Published in final edited form as:

Bioorg Med Chem Lett. 2013 January 1; 23(1): 323–326. doi:10.1016/j.bmcl.2012.10.089.

A Rhodamine-Labeled Citalopram Analogue as a High-Affinity Fluorescent Probe for the Serotonin Transporter

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Abstract

A novel fluorescent ligand was synthesized as a high-affinity, high specificity probe for visualizing the serotonin transporter (SERT). The rhodamine fluorophore was extended from an aniline substitution on the 5- position of the dihydroisobenzofuran ring of citalopram (**2**, 1-(3-(dimethylamino)propyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile), using an ethylamino linker. The resulting rhodamine-labeled ligand **8** inhibited [³H]5-HT uptake in COS-7 cells ($K_i = 225$ nM) with similar potency to the tropane-based JHC 1-064 (**1**), but with higher specificity towards the SERT relative to the transporters for dopamine and norepinephrine. Visualization of the SERT with compound **8** was demonstrated by confocal microscopy in HEK293 cells stably expressing EGFP-SERT.

Keywords

Fluorescent ligand; citalopram; SERT; rhodamine

The serotonin transporter (SERT) is located in the membranes of presynaptic serotonergic neurons, and functions to transport excess serotonin into the cell from the synaptic cleft to terminate serotonergic signaling.¹ Although selective serotonin reuptake inhibitors (SSRIs) are used clinically for the treatment of anxiety and depression, drug-protein interactions and long-term effects of these drugs on SERT trafficking and function that affect clinical efficacy remain largely unknown. Fluorescent probes can be powerful tools for the detection of and structure-function studies on membrane proteins.^{2–15}

A fluorescently labeled ligand that selectively binds to a transporter or receptor protein can be used to localize protein distribution in tissue and monitor its trafficking, including internalization in live cells or tissue.^{3, 4} In addition, these tools can be used to study protein

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Supplementary data

Supplementary data associated with this article can be found in the online version.

Supporting Information Available: Experimental methods can be found in the online version.

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function, such as upstream and downstream molecular regulation,^{2, 10, 11} protein oligomerization,⁵ or potentially, clinical efficacy for certain pathological conditions.¹⁵ Combined with other techniques, these structure-function studies can aid in understanding ligand-protein interactions, such as protein conformational changes caused by ligand binding.^{7, 9} and also facilitate protein structure analysis.^{13–14}

Previously, fluorescently tagged 2 β -carbomethoxy-3 β -(3,4-dichlorophenyl)tropane analogues (e.g. **JHC 1-064**, **1**, Fig. 1) were synthesized and used to visualize dopamine transporter (DAT) trafficking in cultured live midbrain dopaminergic neurons.^{3, 4, 16} DAT, along with the norepinephrine transporter (NET) and SERT are three highly homologous transporters belonging to the neurotransmitter: sodium symporter (NSS) family, which share a common protein tertiary structure with twelve transmembrane (TM) domains.¹ Since the tropane-based analogues reported previously bind to all three transporters with similar affinities,¹⁶ a fluorescent molecule, based on a SERT-selective ligand, could be an important addition to the armamentarium of tools to study the structure and function of the SERT.

The antidepressants citalopram (**2**, Fig. 1) and its S-enantiomer are selective serotonin reuptake inhibitors (SSRI) that bind with high affinity to SERT (K_i = 1.94 nM, 0.89 nM, respectively) and selectively over NET (ratio = 3070, 11800 respectively) and DAT (ratio = 4780, 9160 respectively).¹⁷ Structure-activity relationship (SAR) studies, based on a series of citalopram (**2**) analogues, have been described wherein modifications at the N-, 4, and 5-positions have identified points of attachment that maintain both high affinity and SERT selectivity.^{17–20} Specifically, most modifications at the 5-position of the dihydroisobenzofuran ring were well tolerated at the SERT (K_i = 1–40 nM), and none of these analogues demonstrated high binding affinity at NET or DAT, which revealed that steric bulk is tolerated at this position.¹⁷ This is important as the fluorophore is a molecule of equal or greater molecular weight compared to the pharmacophore and thus must be attached to a position where steric bulk does not significantly diminish binding affinity. Among these compounds, the 5-(3'-NH₂-Ph) analogue **3** (K_i = 10.4 nM)¹⁷ was chosen as the template for synthesis of the first citalopram-based fluorescent ligand, as it contained a potentially modifiable NH₂ group.

In this study, we chose rhodamine as the fluorophore, as previous studies with Rhodamine RedX-NHS ester established that it was ideally suited for straightforward synthesis and visualization studies.¹⁶ In addition, rhodamine is widely used for fluorescence studies in biological systems due to its photostability and high quantum yield. It also has a suitable excitation spectrum for the use of relatively inexpensive lasers and is outside the intrinsic fluorescence from protein tryptophans.

The first synthetic strategy developed was to link compound **3** directly to Rhodamine RedX-NHS ester. Unfortunately, the aniline group was not sufficiently reactive, under basic reaction conditions, either at room temperature or at 45°C to couple to the rhodamine reagent, hence, an ethylamino linker was introduced.¹⁶ However, no reaction was detected under basic conditions using 2-bromoethylphthalimide as a reagent, even when the temperature reached >100°C. Another published method also failed to condense the aniline group of compound **3** with N-protected alanine under the condition of N-methylmorpholine and isobutylchloroformate.²¹ In addition, reaction between compound **3** and N-protected alanine anhydride²² failed and resulted in a salt form of **3**.

The strategy shown in Scheme 1 wherein compound **3** was reacted with freshly prepared acyl chloride **5**, made from the N-protected alanine **4**, following a modified procedure²³ was successful. The resulting compound **6** was deprotected with hydrazine to give the free amine

7, which was reacted with rhodamine red-X NHS ester to give the desired fluorescent compound **8**.

The binding affinity and SERT selectivity of compound **8** was determined measuring inhibition of [³H]5-HT uptake into COS-7 cells transiently expressing the hSERT and [³H]dopamine ([³H]DA) uptake into COS-7 cells transiently expressing the hDAT and hNET. These data were compared to the inhibition potency of **1** as determined previously¹⁶ (Table 1).

The fluorescent compound **8** showed a similar potency to **1** for inhibition of [³H]5-HT uptake and was selective for SERT (~50-fold) over DAT and NET, although less selective than the parent molecule **2**.

The fluorescent properties of compound **8** were determined as shown in Figure 2. The maximal excitation and emission wavelengths are at 570 and 592 nm, respectively, (*I*_F, fluorescence intensity. a.u., arbitrary units.)

To demonstrate specific labeling of SERT with compound **8**, we used a HEK293 cell line stably expressing hSERT with EGFP fused to the N-terminus. Cells were incubated with compound **8** for 60 min at 37°C with or without the specific SERT inhibitor paroxetine. As shown in Figure 3, live cell imaging using confocal microscopy revealed a uniform distribution of compound **8** (red) at the cell surface overlapping with the distribution of EGFP-SERT (green). In the presence of paroxetine, no fluorescence was observed at the cell surface in the red channel. The same result was observed when incubation of compound **8** was performed in the presence of 1 μM S-citalopram or 100 μM 5-HT (data not shown). These data demonstrate that compound **8** labels SERT with high specificity and enables SERT visualization in live cells.

In summary, a novel fluorescent ligand selective for hSERT was designed and synthesized using citalopram (**2**) as the SERT-selective pharmacophore. The resulting rhodamine-labeled ligand **8** demonstrated suitable serotonin uptake kinetics (*K*_i = 225 nM) and selectivity over DAT and NET. Specific labeling of the SERT with compound **8** was visualized by confocal microscopy in HEK293 cells stably expressing EGFP-SERT. Further studies comparing compound **8** and **1** in labeling SERT in living neuronal cells are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources: This work was funded by the NIDA-Intramural Research Program (A.H.N.), the Center for Pharmacogenomics, University of Copenhagen, NIH Grant P01 DA 12408, The Lundbeck Foundation and University of Copenhagen Program of Excellence (C.J.L.). The Danish Council for Independent Research, Sapere Aude Program (C.J.L.)

A special thanks to Erick Garcia-Gorbea for the HPLC analysis of compound **8** and to Dr. Amina Woods for the MALDI-TOF analysis of compound **8**.

Abbreviations

SERT   serotonin transporter

NET	norepinephrine transporter
DAT	dopamine transporter
NSS	neurotransmitter:sodium (Na^+/Cl^-) symporter
SSRI	selective serotonin reuptake inhibitor
NBD	nitrobenzoxadiazolealanine
NMM	N-methylmorpholine
NHS	N-hydroxysuccinimide
EGFP	enhanced green fluorescent protein

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Figure 1.
JHC 1-064 (1) Citalopram (2) and its 5-anilino analogue (3).

Figure 2.
Excitation (black) and emission (red) wavelength spectra of compound 8.

Figure 3.

Visualizing **8** binding to hSERT in transfected HEK293 cells. HEK293 cells stably expressing EGFP-hSERT were preincubated in buffer with or without 1 μ M paroxetine for 30 min before one hour incubation with 20 nM **8** at 37°C. **8** labeling was visualized by confocal microscopy. Pictures are representatives of more than 3 individual experiments. Scale bar, 10 μ m.

Scheme 1.

Synthesis of SERT fluorescent compound **8**. Reagents and conditions: (a) 150°C , 2 h; (b) thionyl chloride, CH_2Cl_2 , reflux, 5 h; (c) NEt_3 , CH_2Cl_2 , rt, overnight; (d) NH_2NH_2 , rt, 5 h; (e) (*i*-pr) $_2$ ethylamine, DMF, rt, overnight.

Table 1

K_i values of novel citalopram analogues to hSERT, hDAT and hNET determined by their inhibition potency of radiolabeled substrates^a

compound	hSERT	hDAT	hNET	n	n	n
	[³ H]5-HT inhib. K_i (nM)	[³ H] DA inhib. K_i (nM)	[³ H] DA inhib. K_i (nM)			
5-HT (K_{M0})	491[416;579]	nd	nd	8		
1, JHC 1-064	392 [286;537]	62 [37;105]	194 [153;244]	3	3	3
2	16[12;22]	>1,000,000	>1,000,000	6	2	4
3	390[313;485]	nd	nd	6		
8	225[208;241]	10900[9300;12900]	11800[10800;13000]	6	3	3
DA (K_M)	-	1010[909;1120]	396[369;425]		4	5

^a Uptake inhibition of [³H]5-HT at hSERT and [³H]dopamine ([³H]DA) at hDAT and hNET (K_i values) (mean [SE interval]). K_i values were determined from uptake inhibition experiments of indicated radiolabeled in COS7 cells stably expressing hSERT, hDAT or hNET. The K_i values were calculated from IC₅₀ values determined by nonlinear regression analysis of uptake data using the equation $K_i = IC_{50} / (1 + (L / K_M))$ where L is the concentration of [³H]5-HT or [³H]DA. The K_M value for 5-HT and DA were determined in parallel. nd, not determined.