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Identification of a red-emitting fluorescent ligand for in vitro visualization of human serotonin 5-HT_{1A} receptors

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ABSTRACT

The 5-HT_{1A} receptor subtype is the most thoroughly studied serotonin receptor subtype. We report here the design, synthesis and characterization of two new fluorescent ligands for the 5-HT_{1A} receptor. The new 1-arylpiperazine-based red-emitting fluorescent compound **6** displayed good binding affinity at the 5-HT_{1A} receptor ($K_i = 35$ nM) and was able to label specifically the human 5-HT_{1A} receptor stably expressed in CHO cells visualized using confocal laser scanning microscopy.

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The serotonin_{1A} receptor subtype (5-HT_{1A}) belongs to the large family of serotonin receptors that comprises at least 14 receptor subtypes. The 5-HT_{1A} receptor is a seven transmembrane domain receptor negatively coupled with adenylyl cyclase. This receptor subtype is the most extensively studied serotonin receptor. This was due to the early availability of a selective agonist (8-OH-DPAT) and also because the 5-HT_{1A} receptor was the first serotonin receptor to be cloned.^{1–3} A large body of literature exists on pathophysiological role of this receptor. The 5-HT_{1A} receptor has been initially implicated in anxiety and depression. More recent studies have shown that 5-HT_{1A} receptors are implicated in neuroprotection, cognitive impairment, and pain.⁴ Fluorescent ligands offer a possibility for exploring multiple information such as the mechanism of ligand binding, the movement and internalization of receptors in living cells, the physical nature of the binding pocket, and the visualization of labeled receptors,⁵ which would help in gaining a comprehensive molecular understanding of a given receptor. For example, fluorescent adenosine A₁ agonists and antagonists have been used to study the pharmacology of the receptor at a single-cell and single-molecule level by application of techniques

such as fluorescence correlation spectroscopy that provides the means by which the diffusion of receptor species within defined membrane microdomains can be monitored in real time.⁶ In view of such potential, we became recently involved in the design and synthesis of fluorescent ligands for the 5-HT_{1A} receptor. The high interest in this area of research is witnessed by the appearance in the literature of other fluorescent probes for the 5-HT_{1A} receptor during the preparation of the present manuscript.^{7,8} We have reported two distinct classes of fluorescent ligands for the 5-HT_{1A} receptor, exemplified by compounds **1** and **2** (Fig. 1). The approach that we employed to design the fluorescent compounds **1** and **2** was different from that commonly used. We envisaged the possibility to incorporate a fluorescent core into the pharmacophore scaffold of 'long-chain' arylpiperazine 5-HT_{1A} ligands, without affecting the affinity for the receptor. Therefore, we selected specific fluorescent moieties that could fit the scope. In this way, we were able to access an entire series of fluorescent ligands with the possibility to optimize the fluorescence properties and receptor affinity at the same time.^{9,10} Compounds **1** and **2** showed good fluorescent properties as well as high affinity for the target receptor. However, they failed to visualize 5-HT_{1A} receptors stably expressed in CHO cell line, because of high non-specific binding. Visualization experiments with compound **2** were reported earlier.¹⁰ The reason of such behavior was not clear. High non-specific binding has been related to high lipophilicity of the probe, but no

Abbreviations: 8-OH-DPAT, 8-hydroxy-*N,N*-dipropylaminotetraline; GPCR, G-protein coupled receptor.

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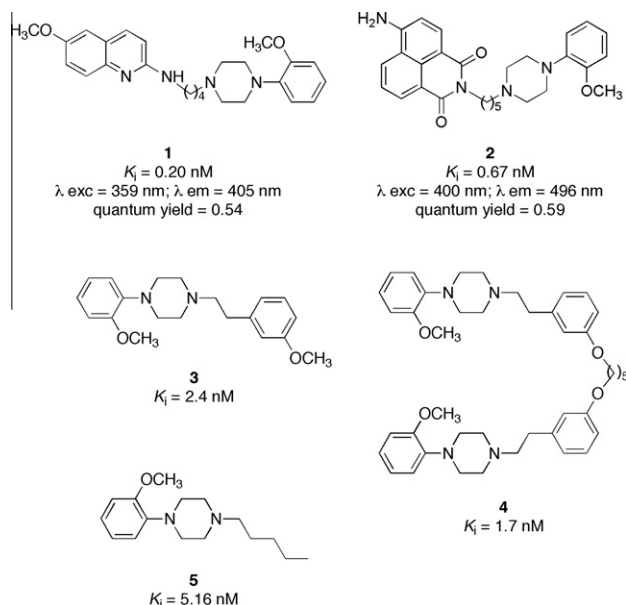


Figure 1. 5-HT_{1A} receptor affinities of compounds 1–5.

clear indications about this aspect emerge from the literature.^{11–13} On the other hand, the possibility that the ligand was bound to a molecular target other than 5-HT_{1A} receptor could not be ruled out. These results forced us to change strategy in the design of fluorescent probes for 5-HT_{1A} receptor.

We then decided to apply a design strategy that has delivered frequently successful results.¹⁴ The general approach to design a fluorescent ligand is to select a pharmacophore molecule with known pharmacological properties and to add a fluorophore to it. One of the main issues with the design of these ligands is knowing where to place the fluorogenic tag in the probe molecule since these tags can be a rather large moiety which needs to satisfy the structure–activity relationships of the original scaffold molecule. Because the binding pocket of G-protein coupled receptors (GPCRs) is buried deeply into transmembrane domains of the receptor protein, the pharmacophore scaffold needs to be properly separated from the fluorescent tag in order to prevent the loss of biological activity. Literature data on fluorescent ligands for GPCRs suggest that a six-atom (or longer) linker can be effectively installed.¹⁵ Therefore, the fluorescent ligand is constituted by three parts: the pharmacophore moiety, the fluorescent dye and a linker.

Keeping in mind these considerations, we have designed our new fluorescent probes for the 5-HT_{1A} receptor. The selection of the pharmacophore has been guided by literature data. In 2007, our research group reported on certain dimeric ligands formally derived from compound 3 that displayed affinity for both 5-HT_{1A} and 5-HT₇ receptors.¹⁶ We found that, in spite of the large size of the substituent in *N*-position of the 1-(2-methoxyphenyl)piperazine core (compound 4, as example), these derivatives displayed high affinity for the 5-HT_{1A} receptor. In 1995 Mokrosz and co-workers reported on some 1-(2-methoxyphenyl)piperazine derivatives that were *N*-substituted by alkyl groups of various length.¹⁷ In particular, the *N*-pentyl derivative 5 still retained high 5-HT_{1A} receptor affinity, suggesting the possibility of further structural manipulation in that position.

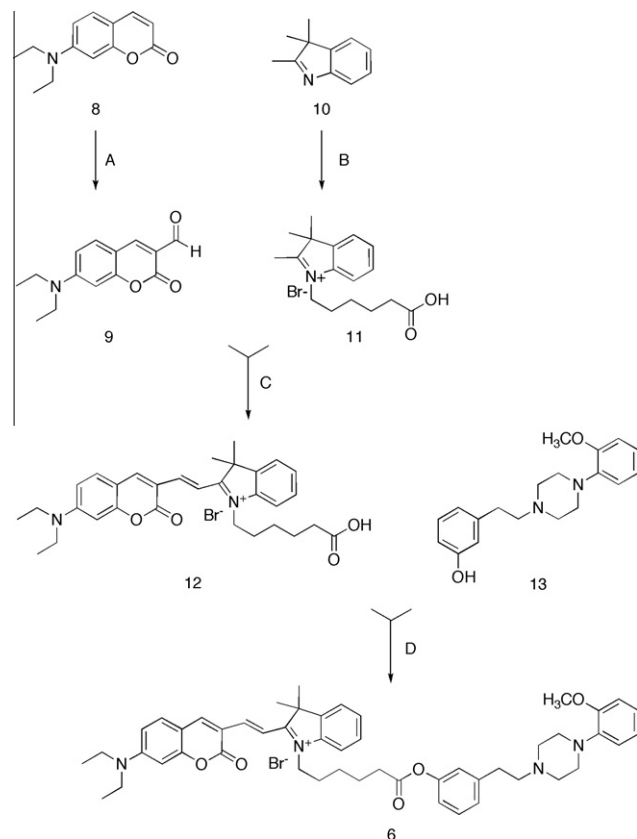
With these data in hands, we decided to synthesize two fluorescent probes on the basis of the structures of compounds 3 and 5. For our purpose, we have selected a red-emitting fluorophore ($\lambda = 650$ nm) and a near-infrared fluorophore ($\lambda = 830$ nm). There are several major advantages of using fluorescent dyes that absorb in the red over those that absorb at shorter blue and green wave-

lengths ($\lambda = 300$ –450 nm). The most important of these advantages is the reduction in background due to light scattering and cell autofluorescence that ultimately improves the sensitivity achievable. Moreover, dyes that absorb and emit within the window between 650 and 900 nm can be used on living cells because these wavelengths do not cause cell damage and also allow deep tissue penetration.¹⁸

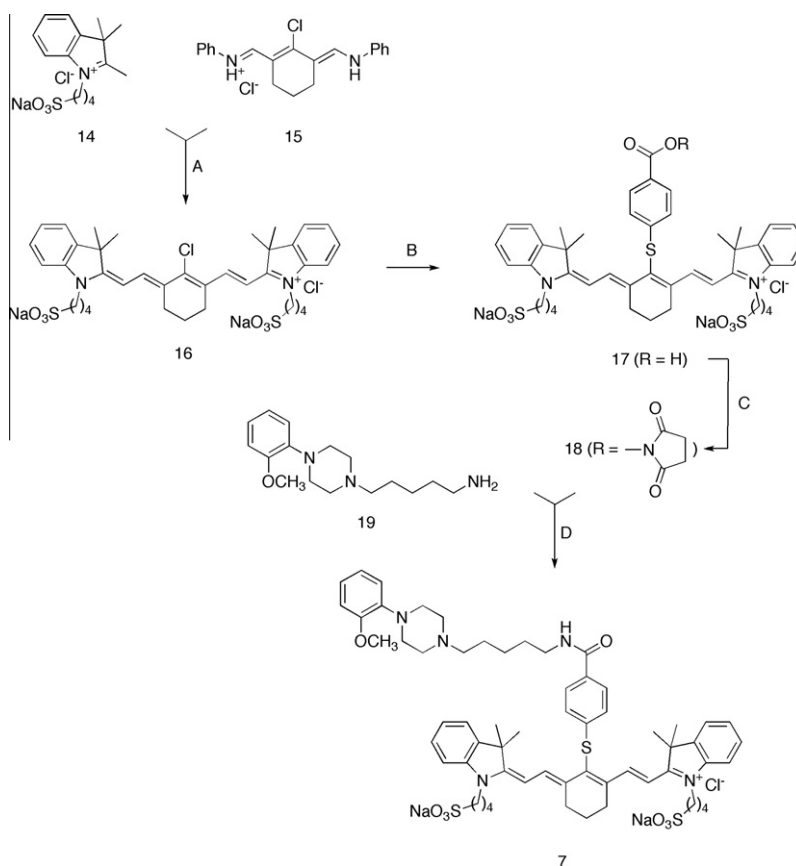
Therefore we have prepared compounds 6 (Scheme 1) and 7 (Scheme 2) that incorporated a red-emitting and a near-infrared fluorophore, respectively.¹⁹

The synthesis of compound 6 is depicted in Scheme 1. For our purpose we synthesized the new red-emitting fluorophore 12 taking advantage from the chemistry developed by researchers at Dyomics.²⁰ The red emitting fluorophore 12 was obtained in high yield starting from easily accessible synthons. Coumarine derivative 8²¹ was formylated with dimethylformamide in the presence of POCl₃ to give the aldehyde 9. The commercially available indolenine 10 was alkylated with 6-bromohexanoic acid to give the intermediate 11.²² This latter intermediate underwent aldol-like condensation with aldehyde 9 to give the fluorophore 12. This latter was reacted with the phenate of 13²³ to afford the final compound 6.²⁴

For the synthesis of the near-infrared tagged ligand we selected from the literature the dye 18²⁵ (Scheme 2) that could be prepared in high yield from easily accessible intermediates. Importantly, no chromatography was used throughout the synthetic route, and simple crystallizations directly from reaction mixtures were adequate to obtain intermediates in an analytically pure form. Trimethyl indoleninium salt 14²⁶ was condensed with iminium salt 15²⁷ to give the intermediate 16. This chloro derivative was reacted



Scheme 1. Reagents and conditions: (A) DMF/POCl₃, then 8, 60 °C, 12 h, 75%; (B) 6-bromohexanoic acid, 80 °C, 12 h, 76%; (C) freshly distilled Ac₂O, 150 °C, 4 h, 70%; (D) NaH and 13, anhydrous toluene, rt, 1 h, then 12, CDI, anhydrous THF, rt, overnight, 30%.



Scheme 2. Reagents and conditions: (A) absolute ethanol, sodium acetate, reflux, 3.5 h, 75%; (B) 4-mercaptobenzoic acid, DMF, rt, 24 h, 60%; (C) disuccinimidyl carbonate, DMAP, DMF, 70 °C, 2 h, 82%; (D) DMF, Et₃N, rt, overnight, 85%.

with 4-mercaptobenzoic acid to give carboxylic acid derivative **17**. The latter was esterified with disuccinimido carbonate to give the dye **18**. Condensation of the dye **18** with the amine **19** afforded the desired fluorescent compound **7**.²⁹

The target compounds were assessed for in vitro affinity at the human cloned 5-HT_{1A} receptor by radioligand binding assays, using [³H]8-OH-DPAT in membranes from transfected HEK-293 cells.³⁰ The inhibition constant *K_i* was calculated from the IC₅₀ value using the Cheng–Prusoff equation.³¹ The binding affinity data are shown in Table 1.

It can be noted that the compound **6** is approximately 10-fold less potent than the parent compound **3** (35 nM vs 2.4 nM). Although the impact of the modification performed on compound **3** was larger as compared to that given by the transformation from compound **3** to the dimer **4** (*K_i* = 1.7 nM), the 5-HT_{1A} receptor affinity of compound **6** was still high enough to perform visualization experiments of 5-HT_{1A} receptor by fluorescent microscopy. Also in the case of compound **7**, incorporation of the large near-infrared fluorophore into the parent compound **5** gave a loss in affinity (12-fold). All in all, the pursued strategy gave good results in term

of 5-HT_{1A} receptor affinities, confirming the validity of our initial assumptions on where to place the fluorophore moiety into the pharmacophore scaffold. Moreover, affinity data indicated that the linker was long enough to effectively separate the pharmacophore and the fluorophore, allowing a reasonably strong interaction between the pharmacophore and the binding crevice.

As far as the spectroscopic properties are concerned (Table 1), both target compounds showed excitation and emission wavelengths and quantum yields in methanol comparable to that reported in the literature for the parent fluorophores. In PBS buffer, the medium used in visualization experiments on cells, compound **6** retained the same fluorescent properties as in methanol, whereas the fluorescent properties of compound **7** were completely attenuated. This behavior, which was not known for this specific fluorophore, points to the importance of an accurate evaluation of the fluorescent properties of the probe in the medium in which it is intended to be used. This was disappointing because the possibility to test compound **7** in fluorescence microscopy experiments to visualize 5-HT_{1A} receptors in vitro was precluded. On the other hand, the potential of compound **6** as probe for visualization of

Table 1
Binding affinity data and spectroscopic properties³² of compounds **6** and **7**

Compd	<i>K_i</i> ± SEM ^a (nM)	MeOH				PBS buffer			
		λ _{exc} (nm)	λ _{em} (nm)	ε (M ⁻¹ cm ⁻¹)	Φ	λ _{exc} (nm)	λ _{em} (nm)	ε (M ⁻¹ cm ⁻¹)	Φ
6	35 ± 2	534	630	4.5 × 10 ⁵	0.3	564	635	4.0 × 10 ⁵	0.2
7	65.8 ± 3.1	800	830	3.0 × 10 ⁵	0.04	ND ^b	ND	ND	ND

^a The values are the mean ± SEM from three independent experiments performed in triplicate.

^b ND: Not Detectable.

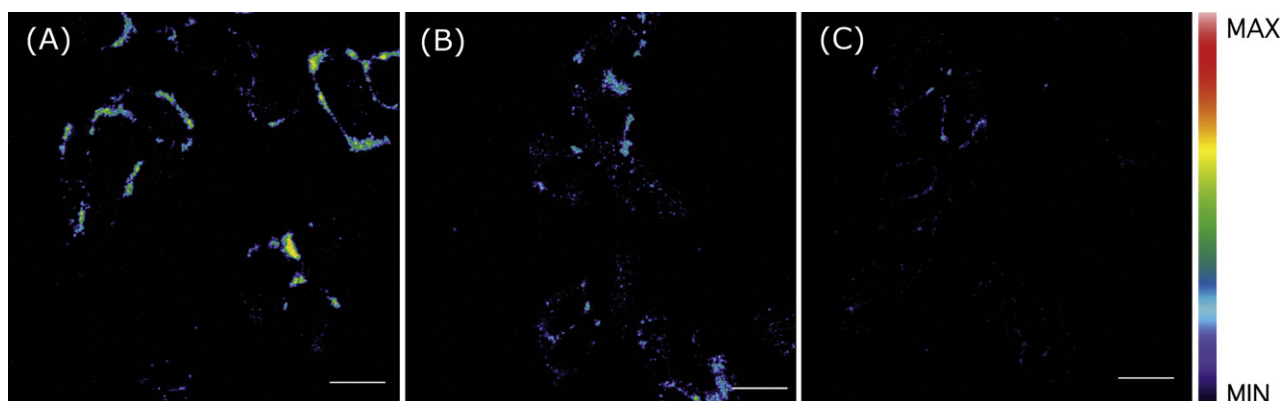


Figure 2. Representative fluorescent micrographs (color coded for intensity scale) of TCHO cells (CHO cells stably expressing the 5-HT_{1A} receptor) in absence (A) and in presence (B) of 2 mM 5-HT and (C) CHO cells labeled with 2 μM of compound **6** at 4 °C. The scale bar represents 20 μm. Compound **6** labeling was significantly lower in wildtype CHO cells (untransfected, i.e., not overexpressing the 5-HT_{1A} receptor) and in TCHO cells upon competition with 1000-fold excess of 5-HT, as compared to TCHO cells labeled with compound **6** alone under similar conditions.

the 5-HT_{1A} receptor in cells was assessed by confocal laser scanning microscopy.³³ The incubation of cells with compound **6** (2 μM) allowed visualization of the 5-HT_{1A} receptor in stably transfected CHO cells (TCHO) using conventional confocal microscopy (λ_{exc} = 561 nm) (Fig. 2A). Appreciable membrane labeling and less internalization was observed when labeling was performed at 4 °C. Moreover, a significant reduction in compound **6** labeling was observed in TCHO cells upon competition with 1000-fold excess of serotonin (Fig. 2B). Wild-type CHO (CHO) were incubated with the same concentration of compound **6** to assess the specificity of the labeling. CHO cells showed significantly less labeling as compared to TCHO cells under similar conditions (Fig. 2C). Considered together, these results indicate that compound **6** is a suitable probe for in vitro visualization of the 5-HT_{1A} receptor.

In conclusion, we have reported here the rational design, synthesis, fluorescent properties, and affinity values of two potential fluorescent probes for 5-HT_{1A} receptor. Of these ligands, compound **6** demonstrated the ability to label specifically 5-HT_{1A} receptors stably expressed in CHO cells without non-specific binding, which is different from our previous fluorescent ligands **1** and **2**. Thus, the change of strategy has been successful. While this manuscript was in preparation, a study has been published reporting the identification of fluorescent ligands for 5-HT_{1A} receptor through a similar approach.⁷ However, the ligands described therein incorporated the small dansyl fluorophore which emits in the green region of spectrum. It can be highlighted here that compound **6** possess fluorescent properties best suited for use in living cell. With this respect, the full potential of the compound **6** is currently under evaluation and results will be presented in due course.

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- yield pure compound as a green solid (0.09 g, yield 85%). ^1H NMR (CD_3OD): δ 1.33–1.39 (m, 2H), 1.45 (s, 12H), 1.52–1.60 (m, 2H), 1.89–2.04 (m, 12H), 2.41–2.47 (m, 6H), 2.62–2.75 (m, 8H), 3.01 (br s, 4H), 3.32–3.35 (m, 2H), 3.83 (s, 3H), 4.17 (app t, 4H), 6.35 (d, 2H, $J = 14$ Hz), 6.85–7.03 (m, 5H), 7.21 (td, 2H, $J = 1.1$, 7.2 Hz), 7.31–7.41 (m, 8H), 7.74 (app d, 2H), 8.72 (d, 2H, $J = 14$ Hz). ESI $^+$ /MS m/z 1104 (MH^+), 1126 (MNa^+), 1148 ($\text{M}(\text{Na}^+)_2$). ESI $^+$ /MS/MS m/z 1104 (100), 555 (13).
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32. Emission spectra of compounds **6** and **7** were determined in methanol or PBS buffer. In all experiments the excitation and the emission bandpass was set at 10 nm. The emission spectra were obtained from 500 to 900 nm with excitation set at the appropriate excitation wavelength. The excitation spectra of compounds **6** and **7** were obtained from 400 to 800 nm with the emission being recorded at the appropriate wavelength. Fluorescence quantum yields were calculated with respect to: rhodamine 6G in H_2O ($\Phi = 0.90$) for compound **6**; ICG in DMSO ($\Phi = 0.013$) for compound **7**. Quantum yields were calculated according to: Demas, J. N.; Crosby, G. A. *Phys. Chem.* **1971**, 75, 991. Absorption spectra were recorded with a PerkinElmer UV–vis–NIR spectrophotometer, fluorescence spectra were obtained with a PerkinElmer LS55 spectrofluorometer.
33. For fluorescence microscopy, stock solution of the compound **6** was prepared in ethanol and then diluted to the final concentration with PBS buffer in order that ethanol content in then labeling solution was <1% (v/v). CHO cells stably transfected with the intronless human genomic clone G-21 (Fargin, A.; Raymond, J. R.; Lohse, M. J.; Kobilka, B. K.; Caron, M. G.; Lefkowitz, R. J. *Nature* **1988**, 335, 358) and heterologously expressing the human 5-HT $_{1A}$ receptor (referred to as TCHO) (Kalipatnapu, S.; Pucadyil, T. J.; Harikumar, K. G.; Chattopadhyay, A. *Biosci. Rep.* **2004**, 24, 101), and CHO cells without the receptor were grown in D-MEM/F-12 (1:1) supplemented with 1.2 g/L of sodium bicarbonate, 10% fetal calf serum, 60 $\mu\text{g/mL}$ penicillin, 50 $\mu\text{g/mL}$ streptomycin, 50 $\mu\text{g/mL}$ gentamycin sulphate and 200 $\mu\text{g/mL}$ Geneticin (for TCHO) in a humidified atmosphere with 5% CO_2 at 37 $^\circ\text{C}$. For labeling studies, cells were washed twice with PBS buffer and labeling was carried out in PBS buffer supplemented with Ca^{2+} and Mg^{2+} . Cells were incubated with 2 μM of compound **6** for 5 min at 4 $^\circ\text{C}$ in order to inhibit endocytosis. To competitively wash off the compound **6**, PBS containing 2 mM 5-HT (1000-fold excess) was used. Cells grown in Lab-Tek chambers (Nunc, Denmark) were observed with a Zeiss LSM 510 Meta NLO confocal microscope (Jena, Germany). The imaging was performed with 63 \times , 1.4 NA, oil immersion objective. Compound **6** was excited at 561 nm with DPSS laser, and emission was collected using a bandpass filter at 575–630 nm.