basal density is altered in several diseases, with the result that the evaluation of its expression levels by means of molecular imaging techniques represents a promising diagnostic approach. Experimental procedures using a labeled ligand often cause loss of the bound probe, and consequently high affinity ligands covalently binding the receptor protein are needed to overcome this problem. We have previously described a series of *N*,*N*-dialkyl-(2-phenylindol-3-yl)glyoxylamides as potent and selective TSPO ligands. Starting from these derivatives, we designed novel TSPO irreversible ligands bearing an electrophilic isothiocyanato group (**7**, **8**), together with an irreversible NBD-fluorescent probe (**18**). The TSPO affinity of the new irreversible ligands was measured on rat tissue homogenates by [^{3}H]Ro 5-4864 radiobinding kinetic assays, all compounds showing high affinities for the target protein. Further biological characterization of the fluorescent irreversible TSPO probe **18** was carried out by using fluorescent spectroscopy in human glioma cells.

Introduction

The 18 kDa translocator protein (TSPO[a]) is an evolutionarily well-conserved protein consisting of 169 amino acids,[1,2] which is mainly located at the contact sites between the outer and inner mitochondrial membranes.[3,4] TSPO is also expressed at low levels in other subcellular compartments such as plasma membranes and the nuclear fraction of cells.[5] Photolabeling studies indicated that this receptor is strictly associated in a trimeric complex with the 32 kDa voltage-dependent anion channel (VDAC) and the 30 kDa adenine nucleotide translocase (ANT),[6] thus forming the mitochondrial permeability transition pore (MPTP).

TSPO is involved in a variety of biological processes including cholesterol transport, steroidogenesis, calcium homeostasis, lipid metabolism, mitochondrial oxidation, cell growth and differentiation, apoptosis induction, and regulation of immune functions.[7,8] Recent evidence suggests that on glial cells, TSPO may regulate the biosynthesis of neurosteroids, leading to the hypothesis of its potential role as a key determinant for the treatment of neuropathological conditions.[9−12]

TSPO basal expression is up-regulated in a number of human pathologies, including a variety of tumors and neuropathologies, such as gliomas and neurodegenerative disorders (Huntington's and Alzheimer's diseases), as well as various forms of brain injury and inflammation.[13−16] Furthermore, changes in TSPO receptor levels have been found in anxiety and mood disorders.[12,17−19]

The evaluation of TSPO expression and distribution, by means of specific molecular probes may thus represent a promising diagnostic marker of the above-listed diseases.[20,21] Typically, a probe is a derivative of a known ligand, designed to allow visualization of the target protein while maintaining the affinity and selectivity of the parent compound. A number of radiolabeled ligands targeting TSPO have been developed as powerful tools to image and measure the expression levels of this protein in both humans and animals.[22−28] On the other hand, fluorescent ligands represent a safer, faster, and less expensive alternative to radioligands for usage in biomedical research.[29−31] In this field, we have recently developed novel highly potent and selective fluorescent probes targeting TSPO with the general formula **II**, designed as derivatives of the highly potent *N*,*N*-dialkyl-2-phenylindol-3-ylglyoxylamide ligands **I**,[32,33] featuring the fluorescent moiety linked to the *N*-alkyl chain (see Chart 1).[31] For these compounds, 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.[31]

Tissue experimental procedures using a labeled ligand often cause the alteration of the chemical equilibrium and the subsequent loss of the bound fluorescent ligand. To overcome this problem, some high affinity ligands which bind covalently to the receptor protein have been developed.[34]

The aim of this work was to design, synthesize, and biologically characterize a series of 2-phenylindol-3-ylglyoxylamides and to test their validity as new TSPO probes. All of these compounds are characterized by the presence of a

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[a]Abbreviations: TSPO, 18 kDa translocator protein; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PBS, phosphate-buffered saline; SEM, standard error of mean.

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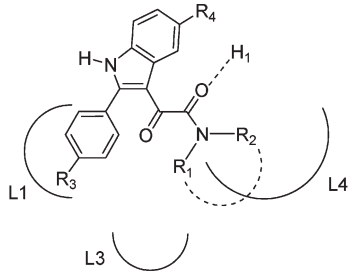
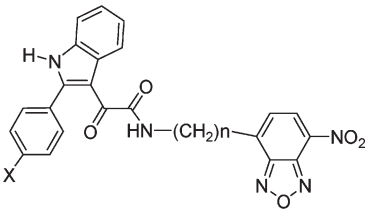
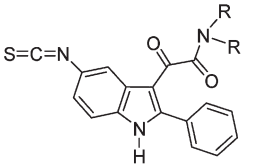
Chart 1. Structures of Known (**I** and **II**) and Novel (**7**, **8**, and **18**) TSPO Ligands



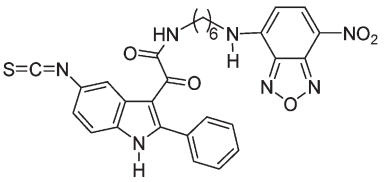
N,*N*-Dialkyl-2-phenylindolylglyoxylamides **I**



II



7 R = *n*-propyl
8 R = *n*-hexyl



18

chemoreactive isothiocyanate group, able to bind the receptor protein irreversibly and covalently (compounds **7**−**8**, Chart 1). Moreover, compound **18** (Chart 1), featuring the NBD-fluorescent moiety, was synthesized to develop an irreversible fluorescent probe. The presence of these two devices (a chemoreactive group and a fluorescent chromophore) on a single molecule may offer a multiplicity of advantages, both in protein purification/characterization and in protein cell visualization/density determination. To the best of our knowledge, no TSPO molecular probes with these characteristics have been described to date.

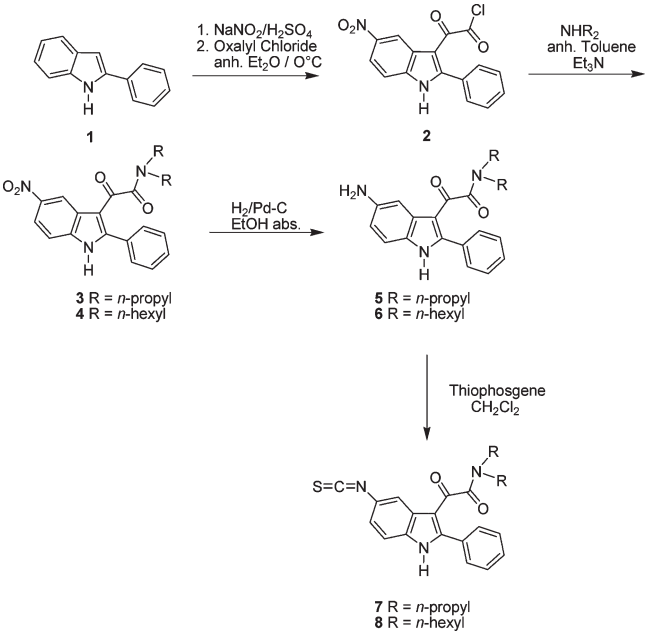
For the rational design of the irreversible derivatives **7**, **8**, and **18**, we take into consideration our pharmacophore/topological model (Chart 1)[31−33] made up of three lipophilic pockets L1, L3, and L4, occupied by the 2-phenyl group, and the arylalkyl substituents on the amide nitrogen, respectively, and an H-bond donor group interacting with the amide carbonyl group. In this view, the position 5 of the indole nucleus seemed suitable to support the chemoreactive group without affecting the affinity or the selectivity for TSPO. Among the possible chemoreactive moieties, the isothiocyanate function has proven to be extremely versatile as an electrophilic moiety for irreversible ligands. It can easily be synthesized from a primary amino group; furthermore, its high reactivity toward amino and sulfhydryl groups, along with its low reactivity toward water and other hydroxyl functions, accounts for its successful applications in receptor studies.[35−40]

In this paper, we describe the synthesis, the spectroscopic, and the biological characterization of novel irreversible 2-phenylindol-3-ylglyoxylamide TSPO ligands **7**, **8**, and **18**.

Chemistry

The target *N*,*N*-dialkyl-5-isothiocyanato-2-phenylindol-3-ylglyoxylamides **7** and **8** were synthesized through the 5-nitro-2-phenylindol-3-ylglyoxylamides **3** and **4**, which in turn were obtained following the experimental procedures described in our previous work (Scheme 1).[33] The 5-nitro-2-phenylindol-3-ylglyoxylamides **3** and **4** were catalytically hydrogenated over

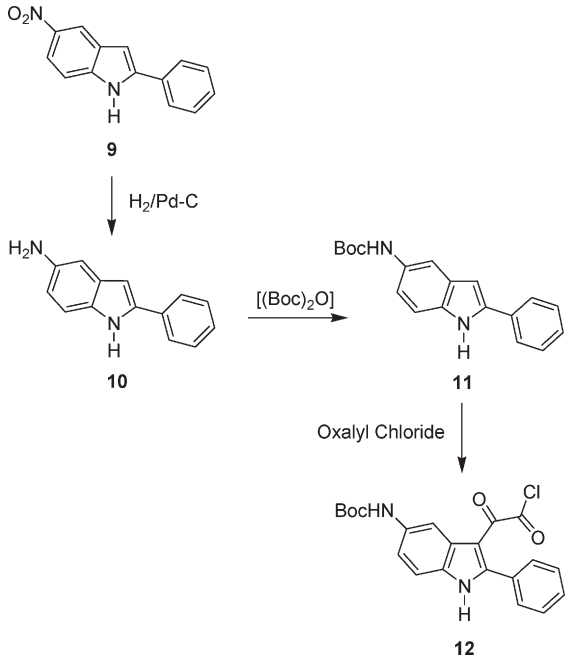
Scheme 1. Synthesis of Irreversible Ligands **7** and **8**



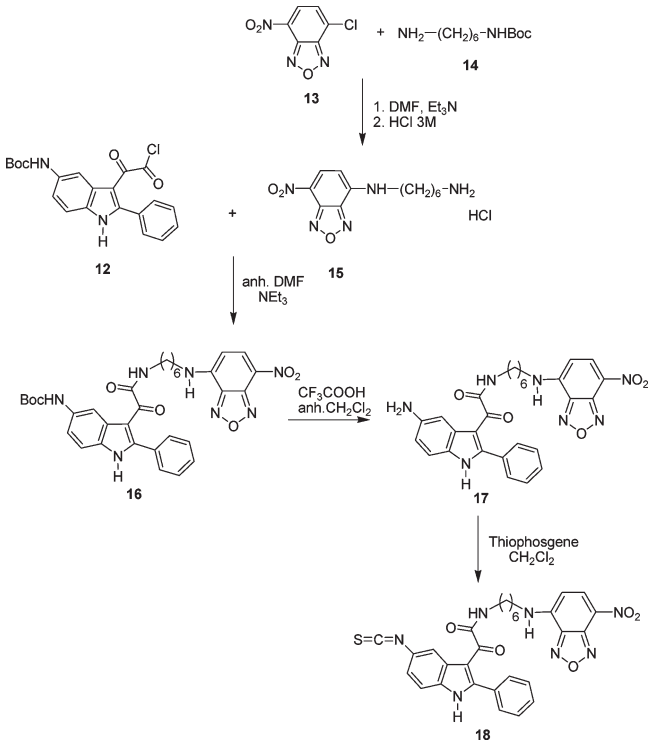
palladium to yield the corresponding amines **5** and **6**, which were then reacted with thiophosgene in a biphasic CH_2Cl_2/aqueous $NaHCO_3$ system to give the desired isothiocyanato derivatives **7** and **8**.

The synthesis of the irreversible fluorescent probe **18** was achieved by the convergent procedure outlined in Schemes 2 and 3. The first key intermediate 5-(*N*-BOC-amino)-2-phenylindol-3-ylgyloxyl chloride **12** was obtained in three steps starting from the 5-nitro-2-phenylindole **9**,[33] (Scheme 2) which was first catalytically hydrogenated to yield the corresponding amine **10**, subsequently protected with the BOC group to give **11**, and then reacted with oxalyl chloride. The *N*-BOC-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)hexamethylendiamine hydrochloride **15**, prepared as described in our previous work,[31] was condensed with compound **12** in anhydrous dimethylformamide in the presence of triethylamine at room temperature,

Scheme 2. Synthesis of Intermediate Glyoxylyl Chloride **12**



Scheme 3. Synthesis of Irreversible and Fluorescent Ligand **18**



yielding the fluorescent compound **16** (Scheme 3). Simple deprotection of the BOC group with trifluoroacetic acid in anhydrous CH_2Cl_2 gave the 5-amino derivative **17**, which was then reacted with thiophosgene, yielding the desired irreversible fluorescent compound **18** (Scheme 3).

Spectroscopic Properties of Irreversible Fluorescent Ligand 18

The ultraviolet absorption and the emission spectra of compound **18** were measured to investigate the spectroscopic properties of the ligand and how these were affected by the

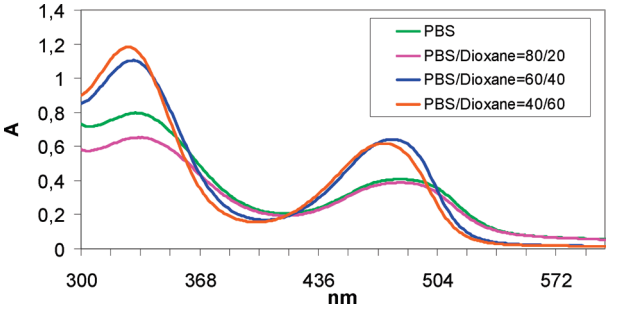


Figure 1. Absorption spectra of compound **18** at 10 μM in solutions varying from aqueous to 60% dioxane–water (v/v) PBS.

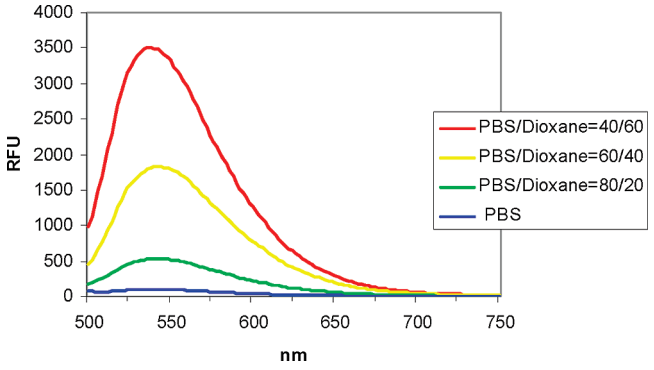


Figure 2. The influence of the polarity of the medium on the fluorescence of compound **18** was investigated by the addition of dioxane (indicated in % v/v) in PBS measured at a λ_{max} of 470 nm.

environment. Product **18**, 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).[31] The percentage of DMSO did not exceed 1% of the final assay volume solution.

Figure 1 shows the absorption spectra of compound **18**. In an aqueous solution, the spectra were characterized by two maximal absorptions at 331 and 482 nm, showing extinction coefficients $\varepsilon = 7963\ M^{-1}\ cm^{-1}$ and $\varepsilon = 4078\ M^{-1}\ cm^{-1}$, respectively. When compound **18** was dissolved in PBS solution containing 20% of dioxane, the spectrum proved to be practically unchanged, with the exception of a slight decrease in the extinction coefficient at the absorption maximum at the lower wavelength from $\varepsilon = 7963\ M^{-1}\ cm^{-1}$ to $\varepsilon = 6523\ M^{-1}\ cm^{-1}$. The further increase in hydrophobicity of the medium caused a small hypsochromic shift of both maximal absorptions, accompanied by a significant hyperchromic effect (40% dioxane–water (v/v) PBS: $\lambda_{max} = 328$ nm $\varepsilon = 11036\ M^{-1}\ cm^{-1}$, $\lambda_{max} = 478$ nm $\varepsilon = 6415\ M^{-1}\ cm^{-1}$; 60% dioxane–water (v/v) PBS: $\lambda_{max} = 327$ nm $\varepsilon = 11832\ M^{-1}\ cm^{-1}$, $\lambda_{max} = 474$ nm $\varepsilon = 6181\ M^{-1}\ cm^{-1}$).

Figure 2 shows the emission spectra of compound **18**. As expected, the decrease in polarity of the environment (from aqueous buffer to 60% dioxane in PBS) induced an approximately 30-fold increase in quantum yield. This phenomenon was accompanied by a slight blue-shift of the emission maximum from 542 to 538 nm.

Biological Studies

The biological characterization of the newly developed irreversible ligands **7**, **8**, and **18** started with the evaluation of their equilibrium binding parameters, followed by the

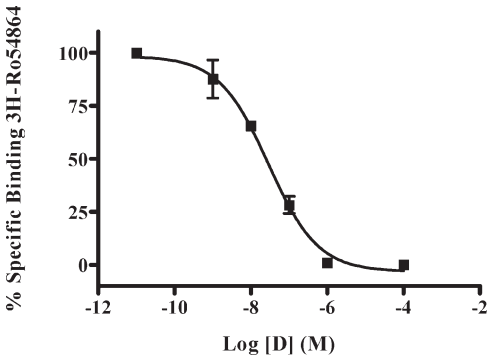


Figure 3. Displacement of [^{3}H]Ro 5-4864 by compound **18** in rat kidney mitochondria membranes. Bound radioactivity is expressed as a percentage of specific binding in the presence of competitor molecule. Points represent the mean values ± SEM of triplicate determinations pooled from three independent experiments.

investigation of the potential irreversibility of their binding to the protein.

The affinity of the newly synthesized indolylglyoxylamides for TSPO was determined in rat kidney membranes by competition experiments against [^{3}H]Ro 5-4864.[41] It has been reported that the interaction between TSPO and irreversible ligands bearing an isothiocyanato group probably takes place in accordance with the following equation (eq 1):[42,43]

$$D + R \overset{K_d}{\rightleftarrows} DR \xrightarrow{K_2} DR_{irrev} \quad (1)$$

The binding process begins with a first reversible step, followed by the formation of an irreversible molecular complex. Kinetic and mathematical analysis of this two-step reaction were developed in order to evaluate the order rate and kinetic constants (K_d and K_2) of our TSPO ligands.[44]

Moreover, the irreversible fluorescent probe **18** was assayed in experiments aimed at labeling TSPO in U87MG human glioma cells by means of fluorescence spectroscopy.

Results

As a first step, the equilibrium binding parameters (IC_{50} and K_i values) of the test compounds were derived by competition experiments against [^{3}H]Ro 5-4864 in rat kidney membranes. Ro 5-4864 was used as the reference standard, showing a K_i value of 23 nM, in accordance with literature data.[45] The curve of [^{3}H]Ro 5-4864 displacement by the irreversible fluorescent ligand **18** is shown in Figure 3, while the analogous graphs for compounds **7** and **8** are included in the Supporting Information. Binding data for the three ligands **7**, **8**, and **18** are listed in Table 1; all compounds showed affinity values in the nanomolar range, comparable to that of the reference standard Ro 5-4864 (K_i from 37.6 to 49.5 nM).

To investigate the potential covalent binding of compounds **7**, **8**, and **18** to TSPO, the samples derived from competition radiobinding assays were diluted more then 10 times with fresh buffer at the end of the incubation time; data did not markedly change with respect to those obtained in the undiluted samples (data not shown). This observation indicates that the TSPO−ligand interaction is irreversible, and it immediately disqualifies the application of the Cheng−Prusoff equation[46] in binding parameter analysis.

With the aim of evaluating the rate order of the binding reaction, a kinetic analysis method was developed, as reported

Table 1. Values of IC_{50} and K_i for Compounds **7**, **8**, and **18** Obtained from Three Independent Equilibrium Binding Assays[a]

compd	IC_{50} (nM)	K_i (nM)
7	45.6 ± 0.4	37.9 ± 0.4
8	59.7 ± 0.6	49.5 ± 0.5
18	42.2 ± 0.4	37.6 ± 0.4
Ro 5-4864		23 ± 3.1

[a] The IC_{50} values were converted to an absolute inhibition constant K_i, using the Cheng−Prusoff equation.[45] Data are shown as values ± SEM.

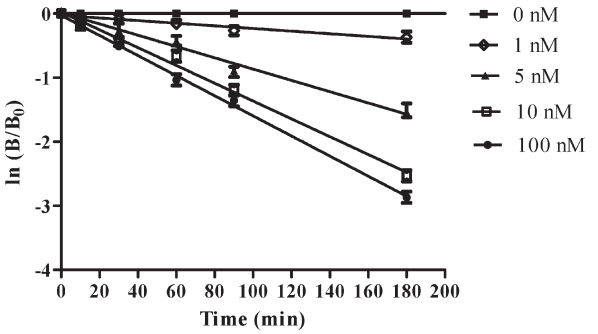


Figure 4. Time course of compound **18** binding to receptor. The semilogarithmic representation of the decrease in [^{3}H]Ro 5-4864 binding to TSPO by pretreatment (0, 10, 30, 60, 90, and 180 min) with various concentrations of compound **18** is shown. Points represent the mean values ± SEM of duplicate determinations pooled from three independent experiments.

in literature.[44,47] Results showed that mitochondrial membrane pretreatment with newly synthesized TSPO ligands inhibited the [^{3}H]Ro 5-4864 binding to TSPO. This inhibition was time- and ligand concentration-dependent, as exemplified in Figure 4, which refers to compound **18** (see Supporting Information for derivatives **7** and **8**), where B is binding after pretreatment of membranes with different concentrations of TSPO ligands for several times, and B_0 is control binding. When the B and B_0 ratio was plotted on a logarithmic scale versus incubation times, the decrease in the ln B/B_0 values appeared to be linearly related to the preincubation time. Therefore, inactivation proceeded according to apparent first-order kinetics. The apparent first-order rate constant (K_{obs}) was defined by eq 2:

$$\ln \frac{B}{B_0} = -K_{obs} \times t \quad (2)$$

Thus, a mathematical analysis was conducted to calculate the dissociation constant (K_d) of the reversible receptor−ligand complex and the pseudo first-order rate constant (K_2) for the irreversible interaction between the receptor and the irreversible ligand, as previously reported by Liu-Chen and colleagues.[44] The mathematical equation describing the process is shown below (eq 3):

$$\frac{1}{K_{obs}} = \frac{K_d}{K_2} \cdot \frac{1}{[D]} + \frac{1}{K_2} \quad (3)$$

K_{obs} is the observed first-order rate constant for irreversible binding, and it is derived from the slope of the different straight lines shown in Figure 4. The plotting of $1/K_{obs}$ versus $1/[D]$ (where D is the ligand concentration) gave a straight line that intercepted the ordinate and abscissa axes at $1/K_2$ and $-1/K_d$, respectively. The slope of the line was the ratio K_d/K_2 (Figure 5).

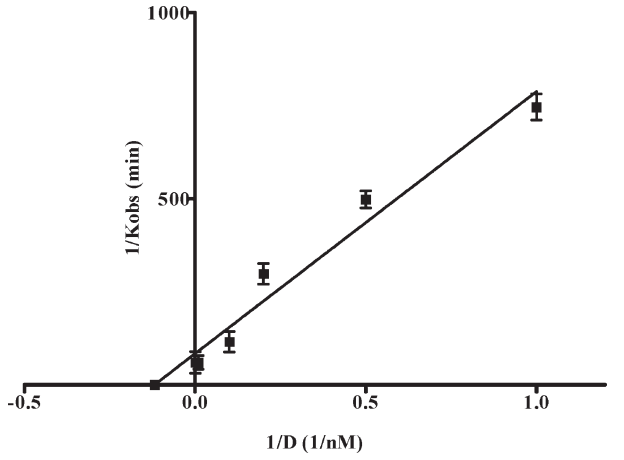


Figure 5. Calculation of K_2 and K_d for ligand **18**. On the basis of data obtained by three different experiments carried out in duplicate, the values were calculated in accordance with eq 2. Data are shown as mean values ± SEM.

Table 2. Values of K_2 and K_d for the Irreversible Binding of Compounds **7**, **8**, and **18** to TSPO[a]

compd	K_2 (nM)	K_d (nM)
7	0.003 ± 0.001	3.17 ± 0.31
8	0.015 ± 0.002	5.07 ± 0.61
18	0.012 ± 0.002	8.31 ± 0.89

[a] Data, obtained from three independent kinetic assays, are shown as values ± SEM.

Table 3. Physicochemical Predicted Properties of Compound **18**[48]

compd	ClogP[a]	ASA[b] (Å)	PSA[c] (Å)	N[d]	bond count	rb[e]	MW[f](Da)
18	4.485	847.530	170.945	67	46	13	583.620

[a] Calculated *n*-octanol/water partition coefficient. [b] Total solvent accessible surface area. [c] Polar surface area. [d] Number of atoms. [e] Number of rotatable bonds. [f] Molecular weight.

Analogous graphs for compounds **7** and **8** are supplied in the Supporting Information. K_2 and K_d values of each compound are listed in Table 2. Interestingly, all the K_d values are in the nanomolar range (K_d from 3.17 to 8.31 nM), confirming an effective interaction with the receptor.

To estimate the ability of compound **18** to permeate cell membranes, a number of its physicochemical properties were calculated and reported in Table 3.[48] Values obtained were consistent with an adequate distribution of **18** into the cell.

The ability of the probe to specifically and irreversibly label TSPO was evaluated in live cells of human glioma, using fluorescence spectroscopy. For this purpose, U87MG glioma cells were incubated with different concentrations of **18** (100, 250, and 500 nM) after pretreatment with buffer (control) or with 10 μM Ro 5-4864. Incubation was performed at 90 min, a time in which the irreversible bound is formed (see eqs 2 and 3), and at a shorter time (20 min). As expected, at the shortest time, the detected values of fluorescence intensity were smaller than the mean values obtained after 90 min (panels A and B of Figure 6, respectively). Interestingly, at 20 min time incubation, Ro 5-4864 was able to displace compound **18** in a similar extent for all the concentrations assayed, demonstrating the specificity of ligand **18**−TSPO binding. Conversely, in the experiments performed at the longest time, Ro 5-4864 showed to be unable to displace **18**, demonstrating the irreversibility of the bond.

Conclusions

We designed novel irreversible TSPO ligands featuring the 2-phenylindol-3-ylglyoxylamide scaffold. The binding of these compounds to the receptor was investigated in detail, characterizing both order rate and kinetic constants. Experimental data showed that all the new derivatives effectively bind the receptor by means of an irreversible covalent interaction. Fluorescence spectroscopy experiments performed on derivative **18**, featuring an NBD fluorophore group, revealed its ability to specifically and irreversibly label TSPO

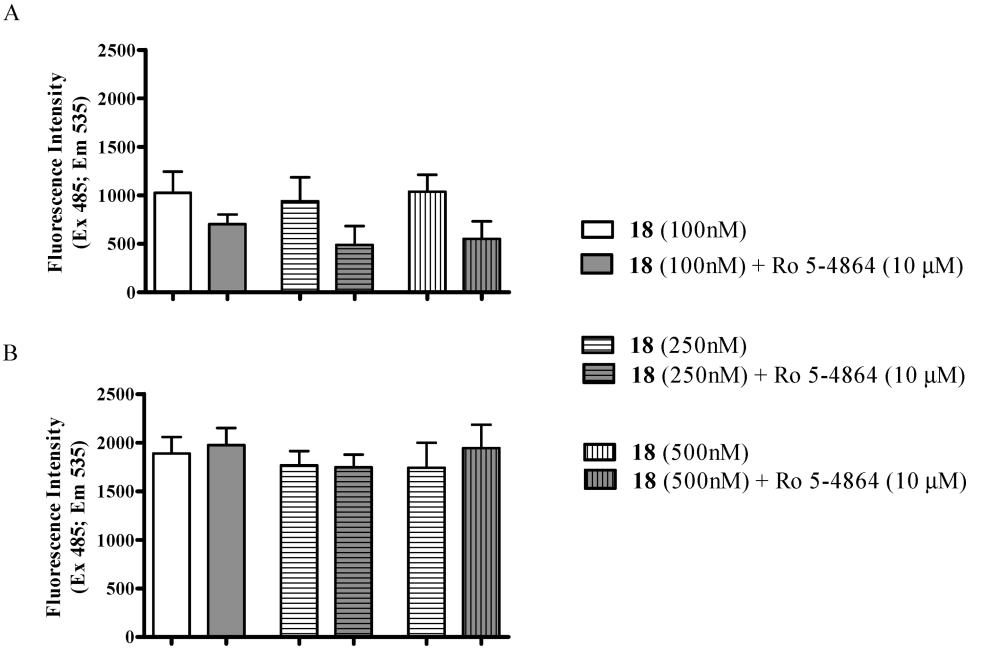


Figure 6. Human glioma cell staining with the irreversibile fluorescent TSPO ligand **18**. The cells were incubated with compound **18** for 20 min (A) and 90 min (B), in the absence or presence of 10 μM Ro 5-4864 (gray bars). Data were obtained from three independent assays and are shown as mean values of fluorescence intensity ± SEM.

in live U87MG glioma cells. All these novel irreversible TSPO ligands represent useful tools in the investigation of the physiological role of TSPO and in the detection of its expression levels.

In particular, the fluorescent probe **18** may be employed as diagnostic marker to evaluate the TSPO expression in peripheral cells, post-mortem, and biopsy tissues from patients affected by diseases in which the TSPO density is altered. Actually, irreversible probes offer the advantage of a lasting detection in visualization techniques that generally require multiple washes to remove the nonspecific signal, steps in which reversible probes may be lost.

Complementary investigations using irreversible and reversible fluorescent TSPO ligands, in in vitro and in vivo studies, respectively, may offer a useful platform for the development of new diagnostic approaches.

Experimental Section

Chemistry. Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Infrared spectra were recorded with a Nicolet/Avatar FT-IR spectrometer in Nujol mulls. Routine nuclear magnetic resonance spectra were recorded in DMSO-d_6 solution on a Varian Gemini 200 spectrometer operating at 200 MHz. Mass spectra were obtained on a ThermoQuest Finnigam GCQplus spectrometer using a direct injection probe and an electron beam energy of 70 eV. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Silica gel 60 (230−400 mesh) was used for column chromatography. Combustion analyses on target compounds were performed by our Analytical Laboratory in Pisa. All compounds showed ≥95% purity. Ultraviolet absorption spectra were measured with a Perkin-Elmer UV/Vis spectrophotometer Lambda 5. Fluorescence spectra were recorded with a Gemini XS Molecular Device Spectramax fluorescence spectrophotometer.

5-Nitro-(2-phenylindol-3-yl)glyoxyl chloride **2**,[33] *N*,*N*-dialkyl-[5-nitro-(2-phenylindol-3-yl)glyoxyl]amide derivatives **3** and **4**,[33] 5-nitro-2-phenylindole **9**,[33] and *N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,6-diaminohexane hydrochloride **15**[31] were prepared in accordance with our previous works.

General Procedure for the Synthesis of *N*,*N*-Dialkyl-[5-amino-(2-phenylindol-3-yl)glyoxyl]amide Derivatives 5 and 6. A mixture of the appropriate *N*,*N*-dialkyl-[5-nitro-(2-phenylindol-3-yl)-glyoxyl]amide derivative **3** and **4** (0.73 mmol) and 10% Pd/C (0.05 g) in 150 mL of absolute ethanol was hydrogenated at room temperature and pressure. Once hydrogen absorption ceased (4−6 h), the catalyst was filtered off and the solution was evaporated to dryness at reduced pressure. The semisolid products **5** and **6** obtained were utilized in the following reaction without any further purification.

***N*,*N*-Dipropyl-[5-amino-(2-phenylindol-3-yl)glyoxyl]amide 5.** Yield 91%. IR (nujol, cm^{-1}): 3365, 3221, 1627, 1214, 1088, 1043. ^{1}H NMR (DMSO-d_6, ppm): 0.64−0.74 (m, 6H, $2CH_3$), 1.08−1.20 (m, 4H, $CH_2CH_2CH_3$), 2.82 (t, 2H, J = 8.0 Hz, NCH_2), 2.98 (t, 2H, J = 8.0 Hz, NCH_2), 5.31 (bs exch, 2H, NH_2), 6.63 (d, 1H, J = 9.0 Hz, Ar-H), 7.15 (d, 1H, J = 7.6 Hz, Ar-H), 7.34 (s, 1H, Ar-H); 7.44−7.48 (m, 5H, Ar-H), 12.04 (s exch, 1H, NH). Anal. Calcd for $C_{22}H_{25}N_3O_2$ (%): C, 72.70; H, 6.93; N, 11.56. Found: C, 72.87; H, 6.83; N, 11.65.

***N*,*N*-Dihexyl-[5-amino-(2-phenylindol-3-yl)glyoxyl]amide 6.** Yield 89%. IR (nujol, cm^{-1}): 3365, 3290, 1627, 1600, 1211, 1094. ^{1}H NMR (DMSO-d_6, ppm): 0.74−0.90 (m; 6H, $2CH_3$), 1.10−1.43 (m, 16H, $8CH_2$), 2.87 (t, 2H, J = 7.2 Hz, NCH_2), 3.02 (t, 2H, J = 7.2 Hz, NCH_2), 4.92 (bs exch, 2H, NH_2), 6.61 (d, 1H, J = 6.6 Hz, Ar-H), 7.14 (d, 1H, J = 8.4 Hz, Ar-H), 7.32 (s, 1H, Ar-H), 7.44−7.48 (m, 5H, Ar-H), 12.00 (bs exch, 1H, NH). Anal. Calcd for $C_{28}H_{37}N_3O_2$ (%): C, 75.13; H, 8.33; N, 9.39. Found: C, 74.98; H, 8.27; N, 9.26.

General Procedure for the Synthesis of *N*,*N*-Dialkyl-[5-isothiocyanato-(2-phenylindol-3-yl)glyoxylamide Derivatives 7 and 8. A stirred solution of **5** and **6** (0.52 mmol) in 10 mL of $NaHCO_3$ 6% was added to 10 mL of CH_2Cl_2. After 20 min of vigorous stirring at 0 °C, thiophosgene (0.03 mL, 0.52 mmol) was added dropwise. The reaction mixture was left under stirring for 24 h at room temperature (TLC analysis), and the organic solvent was removed under reduced pressure and the crude residue was purified by washing with cold ether to afford derivatives **7** and **8**.

***N*,*N*-Dipropyl-[5-isothiocyanato-2-phenylindol-3-yl)glyoxyl-amide 7.** Yield 71%; mp 77−79 °C. IR (nujol, cm^{-1}): 3187, 2095, 2047, 1617, 1211, 1088. ^{1}H NMR (DMSO-d_6, ppm): 0.68−0.79 (m, 6H, $2CH_3$), 1.18−1.29, (m, 2H, CH_2CH_2-CH_3), 1.42−1.52 (m, 2H, $CH_2CH_2CH_3$), 2.91 (t, 2H, J = 7.6 Hz, NCH_2), 3.04 (t, 2H, J = 7.6 Hz, NCH_2), 7.34 (d, 1H, J = 8.4 Hz, Ar-H), 7.53−7.57 (m, 6H, Ar-H), 8.33 (s, 1H, Ar-H), 12.79 (s exch, 1H, NH). MS m/e %: 405 (M^+, 5), 277 (10), 129 (15), 83 (100). Anal. Calcd for $C_{23}H_{23}N_3O_2S$ (%): C, 68.12; H, 5.72; N, 10.36. Found: C, 68.29; H, 5.65; N, 10.47.

***N*,*N*-Dihexyl-[5-isothiocyanato-2-phenylindol-3-yl)glyoxylamide 8.** Yield: 80% oil. IR (nujol, cm^{-1}): 3180, 2116, 2061, 1617, 1258, 1091, 746. ^{1}H NMR (DMSO-d_6, ppm): 0.73−0.90 (m; 6H, $2CH_3$), 1.09−1.43 (m, 16H, $8CH_2$), 2.97−3.08 (m, 4H, $2NCH_2$), 7.35 (d, 1H, J = 8.4 Hz, Ar-H), 7.53−7.60 (m, 6H, Ar-H), 8.00 (s, 1H, Ar-H), 12.78 (s exch, 1H, NH). MS m/e %: 489, (M^+, 5), 277 (100), 85 (15). Anal. Calcd for $C_{29}H_{35}N_3O_2S$ (%): C, 71.13; H, 7.20; N, 8.58. Found: C, 70.75; H, 7.03; N, 8.63.

5-Amino-2-phenylindole 10. A mixture of 5-nitro-2-phenylindole **9** (0.543 g, 4.0 mmol) and 10% Pd/C (0.28 g) in 150 mL of absolute ethanol was hydrogenated at room temperature and pressure. Once hydrogen absorption ceased (3−4 h), the catalyst was filtered off and the solvent was evaporated to afford a violet-colored amorphous solid. The residue was purified by crystallization from toluene to afford 0.666 g (80%) of **10**, mp 223−225 °C. IR (nujol, cm^{-1}): 3347, 3413, 1631, 1537, 1071, 757. ^{1}H NMR (DMSO-d_6, ppm): 4.87 (s exch, 2H, NH_2), 6.52 (d, 1H, J = 8.2 Hz, Ar-H), 6.63 (s, 1H, Ar-H), 6.70 (s, 1H, Ar-H), 7.10 (d, 1H, J = 8.4 Hz, Ar-H), 7.22−7.29 (m, 1H, Ar-H), 7.38−7.45 (m, 2H, Ar-H), 7.78 (d, 2H, J = 7.4 Hz, Ar-H), 11.06 (bs exch, 1H, NH). Anal. Calcd for $C_{14}H_{12}N_2$ (%): C, 80.74; H, 5.81; N, 13.45. Found: C, 80.60; H, 5.83; N, 13.26.

5-*N*-Boc-amino-2-phenylindole 11. Di-*tert*-butyldicarbonate (0.348 g, 1.6 mmol) was added to a stirred solution, cooled at 0 °C, of 5-amino-2-phenylindole **10** (0.330 g, 1.6 mmol) in a mixture of acetone (6 mL) and H_2O (3 mL). The reaction mixture was stirred at room temperature for 24 h (TLC analysis). The solvent was reduced to half volume under reduced pressure, and the precipitate that formed was collected and purified by crystallization from toluene (67%); mp 165−167 °C. IR (nujol, cm^{-1}): 3437, 3326, 3289, 1686, 1531, 1050, 754. ^{1}H NMR (DMSO-d_6, ppm): 1.48 (s, 9H, $C(CH_3)_3$), 6.81 (s, 1H, Ar-H), 7.10−7.15 (m, 1H, Ar-H), 7.23−7.32 (m, 2H, Ar-H), 7.40−7.47 (m, 2H, Ar-H), 7.65 (s, 1H, Ar-H), 7.80−7.85 (m, 2H, Ar-H), 9.05 (s exch, 1H, CONH), 11.36 (s exch, 1H, NH). Anal. Calcd for $C_{19}H_{20}N_2O_2$ (%): C, 74.00; H, 6.54; N, 9.08. Found: C, 74.20; H, 6.63; N, 9.26.

5-*N*-Boc-amino-2-phenylindol-3-ylglyoxyl chloride 12. Oxalyl chloride (0.07 mL, 0.8 mmol), in 2 mL of anhydrous THF, was added dropwise over 10−15 min to a cooled (0 °C) stirred solution of **11** (0.184 g, 0.6 mmol) in 10 mL of freshly distilled THF. The mixture was maintained at room temperature for 2 h. The organic solvent was removed under reduced pressure, and the residue was washed with three portions of dry distilled THF. The glyoxyl chloride **12** obtained was particularly unstable, and thus it was utilized in the following reaction without any further purification.

***N*-[5-(*N*-Boc-amino)-2-phenylindol-3-ylglyoxyl]-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,6-diaminohexane 16.** A solution of amine

15 (0.084 g, 0.3 mmol) in 2−3 mL of dry DMF was added dropwise to a stirred solution, cooled at 0 °C, of glyoxyl chloride **12** (0.199 g, 0.5 mmol) in 3 mL of the same solvent, followed by addition of a solution of triethylamine (0.16 mL, 1.2 mmol). The reaction mixture was allowed to warm to room temperature, stirred for 12 h (TLC analysis), and then filtered to eliminate the triethylamine hydrochloride formed. The organic solvent was evaporated to dryness, and the residue was washed with water. The suspension was filtered, and the brown-colored amorphous solid obtained was washed with cold diethyl ether to afford 0.253 g (80%) of **16**; mp 153−155 °C. IR (nujol, cm^{-1}): 3324, 1696, 1580,1290, 1153, 897, 726. ^{1}H NMR (DMSO-d_6, ppm): 1.06−1.80 (m, 19H, $CH_2(CH_2)_4$-CH_2, $C(CH_3)_3$, $CONHCH_2$), 2.68−2.74 (m, 2H, CH_2NH), 6.42 (d, 1H, J = 9.2 Hz, 5′-H), 7.34−7.53 (m, 7H, Ar-H), 8.28 (s, 1H, Ar-H), 8.42 (bs exch, 1H, COCONH), 8.51 (d, 1H, J = 9.0 Hz, 6′-H), 9.28 (s, 1H, Ar*NH*CO), 9.57 (bs exch, 1H, CH_2NH), 12.21 (s exch, 1H, NH). Anal. Calcd for $C_{28}H_{37}N_3O_2$ (%): C, 61.77; H, 5.50; N, 15.28. Found: C, 61.90; H, 5.63; N, 15.16.

***N*-(5-Amino-2-phenylindol-3-ylglyoxyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,6-diaminohexane 17.** Trifluoroacetic acid (2 mL, 20 mmol) was added dropwise to a stirred solution of derivative **16** (0.192 g, 0.3 mmol) in 10 mL of anydrous CH_2Cl_2, cooled at 0 °C. The mixture was stirred at room temperature for 12 h (TLC analysis). Then the reaction mixture was concentrated under reduced pressure. The resulting solid residue was taken up with water and cooled in an ice bath. Upon the addition of a 5% solution of $NaHCO_3$, a precipitate formed which was filtered to provide 0.148 g (91%) of **17**; mp 195−197 °C. IR (nujol, cm^{-1}): 3338, 3242, 1621, 1587, 1135, 801, 723. ^{1}H NMR (DMSO-d_6, ppm): 1.04−1.36 (m, 8H, $CH_2(CH_2)_4CH_2$), 1.60−1.69 (m, 2H, $CONHCH_2$), 2.68−2.74 (m, 2H, CH_2NH), 6.42 (d, 1H, J = 9.6 Hz, 5′-H), 6.76−6.80 (m, 1H, Ar-H), 7.25−7.55 (m, 9H, Ar-H, NH_2), 8.36 (bs exch, 1H, COCONH), 8.52 (d, 1H, J = 9.8 Hz, 6′-H), 9.58 (bs exch, 1H, CH_2NH), 12.11 (s exch, 1H, NH). Anal. Calcd for $C_{28}H_{27}N_7O_5$ (%): C, 62.10; H, 5.03; N, 18.10. Found: C, 62.27; H, 4.83; N, 18.19.

***N*-(5-Isothiocyanato-2-phenylindol-3-ylglyoxyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,6-diaminohexane 18.** A stirred solution of **17** (0.162 g, 0.3 mmol) in 20 mL of 6% $NaHCO_3$ was added to 20 mL of CH_2Cl_2. After 20 min of vigorous stirring at 0 °C, thiophosgene (0.1 mL, 1 mmol) was added dropwise. The reaction mixture was stirred for 4 h. A precipitate formed which was collected by vacuum filtration and washed with cold ether to afford 0.117 g (67%) of **18**; mp 152−154 °C (dec). IR (nujol, cm^{-1}): 3345, 2123, 2041, 1648, 1614, 1586, 1177, 1105, 811, 719. ^{1}H NMR (DMSO-d_6, ppm): 1.15−1.36 (m, 8H, $CH_2(CH_2)_4CH_3$), 1.60−1.69 (m, 2H, $CONHCH_2$), 2.72−2.80 (m, 2H, CH_2NH), 6.41 (d, 1H, J = 7.8 Hz, 5′-H), 7.31−7.56 (m, 8H, Ar-H,CONH), 8.01 (s, 1H, Ar-H), 8.50 (d, 1H, J = 7.2 Hz, 6′-H), 9.57 (bs exch, 1H, CH_2NH), 12.68 (s exch, 1H, NH). MS *m*/*e* %: 589, (M^+, 5), 277 (5), 83 (100). Anal. Calcd for $C_{29}H_{25}N_7O_5S$ (%): C, 59.68; H, 4.32; N, 16.80. Found: C, 59.81; H, 4.13; N, 17.06.

Biological Methods. Materials. [^{3}H]Ro 5-4864 (sa 70.0 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. Ro 5-4864 powder was obtained from Sigma-Aldrich. Cell culture media and growth supplements were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and Bio-Rad (Hercules, CA). Nonessential amino acids (1%) were from GIBCO (Milan, Italy). All other reagents were from standard commercial sources.

Cell Culture. The human glioblastoma cell line U87MG was obtained from the National Institute for Cancer Research (ICLC) of Genoa. It was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% nonessential amino acids at 37 °C in a humidified atmosphere composed of 5% CO_2 and 95% O_2.

[^{3}H]Ro 5-4864 Binding to Rat Kidney Mitochondrial Membranes: Reversible Binding. The binding studies were carried out essentially as previously described.[31,49]

Time Course of Binding of the New Ligands to the TSPO Receptor: Irreversible Binding. The binding studies were carried out essentially as previously described.[47] Briefly, rat kidney membranes were incubated with each compound (concentration range: 1 nM to 1 μM) for different times (0, 10, 30, 60, 90, and 180 min) in binding assay buffer. Incubations were stopped by the addition of 1 mL of ice-cold 50 mM Tris/HCl buffer (pH 7.4), and then the samples were centrifuged for 15 min at 13000*g* at 4 °C. Pellets were washed twice with cold binding buffer. Then, samples were processed for determination of protein content. [^{3}H]Ro 5-4864 binding assays were performed in a final volume of 500 μL of binding assay buffer containing membranes (65 μg of protein/tube), as mentioned above.

Fluorescent Labeling of Human Glioma Cells. U87MG cells were cultured in 96-well plates (4×10^4 cells/well) in RPMI 1640 complete medium. After 24 h, the cells were incubated with different concentrations of **18** (100, 250, and 500 nM) in cell culture medium, for 20 and 90 min, under 5% CO_2 at 37 °C. In parallel, some samples were incubated with Ro 5-4864 (10 μM) prior to add compound **18**. To remove the excess of unbound compound, the cells were washed four times with cell medium with cysteine, a molecule able to react with the isothiocyanate group. After washing, PBS was added to the cells and the fluorescence intensity (Ex: 485 nm; Em: 535 nm) from each sample was measured by Victor Wallac 2 (Perkin-Elmer, Boston, MA). All data are presented as means ± SEM, derived from at least three independent experiments done in duplicate.

Data Analyses. Data were analyzed by use of the GraphPad Prism software (GraphPad Software, version 4.0; San Diego, CA). Statistical analyses were performed by one-way ANOVA (with post hoc Bonferroni test).

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Supporting Information Available: Displacement of [^{3}H]Ro 5-4864 by compounds **7** and **8** in rat kidney mitochondria membranes, Time course of compounds' binding to the receptor, and the calculation of K_2 and K_d for ligands **7** and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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