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Development of Fluorescent Ligands for the Human 5-HT$_{1A}$ Receptor

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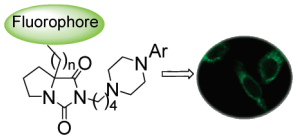
ABSTRACT In this work, we report the design and synthesis of a set of fluorescent probes targeting the human 5-HT$_{1A}$ receptor (h5-HT$_{1A}$R). Among the synthesized compounds, derivative **4** deserves special attention as being a high-affinity ligand (K_i = 2 nM) with good fluorescent properties (I_{em} > 1000 au and a fluorescence quantum yield, Φ_f, of 0.26), which enables direct observation of the h5-HT$_{1A}$R in cells. Thus, it represents the first efficacious fluorescent probe for the specific labeling of h5-HT$_{1A}$R in cells. Our results provide the basis for the introduction of a variety of tags in scaffolds of G protein-coupled receptor (GPCR) ligands that enable visualization, covalent binding, or affinity pull-down of receptors. These strategies should contribute to the optimization of the therapeutic exploitation of known or new members of the GPCR superfamily by providing valuable information about their location or level of expression.

KEYWORDS G protein-coupled receptors (GPCRs), serotonin (5-HT), 5-HT$_{1A}$ receptor, probes, fluorescent ligands, receptor visualization



The development of compounds that enable direct observation of subfractions of the proteome has become of paramount importance in the quest to understand biological processes at the systems level. In this context, activity-based protein profiling (ABPP) can be considered as one of the major contributions. This approach has been successfully applied to the study of different enzyme families[1] and has been recently extended to the nicotinic acetylcholine receptor ion channel.[2] However, the superfamily of G protein-coupled receptors (GPCRs), which account for more than the 50% of the druggable genome,[3] remains to be addressed.[1] The concept of ABPP probes for GPCRs is more complex than for enzymes or ion channels due to the different GPCR conformations that coexist in dynamic equilibrium and also because GPCR ligand binding can switch conformations between active and inactive state(s).[4,5] This fact might account for the lack of molecular probes able to generate knowledge on GPCRs in a high-throughput manner. Usually, information about the presence of a given GPCR is derived from detection of its cognate RNA, which might not correlate with the actual levels of protein.

Therefore, the development of probes for visualization of GPCRs can be considered as the first step in the application of ABPP principles to this field. With this broad objective in mind, we have initiated a project aimed at the search of fluorescent probes that enable direct visualization of GPCRs in complex biological systems. Although some GPCR fluorescent ligands have been recently described, their suitability for application in cell systems has proven difficult.[6] Moreover, the introduction of alternative tags other than fluorophores to obtain additional information in a high-throughput manner comparable to ABPP approaches in the field of enzymes is still lacking. We have focused on the 5-HT$_{1A}$ receptor (5-HT$_{1A}$R), one of the most important GPCRs from a therapeutic point of view. This serotonin (5-HT) receptor is involved in the regulation of key processes in neurobiology, including excitotoxicity, pain, and anxiety.[7] Furthermore, variation of 5-HT$_{1A}$R levels has been linked to the pathogenesis of a number of diseases.[8] Therefore, the availability of agents able to directly visualize the 5-HT$_{1A}$R would be of importance. In fact, some potent fluorescent 5-HT$_{1A}$R ligands have been recently reported,[9] although their use in cell systems was not optimized. In this work, we report the design of fluorescent probes based on the introduction of a fluorophore in the scaffold of our previously characterized 5-HT$_{1A}$R agonists without affecting affinity. Among the synthesized compounds, derivative **4** deserves special attention

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as being a potent 5-HT$_{1A}$R ligand (K_i = 2 nM) with good fluorescent properties (I_{em} > 1000 au and a fluorescence quantum yield, Φ_f, of 0.26), which enables direct observation of human 5-HT$_{1A}$R (h5-HT$_{1A}$R) in cells. These results support the validity of our approach and represent the starting point for further optimization in terms of other fluorophores or tags, experiments that are under way in our laboratory.

The design of the new fluorescent probes for the 5-HT$_{1A}$R was based on our previously reported potent agonists **1** (UCM-310590)[10] and **2** (UCM-2550)[11] (K_i = 5.5 and 2.4 nM, respectively; Figure 1). Using these arylpiperazines as scaffolds, we have explored different structural possibilities for the introduction of the fluorescent tag. Extensive structure−activity relationship (SAR) studies together with computational models of 5-HT$_{1A}$R−ligand interactions previously performed in our group (see ref 11 and references cited therein) have allowed us to identify the molecular determinants of the ligand−receptor interaction. On the basis of this knowledge, we envisioned that introduction of the fluorescent tag at position 7a of the bicyclohydantoin should not notably affect 5-HT$_{1A}$R affinity,[12] whereas modifications in the aryl substituent of the piperazine moiety might involve deeper changes in the affinity and selectivity of the ligands.[13] Therefore, we have considered two possibilities: (i) attachment of the tag to position 7a of the bicyclohydantoin using an aliphatic chain as spacer (compounds **3**−**8**) and (ii) replacement of the aromatic moiety of **1** and **2** (compounds **9**−**12**) by the tag (Figure 1).

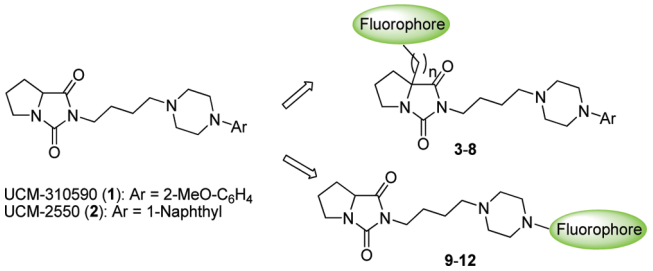


Figure 1. Design of fluorescent probes for the h5-HT$_{1A}$ receptor.

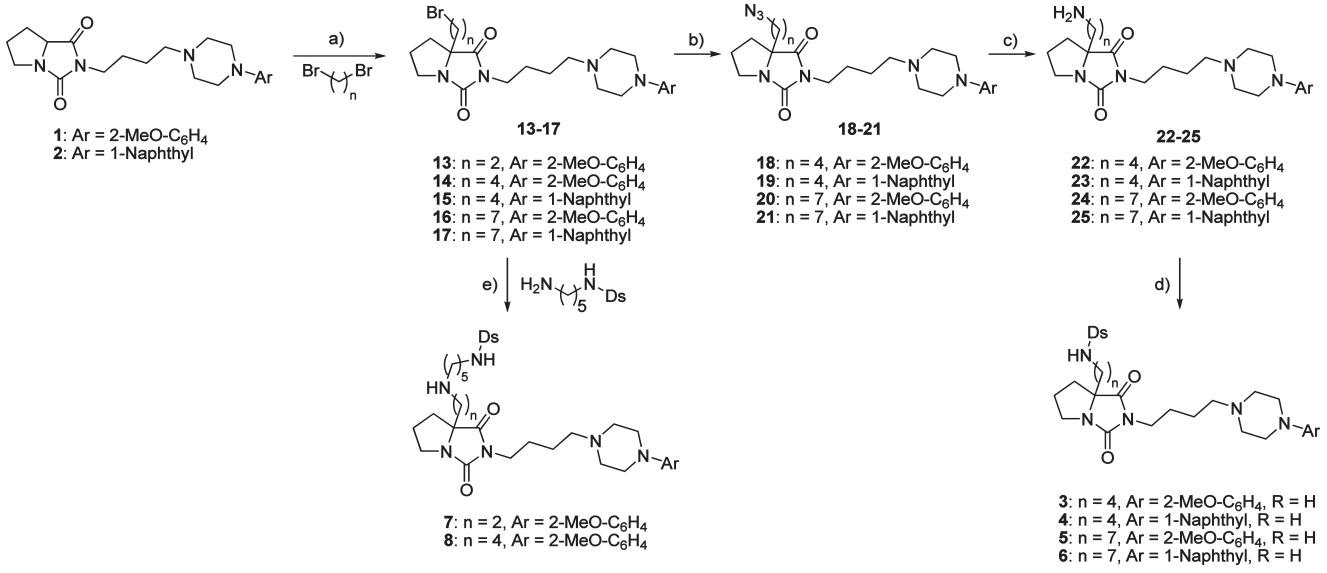
We have selected the dansyl (Ds) group [(5-dimethylamino)naphthalene-1-sulfonyl] as the fluorescent tag since it is of moderate size and it is commercially available as sulfonyl chloride; therefore, it can be readily introduced into the structures of compounds **1** and **2**, and it has satisfactory fluorescent properties to be used in a cellular environment.[14]

The new arylpiperazine derivatives **3**−**12** were synthesized following standard methodologies (Schemes 1 and 2). Bicyclohydantoins **1**,[10] **2**,[11] and **26**[10] were prepared according to previously described procedures. Detailed synthetic procedures for all new compounds are described in the Supporting Information.

Target compounds **3**−**12** were assessed for in vitro affinity at the h5-HT$_{1A}$R by radioligand binding assays, using [^{3}H]-8-hydroxy-DPAT in membranes from transfected HEK-293 EBNA cells (see the Supporting Information for details). The inhibition constant K_i was calculated from the IC$_{50}$ value using the Cheng−Prusoff equation,[15] and the values in Table 1 are the means of 2−4 independent experiments. The fluorescent properties of compounds **3**−**12** (Table 1) were measured as 10 μM solutions of the corresponding compound in the same buffer solution used for the binding assays.

High 5-HT$_{1A}$R affinity values were obtained when the Ds tag was attached by means of a spacer to the tertiary carbon of the bicyclohydantoin of **1** and **2** (compounds **3**−**8**). These results are consistent with our previous SAR studies and

Scheme 1. Synthesis of Compounds **3**−**8**[a]



[a] Reagents and conditions: (a) LDA, THF, 0 °C to room temperature, 24 h, 46−89%. (b) NaN_3, H_2O/DMF, 50 °C, 24 h, 87−92%. (c) PPh_3, H_2O/THF, room temperature, 24 h, 84−92%. (d) DsCl, Et_3N, CH_2Cl_2, room temperature, 16 h, 55−82%. (e) NaI, Et_3N, DMF, 50 °C, 16 h, 40−80%.

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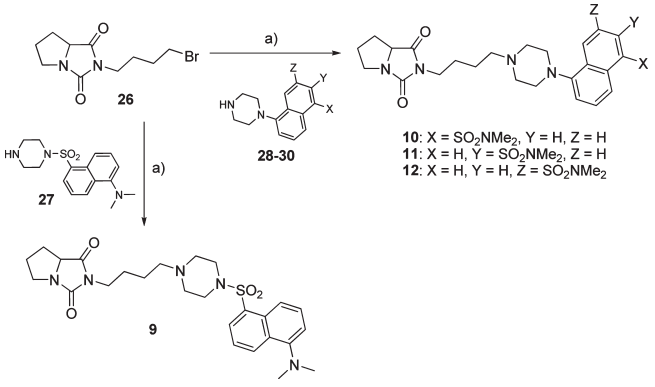
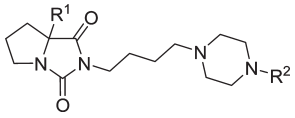
receptor−ligand interaction models, which indicated that position 7a admits voluminous substituents without affecting receptor binding. In general, derivatives with R^2 = 1-naphthyl showed higher affinities than their corresponding 2-methoxyphenyl counterparts, as seen, for example, in compound **4** vs **3** (K_i values of 2 and 11 nM, respectively) or in compound **6** vs **5** (K_i values of 5 and 15 nM, respectively). Further elongation of the spacer did not significantly increase binding affinity, as shown by the 2-methoxyphenyl derivatives **3** vs **5** and **7** vs **8** (K_i values of 11 vs 15 and 7 vs 9 nM, respectively). In particular, the new ligands **4** and **6**, with K_i values of 2 and 5 nM, respectively, showed affinities comparable to their parent compounds **1** and **2** (K_i of 5.5 and 2.4 nM, respectively). On the other hand, when the aromatic moiety of **1** or **2** was replaced by the fluorescent tag (compounds **9**−**12**), a variety of K_i values were obtained. Direct attachment of the Ds group to the piperazine (compound **9**) led to a complete lack of 5-HT_{1A}R affinity (K_i > 1000 nM). However, when the *N*,*N*-dimethylaminosulfonyl-substituted naphthalene ring is used as a Ds surrogate, affinity was restored. The position of the *N*,*N*-dimethylaminosulfonyl function in the naphthalene ring was found to be crucial for affinity, as illustrated by the diversity of K_i values obtained for ligands **10**−**12**, with K_i values ranging from 2.4 to

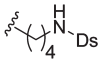
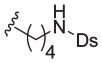
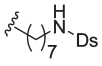
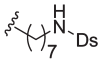
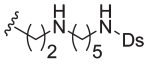
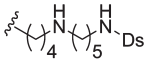
Scheme 2. Synthesis of Compounds **9**−**12**[a]



[a] Reagents and conditions: (a) Et_3N, CH_3CN, 60 °C, 24 h, 53−99%.

Table 1. 5-HT_{1A}R Affinity and Fluorescence Values for Compounds **1**−**12**



compd	R^1	R^2	K_i (nM)[a]	Fluorescence[b] λ_{ex}[c] (nm)	λ_{em}[c] (nm)	I_{em} (au) (em slit, nm)	Φ_f[d]
1	H	2-methoxyphenyl	5.5[e]	280	364	462 (5)	n.d.[f]
2	H	1-naphthyl	2.4[g]	330	416	353 (5)	n.d.
3		2-methoxyphenyl	11 ± 3	328	526	218 (5)	n.d.
4		1-naphthyl	2.0 ± 0.6	320	484	>1000 (2.5)	0.26
5		2-methoxyphenyl	15 ± 3	350	480	>1000 (5)	0.18
6		1-naphthyl	5 ± 3	320	480	>1000 (2.5)	0.24
7		2-methoxyphenyl	7 ± 2	350	527	169 (5)	n.d.
8		2-methoxyphenyl	9 ± 1	350	532	144 (5)	n.d.
9	H	dansyl	>1000	350	532	66 (5)	n.d.
10	H	5-dimethylaminosulfonyl-1-naphthyl	350 ± 20	350	509	930 (5)	n.d.
11	H	6-dimethylaminosulfonyl-1-naphthyl	98 ± 11	350	465	800 (5)	n.d.
12	H	7-dimethylaminosulfonyl-1-naphthyl	2.4 ± 0.1	350	470	>1000 (5)	0.10

[a] The values are the means ± SEMs from two to four independent experiments performed in triplicate. [b] Fluorescence values were determined at 10 μM in binding assay buffer (50 mM Tris-HCl and 0.5 mM $MgSO_4$, pH 7.4) at 25 °C. [c] Wavelengths for the maximal absorption and emission. The excitation slit was 2.5 nm in all cases. [d] The Φ_f values were determined in binding assay buffer at 25 °C. [e] Value from ref 10. [f] n.d., not determined. [g] Value from ref 11.

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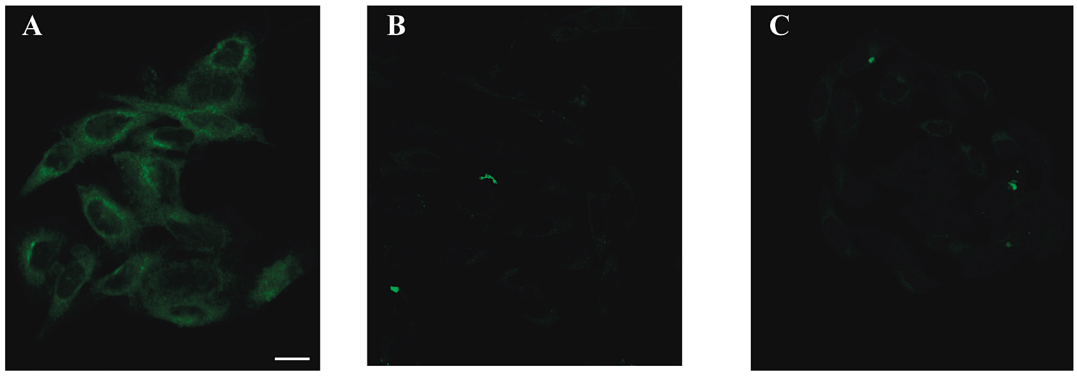

Figure 2. Labeling of CHO cells stably transfected with h5-HT_{1A}R with fluorescent compound **4**. Cells were incubated in the presence of **4** (250 nM) for 10 min, washed, fixed, mounted, and then observed by confocal microscopy (A). To assess specificity, cells were labeled under the same conditions with **4** (250 nM) in the presence of an excess (50 μM) of a nonfluorescent 5-HT_{1A}R ligand of the same series (R^1 = H, R^2 = 3-benzoylphenyl) (B) or in the absence of probe (C). The scale bar represents 20 μm. Preparations were visualized under a SP2 Leica confocal microscope with the 63× objective with a constant laser beam and photodetector sensitivity and are representative of two or three independent experiments.

350 nM. Among them, analogue **12** deserves special attention since it keeps the high affinity value of parent compound **2** [K_i (**12**) = 2.4 nM and K_i (**2**) = 2.4 nM].

A study of the fluorescent properties of all synthesized compounds with a constant excitation slit (2.5 nm) showed that, in general, 1-naphthyl derivatives bearing the tag in position 7a of the bicyclohydantoin exhibit the highest intensity emission I_{em} values (compounds **4** and **6**, with I_{em} > 1000 au for an emission slit of 2.5 nm). With respect to the spacer between the scaffold and the tag, the results suggest that its elongation increases the fluorescence intensity as shown in methoxyphenyl derivatives **5** vs **3** (I_{em} > 1000 au vs 218 au, respectively, for an emission slit of 5 nm). Nevertheless, the presence of an amine in the spacer was detrimental for the fluorescent properties, since derivatives **7** and **8** were poorly fluorescent (I_{em} values of 169 au and 144 au for an emission slit of 5 nm, respectively). Compounds **9**−**12**, bearing the fluorescent moiety attached to the piperazine, showed a diversity of fluorescence intensities, with I_{em} values ranging from low to moderate (I_{em} from 66 to above 1000 au for an emission slit of 5 nm). Additionally, Φ_f values were determined for those compounds with I_{em} > 1000 au for an emission slit of 5 nm (Table 1).

On the basis of their 5-HT_{1A}R affinity and fluorescence profiles, compounds **4** and **6** were selected as suitable candidates for cell visualization experiments (K_i values of 2 and 5 nM and Φ_f of 0.26 and 0.24, respectively). These compounds also showed a high Stokes shift, with an excitation wavelength of 320 nm and maximal emission around 480 nm, with both parameters in the suitable range for these experiments. Although both ligands are similar in terms of affinity and fluorescence profiles, during optimization of the experimental conditions for cell labeling, we observed slightly better results with probe **4** than with probe **6**; therefore, **4** was selected for further experiments. Selectivity of this derivative for 5-HT_{1A}R vs other metabotropic 5-HT receptors was assessed by a screening radioligand binding assay that included 5-HT_{2A}, 5-HT_{4e}, 5-HT_{5a}, 5-HT_6, and 5-HT_7 receptors. Compound **4** (at a concentration of 1 μM) displaces more than the 80% of the corresponding radioligand at 5-HT_{2A}, 5-HT_{5a}, and 5-HT_7 receptors, and it does not significantly bind to 5-HT_{4e} or 5-HT_6 receptors. These results are in agreement with the in vitro profile of the parent compound **2** and support the initial hypothesis that it is possible to introduce a tag in the 7a position of the bicyclohydantoin of these ligands without dramatically changing their in vitro profiles.

To assess the full potential of this type of probe for visualization of the 5-HT_{1A}R in cells, **4** was used in in vitro cell labeling experiments. The incubation of cells with probe **4** (250 nM) allowed direct observation of the h-5HT_{1A}R in stably transfected CHO cells using conventional confocal microscopy (λ_{exc} = 405 nm) (Figure 2A). To assess the specificity of the labeling, transfected cells were incubated with compound **4** in the presence of an excess of the high-affinity (K_i = 38 ± 9 nM) and nonfluorescent ($I_{em} \cong 0$ au, λ_{exc} = 350 nm for excitation/emission slits of 2.5/10 nm) 5-HT_{1A}R ligand belonging to the same series (R^1 = H, R^2 = 3-benzoylphenyl; see the Supporting Information), which essentially eliminated fluorescent labeling (Figure 2B). Figure 2C shows cellular autofluorescence in the absence of probe. Considered together, all of these results confirm the suitability of **4** as a probe for in vitro visualization of the h5-HT_{1A}R.

In summary, the synthesized compounds described herein represent the first efficacious fluorescent probes for the labeling of the h5-HT_{1A}R in cells. Moreover, most of the tagged ligands showed nanomolar affinity for the h5-HT_{1A}R, supporting the generality of our approach. These results are the starting point for introducing a variety of tags in these hydantoin-arylpiperazine ligands for further optimization of the probes in terms of binding selectivity and fluorescence (other fluorophores might be better in terms of wavelength emission, intensity, or both) and for incorporation of other tags that enable covalent binding or affinity pull-downs. These strategies should contribute to an optimized therapeutic exploitation of known or new members of the GPCR superfamily by providing valuable information about their location or level of expression. All of these experiments are currently under way in our laboratory and will be reported in due course.

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SUPPORTING INFORMATION AVAILABLE Full synthetic procedures, analytical and spectral characterization data of the synthesized compounds including elemental analyses, experimental details for the determination of binding affinity, and experimental protocols for spectroscopic measurements, cell labeling, and visualization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions: ‖ These authors contributed equally to this work.

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ABBREVIATIONS 5-HT, serotonin; ABPP, activity-based protein profiling; au, arbitrary units; Ds, dansyl; Φ_f, fluorescence quantum yield; GPCR, G protein-coupled receptor; h5-HT_{1A}R, type 1A of the human 5-HT receptor; *I*, intensity; K_i, ligand−receptor equilibrium dissociation constant; SAR, structure−activity relationship.

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