Synthesis and Characterization of Environment-Sensitive Fluorescent Ligands for Human 5-HT1A Receptors with 1-Arylpiperazine Structure†

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A series of “long-chain” 1-(2-methoxyphenyl)piperazine derivatives containing an environment-sensitive fluorescent moiety (4-amino-1,8-naphthalimide, 4-dimethylaminophthalimide, dansyl) was synthesized. The compounds displayed very high to moderate 5-HT1A receptor affinity and good fluorescence properties. 6-Amino-2-[5-[4-(2-methoxyphenyl)-1-piperazinyl]pentyl]-1H-benzo[de]isoquinoline-1,3(2H)-dione (4) combined very high 5-HT1A receptor affinity (Kᵢ = 0.67 nM), high fluorescence emission in CHCl₃, and undetectable fluorescence emission in aqueous solution. It was evaluated for its ability to visualize 5-HT1A receptors overexpressed in CHO cells by fluorescence microscopy.

Introduction

The serotonin1A (5-HT₁A) receptor is an important member of the large family of serotonin receptors and is perhaps the most extensively studied for several reasons. One is the availability of the selective agonist 8-OH-DPAT⁶, which has allowed extensive biochemical, physiological, and pharmacological characterization of the receptor. The 5-HT₁A receptor was the first serotonin receptor to be cloned and sequenced. Human, rat, and mouse 5-HT₁A receptors have been cloned and their amino acid sequences deduced. More importantly, the receptor has been stably expressed in a number of neural and non-neural cell lines. It is generally accepted that 5-HT₁A receptor is involved in anxiety and depression. Recently, it has been suggested that 5-HT₁A receptor agonists have neuroprotective properties, whereas 5-HT₁A receptor antagonists could be useful in the treatment of cognitive disorders related to Alzheimer’s disease.² Transfection of the cDNA of this receptor into cells has resulted in the acquisition of large amounts of information regarding signal transduction pathways linked to the receptor (adenylyl cyclase inhibition, coupling to phospholipase, regulation of ion channels) and pharmacological properties of the receptor. The realization that the 5-HT₁A receptor couples to multiple signaling pathways in cells and tissues in which it is normally expressed reflects the promiscuity of GPCR signaling pathways and the fact that single receptor subtypes might be linked to various second messengers in a single cell system.³ It is likely that the pharmacology of GPCRs differs markedly depending on their membrane localization and the signaling proteins present within that locality. This might have important implications for the orchestration of drug action within a given cell type and the potential for cross-talk with other signaling pathways.⁴ The study of receptor pharmacology at single cell level may benefit from use of fluorescent ligands. They offer a multiplicity of information such as the mechanism of ligand binding, the movement and internalization of receptors in living cells, the distances between ligands and fluorescently labeled amino acids, the physical nature of the binding pocket, and the visualization of labeled receptors.⁵ Thus, the identification of fluorescent ligands for 5-HT₁A receptor would be of interest. Fluorescent ligands are usually designed starting from the selection of a pharmacophoric moiety that is conjugated with a fluorescent tag through an appropriate linker. For GPCRs activated by biogenic amines, the ligand binding sites are buried within the transmembrane regions of the receptor and interact with small molecules. Consequently, appropriate positioning of the fluorophore within the final conjugate is critical to retain both receptor binding affinity and efficacy. Thus, this structural modification could significantly affect the pharmacological properties of the parent ligands.⁶ As an alternative strategy, we have recently designed fluorescent probes for 5-HT₁A receptor by incorporating the fluorophore moieties into the pharmacophoric part of the ligand. In other words, the fluorescent moiety itself had structural characteristics that could be well-tolerated in the interaction with the target receptor. In this way, a series of fluorescent ligands was generated, with the possibility of optimizing the fluorescence properties and receptor affinity at the same time. In particular, we described a series of fluorescent N-[o-[4-(2-methoxyphenyl)-1-piperazinyl]alkyl]-2-quinolinamines (Chart 1, structure I) characterized by nanomolar affinity at 5-HT₁A receptor, along with acceptable fluorescence properties.⁷ However, the ligands showed excitation wavelengths in the near UV region of the spectrum (340–380 nm) and this could limit their use to visualizing 5-HT₁A receptors overexpressed in cell lines by conventional confocal laser scanning microscopy due to cell...
autofluorescence. To overcome this, we have replaced the 2-quinolinamine with three fluorescent labels possessing different fluorescent properties. The fluorophores were 4-amino-1,8-naphthalimide (6-amino-1,3-dioxo-1H,3H-benzof[de]isoquinolinyl), 4-dimethylaminophthalimide (5-dimethylamino-1H-isooindole-1,3(2H)-dione), and dansyl (5-dimethylaminonaphthalene-1-sulfonyl), which are known to have different excitation wavelengths (315 nm for the dansyl moiety, and 450–470 nm for the others). Moreover they possess the additional feature of being environment-sensitive. These fluorophores exhibit a low quantum yield in aqueous solution but become highly fluorescent in nonpolar solvents or when bound to a hydrophobic site in proteins or membranes. Environment-sensitive molecules may be useful in fluorescence microscopy studies, where fluorescence will be higher for ligand–receptor interaction and lower for the unbound ligand in aqueous environment.

Here we report the design, synthesis, and binding properties for the human 5-HT\textsubscript{1A} receptor of the new ligands 1–12. The fluorescent properties of the ligands were determined in CHCl\textsubscript{3}, PBS buffer solution, and in the presence of cell membranes overexpressing the receptor. We also report some results of visualization of fluorescent ligand-labeled cells using fluorescence microscopy.

**Chemistry.** The synthesis of the final compounds 1–12 (Scheme 1) required the key amines 13a–d, which were prepared according to the literature as detailed in Supporting Information (SI). For target compounds 1–4, key amines 13a–d were condensed with 4-nitro-1,8-naphthalic anhydride to give nitro derivatives 14a–d, which were subsequently reduced by catalytic hydrogenation with hydrazine hydrate in the presence of 10% Pd/C. The other target compounds were prepared from the condensation of 13a–d with 4-(dimethylamino)phthalic acid in the presence of 1,1-carboxyldiimidazole (5–8) or with dansyl chloride (9–12).

**Results and Discussion**

The affinity values for 5-HT\textsubscript{1A} receptor and fluorescent properties of the target compounds 1–12 are displayed in Table 1.

To develop the new series of fluorescent probes for 5-HT\textsubscript{1A} receptors, we took advantage of existing structure–activity relationship studies on 5-HT\textsubscript{1A} receptor ligands with N1-substituted N4-arylpyperazines (the “long-chain” ary1pyperazines). The general formula of these compounds presents a 1-arylpiperazine linked through an alkyl chain to a terminal fragment, belonging to one of the following four structural classes: (i) imides, (ii) amides, (iii) alkyl, arylalkyl, or heteroaryalkyl derivatives, and (iv) tetralins.\textsuperscript{8} In particular, we focused our study on 1-(2-methoxyphenyl)piperazine derivatives because they frequently display high affinity for the 5-HT\textsubscript{1A} receptor as for the antagonists 15 (WAY-100635)\textsuperscript{9} and 16 (NAN-190)\textsuperscript{10} (Chart 1). As outlined above, several examples from the literature indicate that various terminal fragments are well-tolerated. Thus, the replacement of 2-quinolinamine nucleus in structure 1 (Chart 1) with the fluorescent moiety 4-amino-1,8-naphthalimide, 4-dimethylaminophthalimide, and dansyl should be tolerated with respect to the 5-HT\textsubscript{1A} affinity. In doing this, we were encouraged by the structural similarity between the terminal fragment of the 5-HT\textsubscript{1A} antagonist 16 and the 4-dimethylaminophthalimide fluorophore. Finally, optimization of the intermediate alkyl chain length was accomplished because it is well-documented that each combination ary1pyperazine/terminal fragment requires a spacer of appropriate length.\textsuperscript{8} Overall, our initial considerations were confirmed by \(K_i\) values of the target compounds, which ranged between 0.47 and 151 nM. In particular, considering the 4-amino-1,8-naphthalimide derivatives 1–4, we note that length of the spacer greatly influence the affinity. The compound 4 with a five methylene spacer displayed subnanomolar affinity (\(K_i = 0.67\) nM). When the linker was shortened, a considerable loss in affinity was observed (> 65-fold), especially for compounds 1 (\(n = 2\)) and 2 (\(n = 3\)), whereas 3 was only 10-fold less potent than 4. A different trend was found in the group of 4-dimethylaminophthalimide derivatives 5–8. The compounds 6 (\(n = 3\)) and 7 (\(n = 4\)) displayed subnanomolar affinity (\(K_i = 0.47\) and 0.50 nM, respectively), whereas 5 (\(n = 2\)) and 8 (\(n = 5\)) were 30- and 15-fold less potent. Compound 7 is formally derived from 16 by introduction of a dimethylamine substituent in the 4-position of the phthalimide moiety. This modification had no influence on affinity: in fact, both compounds displayed \(K_i\) values in the nanomolar range (0.50 and 0.60 nM, respectively).\textsuperscript{10} The use of dansyl as terminal fragment was also tolerated, although none of the derivatives showed subnanomolar affinity at 5-HT\textsubscript{1A} receptor. \(K_i\) values of compounds 9–12 were within a narrow range.
It is likely that the different spatial orientation of dansyl group with respect to that of phthalimido or naphthalimido is less favorable for interaction with the 5-HT\textsubscript{1A} receptor. Overall, affinity data showed that various terminal fragments are well-tolerated with respect to the 5-HT\textsubscript{1A} receptor affinity and that the optimization of the intermediate alkyl chain length is a key step when studying “long-chain” arylpiperazines. Considering the fluorescent properties in ethanol of the target compounds 1–12, we note that the structural modification of the fluorescent core has little or no influence on either excitation or emission wavelengths.\textsuperscript{11,12} The excitation wavelengths of the dansyl derivatives 9–12 are more shifted toward the UV region of the spectrum as compared to the 4-dimethylaminophthalimide derivatives 5–8 (400–420 nm) and the 4-amino-1,8-naphthalimides 1–4 (440–441 nm). All the target compounds showed high difference of excitation to emission maximal wavelengths (Stokes shift). To probe the environment affecting the sensitivity of the fluorescent ligand, the quantum yield (\(\Phi\)) in PBS buffer and apolar solvent CHCl\textsubscript{3} were determined (Table 1). 1–12 exhibited very low fluorescence in PBS buffer but became fluorescent in CHCl\textsubscript{3}. In particular, compounds 1, 5, and 9, characterized by a two methylene spacer, showed the lower \(\Phi\) value within each series. Compounds 2, 4, and 7 displayed the highest \(\Phi\) values in CHCl\textsubscript{3} (\(\Phi = 0.67, 0.59, 0.86\), respectively). Taken together, 5-HT\textsubscript{1A} receptor affinity data and fluorescence properties showed that the pursued optimization strategy was successful, especially for derivatives 4 and 7, which combined very high 5-HT\textsubscript{1A} receptor affinity \((K_i = 0.67 \text{ and } 0.50 \text{ nM, respectively, high } \Phi \text{ values in CHCl}_3\) and excitation and emission wavelengths in a suitable range for visualization experiments by confocal laser scanning microscopy. On this basis, compounds 4 and 7 were incubated with cell membranes overexpressing human 5-HT\textsubscript{1A} receptor to evaluate how the interaction of the fluorescent ligand with the cell membranes overexpressing human 5-HT\textsubscript{1A} receptor to evaluate how the interaction of the fluorescent ligand with the cell membranes overexpressing human 5-HT\textsubscript{1A} receptor to evaluate how the interaction of the fluorescent ligand with the

![Figure 1. Spectrofluorimetric determination of the fluorescence of 4 (A) or 7 (B) in the presence of cell membranes overexpressing 5-HT\textsubscript{1A} receptors (c) in comparison with cell autofluorescence (b) and PBS buffer alone (a).](image)

Table 1. 5-HT\textsubscript{1A} Receptor Affinities and Fluorescence Properties of the Final Compounds 1–12\textsuperscript{a}

<table>
<thead>
<tr>
<th>comp</th>
<th>R</th>
<th>n</th>
<th>(K_i) nM ± S.E.M.</th>
<th>(\lambda_{\text{exc}}) (nm)</th>
<th>(\lambda_{\text{em}}) (nm)</th>
<th>(\Phi) (PBS)</th>
<th>(\Phi) (CHCl\textsubscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>124 ± 2</td>
<td>441</td>
<td>520</td>
<td>406</td>
<td>500</td>
<td>~ 0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>167 ± 7</td>
<td>440</td>
<td>520</td>
<td>403</td>
<td>497</td>
<td>~ 0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>6.8 ± 0.5</td>
<td>440</td>
<td>520</td>
<td>403</td>
<td>495</td>
<td>0.035</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.67 ± 0.1</td>
<td>440</td>
<td>520</td>
<td>400</td>
<td>496</td>
<td>0.012</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>15 ± 1.4</td>
<td>400</td>
<td>512</td>
<td>395</td>
<td>481</td>
<td>~ 0</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>0.47 ± 0.5</td>
<td>400</td>
<td>512</td>
<td>395</td>
<td>488</td>
<td>0.010</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.50 ± 0.1</td>
<td>406</td>
<td>520</td>
<td>397</td>
<td>490</td>
<td>~ 0</td>
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<tr>
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<td>5</td>
<td>7.8 ± 0.4</td>
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<td>520</td>
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<td>341</td>
<td>480</td>
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</tr>
<tr>
<td>11</td>
<td>4</td>
<td>151 ± 12</td>
<td>335</td>
<td>507</td>
<td>340</td>
<td>482</td>
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</tr>
<tr>
<td>12</td>
<td>5</td>
<td>54 ± 7</td>
<td>335</td>
<td>507</td>
<td>344</td>
<td>482</td>
<td>0.020</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The values are the mean ± SEM from three independent experiments in triplicate.
receptor reflects on the fluorescence emission. Figure 1 shows typical fluorescence curves representing the fluorescence emission of the ligands in PBS, the autofluorescence of cell membrane alone, and the total observed fluorescence due to the membranes incubated with 4 and 7, respectively. The incubation of the fluorescent ligands with cell membranes led to a marked increase in fluorescence emission intensity when compared to PBS solution for both compounds. The naphthalimide derivative 4 determined a 3-fold increase of fluorescence emission when compared to cell membrane autofluorescence, whereas 7 determined only a slight increase in fluorescence emission. Therefore, compound 4 was selected for labeling 5-HT1A receptor in CHO cells. The experiments were performed by conventional confocal laser scanning microscopy at a λexc = 458 nm for single photon excitation and at 900 nm for multiphoton excitation. In the first attempt to visualize 5-HT1A receptors, stably transected CHO cells (TCHO) were incubated with 4 at different concentrations (0.125, 0.5, and 1 μM) at ∼23 °C. In parallel, the same experiments were performed on wild-type CHO cells to evaluate if the binding of the fluorescent ligand was specific. Compound 4 exhibited reasonably bright fluorescence when imaged under these experimental conditions. Representative fluorescence micrographs under various conditions are shown in Figure 2. Unfortunately, 4 appeared to undergo almost instantaneous nonspecific aggregation as well as internalization upon addition to cells. The patterns of labeling exhibited are similar in both TCHO and CHO cells, suggesting predominantly nonspecific interactions. To inhibit endocytosis, labeling was also performed at 4 °C without appreciable improvement in the labeling pattern. Moreover, competition with 5-HT did not induce any perceivable change in the labeling pattern. Therefore, 4 appeared to be unsuitable for visualizing 5-HT1A receptors specifically in CHO cells. An explanation for the observed high nonspecific binding is difficult to address. It has been proposed that high lipophilicity is a factor contributing to the nonspecific binding of some fluorescent probes.13–15 However, literature data do not suggest an optimal lipophilicity range that might be associated with low nonspecific binding for fluorescent ligands. Also, specific interaction of compound 4 with molecular targets other than 5-HT1A receptors present in CHO cells cannot be ruled out. For instance, it has been reported that 1,8-naphthalimide derivatives can strongly interact with DNA.16 Clearly, these possibilities cannot be properly evaluated during the design process.

Conclusions

We have identified a series of environment-sensitive fluorescent ligands for 5-HT1A receptors by inserting an environment-sensitive moiety in the N-[4-(2-methoxyphenyl)-1-piperazinyl]alkyl framework. Several of the newly prepared ligands displayed nanomolar affinity at 5-HT1A receptor and fluorescent properties suitable for use in fluorescence microscopy. In particular, the fluorescent ligand 4 showed a favorable combination of 5-HT1A receptor affinity (Ki = 0.67 nM), Stokes shift (excitation wavelength = 400 nm, emission wavelength = 496 nm), and quantum yield in CHCl3 (Φ = 0.59). In spite its favorable profile, compound 4 was unable to specifically label the human 5-HT1A receptor overexpressed in CHO cell lines.

Experimental Section

General Procedure for Preparation of Nitro Derivatives 14a–d. A mixture of 4-nitro-1,8-naphthalic anhydride (0.48 g, 2.0 mmol) and the appropriate amine 13a–d (2.2 mmol) in absolute EtOH (20 mL) was refluxed for 3 h. After cooling, the solvent was evaporated to dryness and the crude residue was chromatographed (CHCl3/MeOH, 19:1, as eluent). The collected organic layers were dried over Na2SO4 and evaporated to dryness. H2O (20 mL) was added to the residue, and the mixture was refluxed for 1 h. After cooling, the catalyst was removed by filtration through celite and the solvent was evaporated to dryness. H2O (20 mL) was added to the residue, and the aqueous phase was extracted with CHCl3 (2 × 30 mL). The collected organic layers were dried over Na2SO4 and evaporated under reduced pressure. The crude residue was chromatographed (CHCl3/MeOH, 19:1, as eluent) to afford pure compound 14a–d as a brown semisolid.

2-[5-[4-(2-Methoxyphenyl)-1-piperazinyl]pentyl]-6-nitro-1H-benzo[de]isoquinoline-1,3(2H)-dione (14d). Yield 40%. 1H NMR (CDCl3) δ 1.41–1.51 (m, 2H), 1.63–1.72 (m, 2H), 1.76–1.85 (m, 2H), 2.47 (t, 2H, J = 7.4 Hz), 2.69 (br s, 4H), 3.11 (br s, 4H), 3.83 (s, 3H), 4.25 (t, 2H, J = 7.4 Hz), 6.83–7.02 (m, 4H), 7.99 (t, 1H, J = 8.2 Hz), 8.42 (d, 1H, J = 8.0 Hz), 8.69 (d, 1H, J = 8.0 Hz), 8.75 (dd, 1H, J = 0.8, 7.4 Hz), 8.84 (dd, 1H, J = 0.8, 8.7 Hz). ESI-MS m/z: 340.0 (M−). ESI+/MS/MS m/z: 340 (72), 297 (82), 255 (100).

General Procedure for Preparation of Final Compounds 1–4. Hydrazine hydrate (0.15 mL, 3 mmol) was added to a solution of nitro derivatives 14a–d (1.0 mmol) in absolute EtOH (20 mL). 10% Pd/C (50 mg) was added portionwise to the warm solution, and the mixture was refluxed for 1 h. After cooling, the catalyst was removed by filtration through celite and the solvent was evaporated to dryness. H2O (20 mL) was added to the residue, and the aqueous phase was extracted with CHCl3 (2 × 30 mL). The collected organic layers were dried over Na2SO4 and evaporated under reduced pressure. The crude residue was chromatographed (CHCl3/MeOH, 19:1, as eluent) to afford pure compound 1–4 as yellow solid.
6-Amino-2-[5-[4-[2-methoxyphenyl]-1-piperazinyl]pentyl]-1H-benz[d]isoquinoline-3(2H)-dione (4). Yield 40%. 1H NMR (CDCl3) δ 1.41–1.51 (m, 2H), 1.59–1.69 (m, 2H), 1.71–1.80 (m, 2H), 2.48 (app t, 2H), 2.69 (br s, 4H), 3.11 (br s, 4H), 3.85 (s, 3H), 4.16 (t, 2H, J = 7.4 Hz), 5.01 (brs, 2H, D2O exchanged), 6.83–7.02 (m, 5H), 7.64 (d, 1H, J = 7.5 Hz), 8.11 (d, 1H, J = 8.5 Hz), 8.40 (d, 1H, J = 8.0 Hz), 8.58 (d, 1H, J = 7.4 Hz). ESI+/MS m/z 473.0 (MH+). ESI+/MS/MS m/z 281 (28), 225 (100); mp 210–212 °C (from CHCl3/n-hexane). Anal. (C28H32N4O3) C, H, N.

Fluorescence Spectroscopy. Emission and excitation spectra of compounds 1–12 were recorded as detailed in SI. Fluorescence quantum yields were calculated in reference to that of quinine sulfate in 0.5 M H2SO4 as a standard (excitation wavelength 350 nm; Φ = 0.546),17 as detailed in SI.

Fluorescent Labeling of Cells. Fluorescence microscopy observation in CHO cells overexpressing human 5-HT1A receptors were performed as detailed in SI.


References

(1) Pucadyil, T. J.; Kalipatnapu, S.; Chattopadhyay, A. The serotonin 

1a receptor: a representative member of the serotonin receptor


(2) Lacivita, E.; Leopoldo, M.; Berardi, F.; Perrone, R. 5-HT1A


(5) Daly, C. J.; McGrath, J. C. Fluorescent ligands, antibodies, and proteins for the study of receptors. Pharmacol. Ther. 2003, 100, 101–118.


(9) Chemel, B. R.; Roth, B. L.; Armbuster, B.; Watts, V. J.; Nichols, D. E. WAY-100635 is a potent dopamine D4 receptor agonist. Psychopharmacology (Berlin) 2006, 188, 244–251.


(12) Grabchev, I.; Chovelon, J.-M.; Qian, X. A copolymer of 4-X-N-

dimethylaminoethylene-N-allyl-1,8-naphthalimide with methyl-

mecrylate as a selective fluorescent chemosensor in homoge-


(13) Komn, A.; Kolb, V. M.; Terenius, L. Prolonged receptor block-


