

Novel and High Affinity Fluorescent Ligands for the Serotonin Transporter Based on (S)-Citalopram

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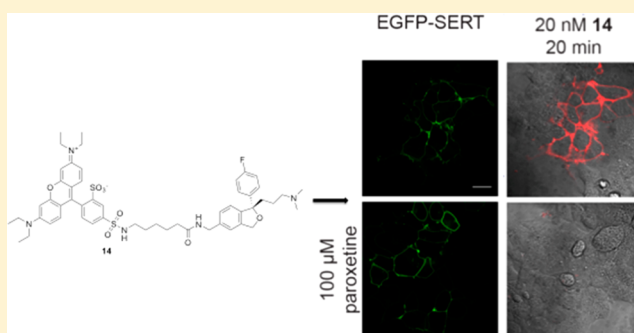
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S Supporting Information

ABSTRACT: Novel rhodamine-labeled ligands, based on (S)-citalopram, were synthesized and evaluated for uptake inhibition at the human serotonin, dopamine, and norepinephrine transporters (hSERT, hDAT, and hNET, respectively) and for binding at SERT, in transiently transfected COS7 cells. Compound **14** demonstrated high affinity binding and selectivity for SERT ($K_i = 3$ nM). Visualization of SERT, using confocal laser scanning microscopy, validated compound **14** as a novel tool for studying SERT expression and distribution in living cells.

KEYWORDS: Fluorescent ligand, SERT, citalopram, rhodamine



The serotonin transporter (SERT) is a member of the Neurotransmitter:Sodium Symporter (NSS) family of transporters and regulates serotonin signaling and homeostasis by mediating reuptake of extracellular serotonin.¹ The important classes of selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) that are prescribed for the treatment of anxiety, and major depressive disorders work through inhibition of serotonin reuptake via SERT. Nevertheless, only a rudimentary understanding of the molecular and cellular mechanisms governing native SERT expression, distribution, and trafficking exists currently. Hence, further investigation is critical for understanding the pathophysiology of these disorders, the effects of chronic drug use, and how to improve long-term treatment.²

One approach to studying native protein function in live cells or brain tissue is to use fluorescently labeled small molecules.^{3,4} Fluorescent ligands that have high affinity and selectivity for their target protein can retain pharmacological properties of the parent molecule and allow concomitant localization and real time monitoring of cellular processes, including distribution, trafficking, sequestering, and postendocytotic sorting.^{4,5} Thus, with the technological advances in fluorescent microscopy, these small molecules can serve as powerful tools for investigating CNS target–ligand interactions and the systems in which they reside and function.

The tropane-based fluorescent ligand, JHC1-64, as well as related analogues that have fluorophores other than rhodamine, have proven highly useful in studying DAT in transfected

heterologous cell systems as well as monitoring endogenous DAT in cultured dopaminergic neurons.^{5–8} However, the lack of selectivity across monoamine transporters and relatively low affinity for SERT decreases the utility of this fluorescent ligand for SERT trafficking studies. Recently, we reported our first attempt at a SERT selective fluorescent ligand ZP 455, using (±)-citalopram as the parent compound (Figure 1).⁹ While ZP 455 demonstrated moderately high affinity and selectivity for SERT ($K_i = 225$ nM) over DAT and NET in HEK293 cells, labeling of SERT in neurons was weak and characterized by

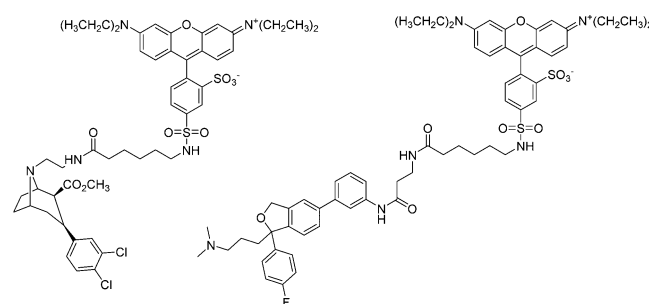


Figure 1. JHC1-64 and ZP 455 (compound 8 in Zhang et al.⁹).

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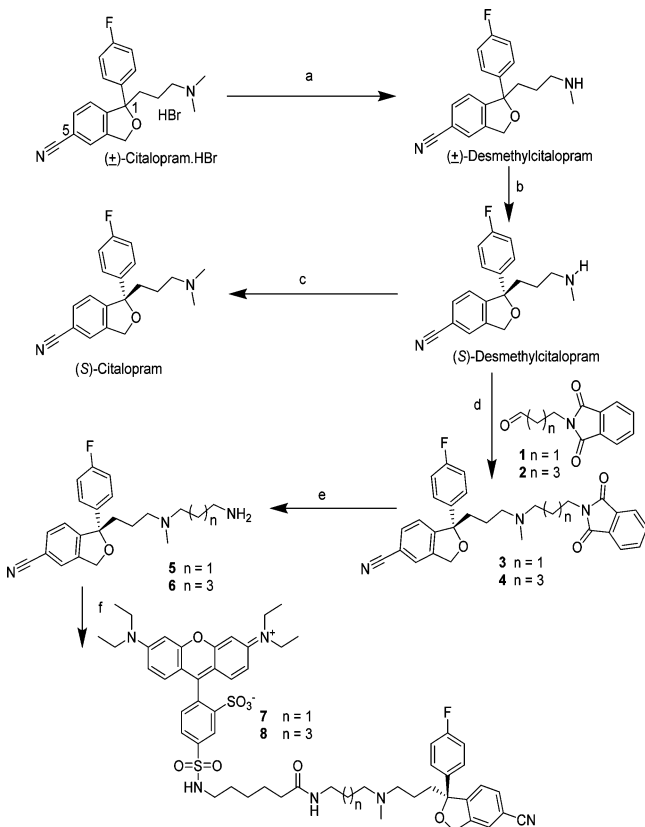
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rapid dissociation of the ligand, possibly due to its relatively low affinity for SERT.

In the present study, using recently developed SAR¹⁰ and chiral separation of (\pm)-citalopram to give the pure enantiomer, (*S*)-citalopram, we developed novel C-1 and C-5 substituted rhodamine labeled ligands with high binding affinity and SERT selectivity.

Scheme 1 outlines the synthetic strategy used for the synthesis of C-1 based fluorescent citalopram analogues 7 and 8^a

Scheme 1. Synthesis of SERT Fluorescent Compounds 7 and 8^a



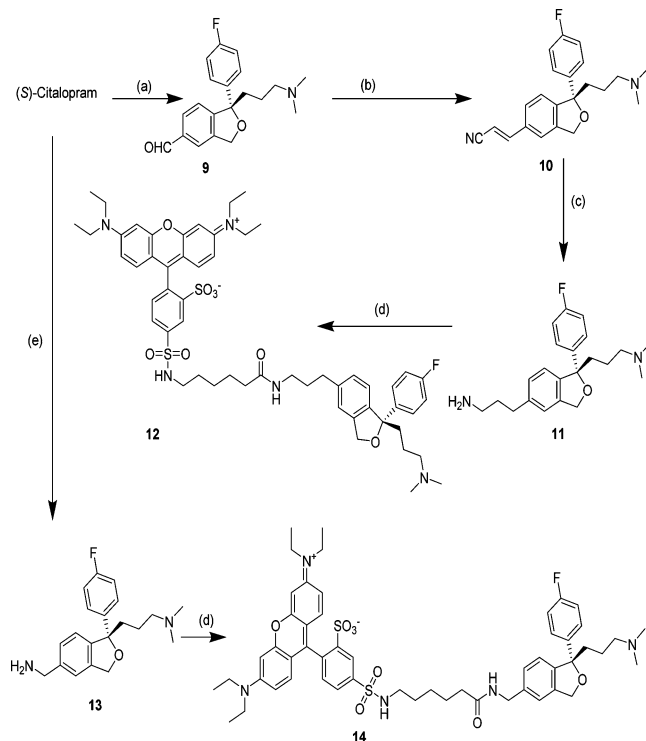
^aReagents and conditions: (a) (i) NH_4OH (ii) ACE-Cl , DCE, 18 h; (b) (+)-DPTTA, MeOH; (c) formaldehyde, formic acid, H_2O , reflux, 5 h; (d) $\text{NaBH}(\text{OAc})_3$, AcOH, DCE, RT, 12 h; (e) hydrazine, EtOH, reflux, 3 h; (f) Rhodamine red-X NHS ester, (*i*-pr)₂ethylamine, DMF, 40–45 °C, 24 h.

8. The HBr salt of (\pm)-citalopram was neutralized and demethylated to afford desmethylcitalopram, as previously described.¹⁰ The (*S*)-enantiomer was separated using (+)-DPTTA ((+)-*O,O'*-di-*p*-toluoyl-D-tartaric acid) as the chiral resolving agent with modifications of a previously reported procedure.¹¹ (*S*)-Desmethylcitalopram was further *N*-alkylated and reduced in the presence of $\text{NaBH}(\text{OAc})_3$ to give intermediates 3 and 4.¹² Deprotection of compounds 3 and 4 using hydrazine gave the free amines 5 and 6, respectively. Rhodamine-NHS ester was coupled to the free amine intermediate 5 and 6 under basic conditions to afford the final products 7 and 8. (*S*)-Desmethylcitalopram was *N*-alkylated to afford (*S*)-citalopram according to the published procedure.¹³

To carry out the synthesis of the C-5 based fluorescent ligand 12, (*S*)-citalopram was oxidized in the presence of an

aluminum–nickel catalyst in formic acid to its aldehyde intermediate 9 (Scheme 2).¹⁰ Compound 9 underwent

Scheme 2. Synthesis of SERT Fluorescent Compounds 12 and 14^a



^aReagents and conditions: (a) Ni–Al alloy, formic acid, reflux, 5 h; (b) cyanoacetic acid, morpholine, DMF, reflux, 5 h; (c) NaBH_4 , CoCl_2 , MeOH, 0 °C to RT, 16 h; (d) rhodamine-NHS ester, (*i*-pr)₂ethylamine, DMF, 40–45 °C, 24 h; (e) LAH, THF, 5 h.

nucleophilic substitution with cyanoacetic acid to afford intermediate 10, which upon reduction provided compound 11.^{14,15} Rhodamine-NHS ester was coupled to 11 under basic conditions to afford compound 12. To synthesize compound 14, (*S*)-citalopram was reduced to intermediate 13¹⁰ and coupled with rhodamine-NHS ester.⁹

All four novel rhodamine analogues (7, 8, 12, and 14) were evaluated for inhibition of serotonin or dopamine uptake at their respective transporters (e.g., hSERT, hDAT, and hNET) and compared to JHC1-64 in transiently transfected COS7 cells using [³H]5-HT for SERT and [³H]dopamine for NET and DAT. On the basis of these assays, compounds 12 and 14 showed the highest apparent affinities at SERT suggesting that substitution at the C-5 position of (*S*)-citalopram resulted in the most well-tolerated analogues at SERT, and the affinities were not significantly influenced by the difference in the linker length between the fluorophore and the parent compound (Table 1). None of the four new fluorescent analogues had any inhibitory effect on the uptake of [³H]dopamine at DAT or NET, at a concentration of 10 μM , in contrast to JHC1-64.

Next, we determined the affinities of the (*S*)-citalopram-based analogues in competition binding assays using [³H](*S*)-citalopram as the radioligand. Because the compounds were highly SERT specific according to the uptake inhibition assay data, only hSERT binding affinities were assessed (Table 2). All four compounds showed moderate to high affinities for SERT; however, compound 14 displayed an affinity similar to that of

Table 1. Uptake Inhibition at hSERT, hDAT, and hNET Expressed in COS7 Cells^a

compd	SERT K_i (nM) [SE interval]	DAT K_i (nM) [SE interval]	NET K_i (nM) [SE interval]
(S)-citalopram	7.4 [3.0–19]	IA	IA
7	140 [110–180]	IA	IA
8	250 [130–450]	IA	IA
12	37 [22–61]	IA	IA
14	34 [19–69]	IA	IA
JHC1-64	94 [72–120]	20 [8–51]	91 [73–110]

^aFor determination of K_i values, IC_{50} values were determined as described in the Supporting Information from nonlinear regression analysis of uptake inhibition assays on transfected COS7 cells using 2,5,6-³H-dopamine for DAT and NET or 5-hydroxy³H-tryptamine for SERT. The K_M values for 2,5,6-³H-dopamine or 5-hydroxy³H-tryptamine were determined in parallel and used for calculating indicated K_i values. The SE interval for each K_i value is indicated and was calculated from $pK_i \pm SE$. Data are means [SE interval] of three to four repeated measurements performed in triplicate. IA = inactive at a conc. of 10 μ M.

Table 2. hSERT Affinities of the Fluorescent Analogues Assessed by Competition Binding^a

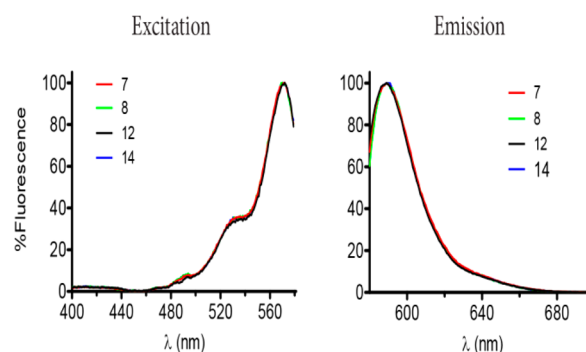
compd	SERT K_i or K_d (nM) [SE interval]
(S)-citalopram	2.6 [1.8–3.8]
7	11 [10–13]
8	180 [160–190]
12	11 [6–19]
14	3.0 [2.9–3.1]

^aFor determination of K_i values, IC_{50} values were determined as described in the Supporting Information from nonlinear regression analysis of competition binding assays using [³H]-S-citalopram (2 nM) as the radioligand. The K_d value for [³H]-S-citalopram was determined in parallel and used for calculating indicated K_i values. The SE interval for each K_i value is indicated and was calculated from $pK_i \pm SE$. Data are means [SE interval] of three to four repeated measurements performed in triplicate.

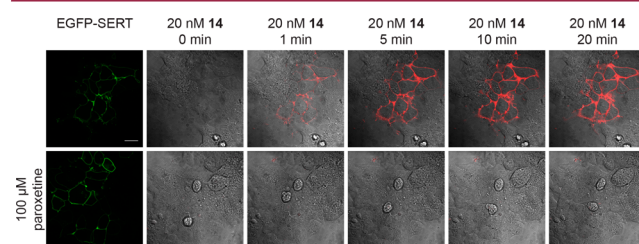
the parent compound, (S)-citalopram; ~4-fold higher affinity than compounds 7 and 12 and 60-fold higher affinity than compound 8 (Table 2). The finding that compound 14 binds with low nanomolar affinity to SERT, and thus with an affinity comparable to the parent compound, is remarkable considering the size of the rhodamine appended to the C-5 position and suggests that it does not interfere with critical binding interactions at the SERT binding site. Our previous fluorescently tagged citalopram ligand, ZP 455, also had rhodamine appended in the C-5 position; however, its SERT binding affinity was ~75-fold lower than for compound 14. In addition to being racemic, ZP 455 possessed an aniline moiety attached to the C-5 position, and this may have contributed to the poorer solubility and binding kinetics of ZP 455 compared to the present set of compounds.

The fluorescent properties for the four compounds 7, 8, 12, and 14 were determined on a fluorescence spectrometer (Figure 2). The compounds had an excitation maximum of 571, 570, 572, and 572 nm, respectively, and all four analogues had an emission maximum of 589 nm. Thus, the different linker lengths or position of substitution had no effect on their fluorescent properties.

To assess whether it was possible to selectively label and visualize SERT in live cells with compound 14, we used HEK293 cells expressing SERT tagged at the N-terminus with

**Figure 2.** Excitation and emission data for compounds 7, 8, 12, and 14. Data are shown as percent of maximum fluorescence and obtained as described in the Supporting Information.

enhanced green fluorescent protein (EGFP-SERT). The cells were incubated with 20 nM 14 and imaged by confocal laser scanning microscopy after 0, 1, 5, 10, or 20 min. As seen in Figure 3, we observed a gradual increase in the fluorescent

**Figure 3.** Compound 14 binds specifically to EGFP-SERT in a time-dependent manner in HEK293 cells. Visualization of SERT with compound 14 using confocal live imaging of HEK293 cells transiently expressing EGFP-SERT. Cells were incubated with 20 nM 14 for 0, 1, 5, 10, or 20 min \pm 100 μ M paroxetine at room temperature. Left panels show fluorescent EGFP signal (EGFP-SERT). Remaining panels show the fluorescent rhodamine signal. Lower panels show imaging of cells coincubated with an excess of paroxetine (100 μ M). The imaging was performed as described in the Supporting Information. The fluorescent rhodamine images are merged with a transmission image in all panels. The experiment shown is representative of three identical experiments.

rhodamine signal that reached maximum after ~10 min and overlapped with the fluorescent EGFP signal in the plasma membrane of EGFP-SERT expressing cells. Importantly, we did not see any labeling of cells without EGFP signal, and moreover, coincubation with an excess of the high affinity SERT inhibitor paroxetine completely blocked labeling of cells with compound 14 (Figure 3).

In summary, we report herein novel fluorescent ligands based on (S)-citalopram with modifications at the C-1 and C-5 positions. We discovered that unlike the tropane-based JHC1-64 that binds to DAT, NET, and SERT, these (S)-citalopram-based fluorescent ligands are highly specific for SERT without any binding to DAT or NET. Moreover, modification at the C-5 position was particularly well tolerated at SERT, and binding affinity was not adversely influenced by the change in linker length between the fluorophore and (S)-citalopram. Compound 14 demonstrated the highest affinity and selectivity for SERT with $K_i = 3.0$ nM, which is comparable to the parent compound ($K_i = 2.6$ nM), indicating that the rhodamine does not interfere with binding of the pharmacophore to the SERT binding site. Application of confocal microscopy demonstrated

furthermore that compound **14** can be used to specifically visualize SERT in the plasma membrane of live cells. Taken together, the combination of compound **14**'s high affinity and selectivity for SERT and the strong signal-to-noise ratio seen in confocal imaging illustrates how well-suited this unique fluorescent tool should be in further studies of SERT expression, localization, and trafficking directly in living cells.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental details for the synthesis and purification of the compounds and their in vitro pharmacological characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitors; DAT, dopamine transporter; NET, norepinephrine transporter; LAH, lithium aluminum hydride; EGFP, enhanced green fluorescent protein; RT, room temperature

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