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Discovery of 1-[2-(2,4-Dimethylphenylsulfanyl)phenyl]piperazine (Lu AA21004): A Novel Multimodal Compound for the Treatment of Major Depressive Disorder

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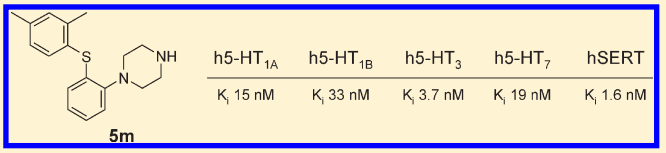
Supporting Information

ABSTRACT: The synthesis and structure−activity relationship of a novel series of compounds with combined effects on 5-HT_{3A} and 5-HT_{1A} receptors and on the serotonin (5-HT) transporter (SERT) are described. Compound **5m** (Lu AA21004) was the lead compound, displaying high affinity for recombinant human 5-HT_{1A} (K_i = 15 nM), 5-HT_{1B} (K_i = 33 nM), 5-HT_{3A} (K_i = 3.7 nM), 5-HT_7 (K_i = 19 nM), and noradrenergic β_1 (K_i = 46 nM) receptors, and SERT (K_i = 1.6 nM). Compound **5m** displayed antagonistic properties at 5-HT_{3A} and 5-HT_7 receptors, partial agonist properties at 5-HT_{1B} receptors, agonistic properties at 5-HT_{1A} receptors, and potent inhibition of SERT. In conscious rats, **5m** significantly increased extracellular 5-HT levels in the brain after acute and 3 days of treatment. Following the 3-day treatment (5 or 10 (mg/kg)/day) SERT occupancies were only 43% and 57%, respectively. These characteristics indicate that **5m** is a novel multimodal serotonergic compound, and **5m** is currently in clinical development for major depressive disorder.



INTRODUCTION

The approximate lifetime prevalence of major depressive disorder (MDD) and anxiety disorders in the United States is 17% and 29%, respectively, and onset is typically in childhood or adolescence.[1] According to the World Health Organization's Global Burden of Disease project, MDD will become the second leading cause of disability worldwide within the next 10 years.[2]

Pharmacotherapy for MDD and anxiety disorders has been available since the late 1950s, when the tricyclic antidepressants (TCAs) and the monoamine oxidase inhibitors (MAOIs) became clinically available. During the 1980s, the first selective serotonin (5-HT) reuptake inhibitors (selective SRIs or SSRIs) were introduced and since then have become the most widely prescribed medication for the treatment of depression and related disorders.[3] SSRIs such as fluoxetine, sertraline, paroxetine, fluvoxamine, and citalopram (**1**) show similar efficacy but clear advantages in terms of improved tolerability and a superior safety profile compared to TCAs and MAOIs.[4] The serotonin and norepinephrine (NE) reuptake inhibitors (SNRIs) venlafaxine, duloxetine, desvenlafaxine, and milnacipran, as well as the allosteric serotonin reuptake inhibitor escitalopram, have been marketed in the 1990s and during the new millennium. In contrast to SSRIs, SNRIs display modest improved efficacy and a slightly faster onset of antidepressant action but lower tolerability.[4,5] Despite more than 5 decades of research, there are still high unmet needs in the treatment of depression. For example, the treatment with currently available antidepressants shows modest efficacy, and the majority of patients with MDD fail to achieve full remission with current therapies.[6] In addition, a relatively long time to onset of symptom relief and issues with relapse characterize marketed antidepressants. Future drugs will also need to address cognitive impairment, pain, and drug-related side effects such as sexual dysfunction, nausea/emesis, weight gain, and potential cardiovascular effects.[4]

For many years, the medical community and pharmaceutical companies have been focusing on developing superior pharmacotherapeutic strategies for the treatment of depression and anxiety.[7,8] Clinical research has demonstrated that the antidepressant activity of SSRIs may be augmented by coadministration of pindolol, an effect that has been attributed to pindolol's partial agonistic effect at the 5-HT_{1A} receptor.[9,10] This observation has inspired many pharmaceutical companies to launch research programs with the aim of discovering designed multiple ligands (DMLs)[11] with dual activity at the serotonin transporter (SERT) and the 5-HT_{1A} receptor. Other profiles such as triple reuptake inhibitors [SERT, NE transporter (NET), and dopamine (DA) transporter (DAT) inhibition] and compounds inhibiting SERT plus various combinations of 5-HT receptor subtypes have also been pursued.[7,12]

At H. Lundbeck A/S, DML programs were pursued with the goal of finding a new generation of antidepressants. These were

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defined prior to initiation of the lead generation program (5-HT_{2C} antagonism and SRI),[13,14] or they emerged from the knowledge that was generated during such programs (5-HT_3 receptor antagonism, 5-HT_{1A} receptor agonism, and SRI). The objective of the current program was to find a compound that combines 5-HT_3 receptor antagonism, 5-HT_{1A} receptor agonism, and SERT inhibition in a single molecule and that will exert superior antidepressant effects in humans compared to marketed drugs. Recently, the term multimodal was coined for compounds that combine at least two separate pharmacological modes of action that complement each other in terms of efficacy or tolerability.[15,16] Here we describe the biological rationale for a novel class of putative multimodal antidepressants, which is proposed to act via different modes of action, namely, blocking ligand-gated ion channels (5-HT_3 receptor) and monoamine transporters (SERT), as well as activating G-protein-coupled receptors (5-HT_{1A} receptor). SERT inhibition is important for antidepressant and anxiolytic activity.[4] The simultaneous use of an efficacious 5-HT_{1A} receptor agonist, together with a SERT inhibitor, is expected to rapidly desensitize the inhibitory somatodendritic 5-HT_{1A} autoreceptors and, at the same time, to mediate part of the antidepressant effect through activation of postsynaptic 5-HT_{1A} receptors.[17,18] The rationale for adding 5-HT_3 receptor antagonism was based on preclinical studies demonstrating that 5-HT_3 receptor antagonists eliminate the inhibitory tonus of 5-HT_3 receptors on the release of NE and acetylcholine (AcCh) in the forebrain, via 5-HT_3 heteroreceptors located on inhibitory γ-aminobutyric acid (GABA)-ergic interneurons.[19−22] These studies indicate that 5-HT_3 receptor antagonists may exert positive effects on mood and cognitive impairment in patients with depression. Adding to this point, 5-HT_{1A} receptor agonists such as buspirone (**2**) are used as anxiolytics in the clinic, whereas 5-HT_3 receptor antagonists such as ondansetron (**3**) are used to treat the nausea associated with chemotherapy and have shown some success in alleviating the gastrointestinal distress associated with SSRIs.[23,24] Interestingly, litoxetine, initially described as an SSRI, was later shown to possess antiemetic properties in ferrets that were ascribed to its moderate 5-HT_3 receptor antagonistic properties.[23] Hence, a combined 5-HT_3 receptor antagonist, 5-HT_{1A} receptor agonist, and SERT inhibitor was hypothesized to have the potential to treat significant clinical unmet needs in MDD and generalized anxiety disorder (GAD).

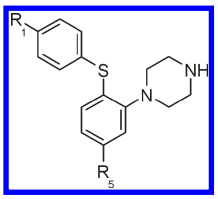
In a focused screening program we identified the arylpiperazine **4b** and a number of structural analogues as a lead series. Arylpiperazines are well-known motifs that are typically found in compounds possessing activities toward a broad range of targets, including various 5-HT receptor subtypes. For example, some 1-(2-phenoxyaryl)piperazines, although structurally similar to our lead series, were recently found to display a slightly different pharmacological profile, such as high 5-HT_{1A} receptor affinity but only modest SERT inhibition.[25] This and other examples indicate that the design of molecules, interacting in a predefined manner with three different target classes (i.e., ligand-gated ion channels, G-protein-coupled receptors, and monoamine transporters), will be challenging, and it was also anticipated that compounds fulfilling our design hypothesis might well interact with additional targets in a subsequent broader target profiling. Indeed, we found that compound **4b** displays moderate human (h) 5-HT_{3A} receptor affinity, very weak h5-HT_{1A} receptor affinity, and potent rat (r) SERT inhibition (Table 1), as well as high affinity (K_i < 100 nM) for h5-HT_{2C} and rα_1 receptors (data not shown). Structurally closely related members of the series displayed substantially higher affinity for h5-HT_{3A} and/or h5-HT_{1A} receptors (Table 1). In contrast to compound **4b**, some of the analogues showed good human microsomal stability and/or minimal cytochrome P450 (CYP450) enzyme inhibition (data not shown). Thus, the series was a useful starting point for a number of DML programs, including the structure−activity relationship (SAR) studies reported herein that led to the discovery of **5m** (Chart 1).

RESULTS

Chemistry. Several different strategies were employed for the synthesis of the target compounds **4**−**6**. The compound class was initially prepared by solid-phase synthesis in order to generate compound libraries for lead discovery programs. The key step of this synthetic strategy was the generation of aromatic carbon−heteroatom bonds through iron-assisted nucleophilic aromatic substitution.[26,27] This was a suitable method for the preparation of focused libraries in a short space of time. However, a different approach was necessary in order to synthesize such molecules in larger quantities and in order to prepare molecules with a more complex substitution pattern on the central benzene ring. For this purpose, the target compounds were synthesized either by two sequential palladium-catalyzed carbon−heteroatom bond formations[28,29] (methods A and B, Schemes 1 and 2) or through cyclization of anilines with bis(2-bromoethyl)amine (method C). In method A, the synthesis started from 2-bromo-substituted *N*-phenylpiperazines **7** that were readily available in multigram quantities through a selective monoamination of dihalogenated benzenes.[30] The thioether linkage was subsequently formed using the method reported by Schopfer and Schlapbach.[31] In the analogous method B, the order of steps was reversed. For both strategies, the required starting materials, halogenated benzenes, were either commercially available or could be readily obtained from the corresponding anilines. Finally, the resulting *tert*-butyloxycarbonyl (Boc)-protected intermediates were deprotected to give the target molecules **4**−**6** as their hydrochloride salts. Overall, method B turned out to be more practical than methods A and C, since the Boc-protected target molecules were easily separated by chromatography from unreacted diaryl sulfides and hydrodebrominated byproducts.

Structure−Activity Relationship Studies. The affinities of target compounds **4**−**6** for the cloned h5-HT_{3A} and h5-HT_{1A} receptors were determined by use of in vitro binding competition assays with membranes prepared from cells expressing the relevant receptors, and data are reported as K_i values (see Supporting Information for pK_i ± SEM values). The inhibition of uptake of [^{3}H]5-HT, [^{3}H]NE, and [^{3}H]DA into rat brain synaptosomes through the monoamine transporters was also measured, and data are reported as mean IC_{50} values (see Supporting Information for pIC_{50} ± SD/SEM values) of at least two independent determinations. These data are shown in Tables 1−3 and 5, along with the structure of the compounds. While affinity measures were used at the cloned h5-HT_{3A} and h5-HT_{1A} receptors, for historical reasons a functional readout was used as the SAR driving assay for assessing potency at the SERT. Internal data and literature data, however, have demonstrated a close correlation between affinity at hSERT and potency in [^{3}H]5-HT uptake assays, suggesting that values are comparable.[32] The compounds were prioritized for further

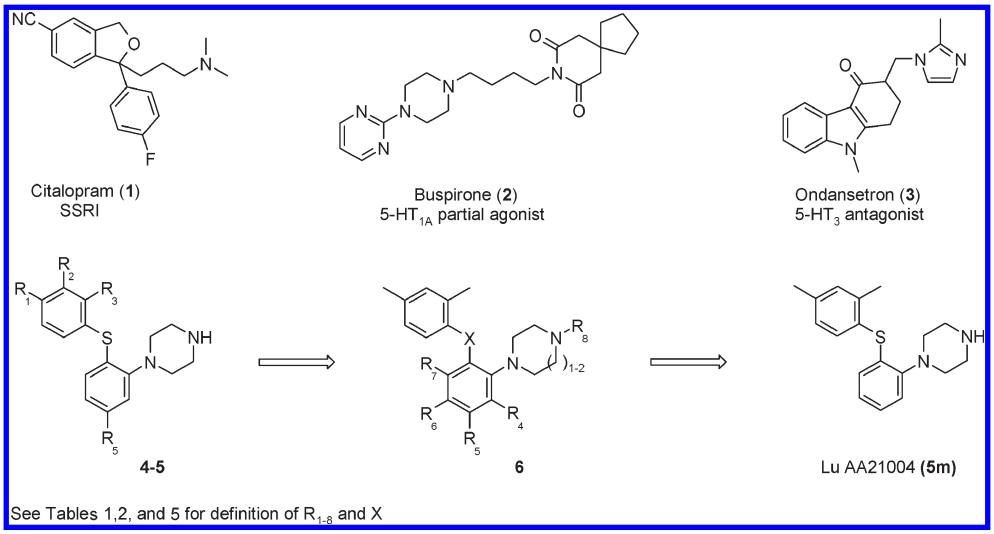
Table 1. Structure−Activity Relationship of Compounds 4a−l Compared with Reference Compounds Citalopram (1), Buspirone (2), and Ondansetron (3) at Human (h) 5-HT_{3A} and h5-HT_{1A} Receptors and the Rat (r) Serotonin Transporter (rSERT)



				K_i (nM)[a]		IC_{50} (nM)[a]
compd	synthesis method	R_1	R_5	h5-HT_{3A}[b]	h5-HT_{1A}[c]	rSERT[d]
4a	B	H	CH_3	310	1100	150
4b	B	OCH_3	CH_3	190	4000	7.9
4c	B	Cl	CH_3	500	2500	9.5
4d	B	F	CH_3	440	1100	68
4e	B	CH_3	CH_3	260	2800	20
4f	A	H	H	64	30	390
4g	A	OCH_3	H	36	130	8.0
4h	A	Cl	H	36	69	13
4i	A	F	H	53	39	67
4j	A	CH_3	H	56	44	18
4k	A	CF_3	H	140	240	6.9
4l	A	$C(CH_3)_3$	H	450	2500	2100
1, citalopram				NT	NT	3.9[e]
2, buspirone				>10000	21	>1000
3, ondansetron				2.8	NT	>2000

[a] Data are the mean of a minimum of two determinations covering 6 decades for each compound. K_i values were calculated from the Cheng−Prusoff equation ($K_i = IC_{50}/(1 + [L]/K_d)$). NT, not tested. [b] [^{3}H]Granisetron binding to cloned 5-HT_{3A} receptors. [c] [^{3}H]5-Carboxamidotryptamine binding to cloned 5-HT_{1A} receptors. [d] [^{3}H]5-HT uptake measured in rat synaptosomes. [e] Reference 59.

Chart 1. Structures of citalopram (1), buspirone (2), ondansetron (3), target compounds (4-6) and 5m



SAR studies based on the ratios between the in vitro affinities/potencies at h5-HT_{3A}, and h5-HT_{1A} receptors and the rSERT. It has been demonstrated in both preclinical and clinical studies that a relatively high hSERT occupancy of around 80−90% is required for SSRIs and SNRIs to exert an antidepressant activity.[33−35] In contrast, the relationship between the in vivo

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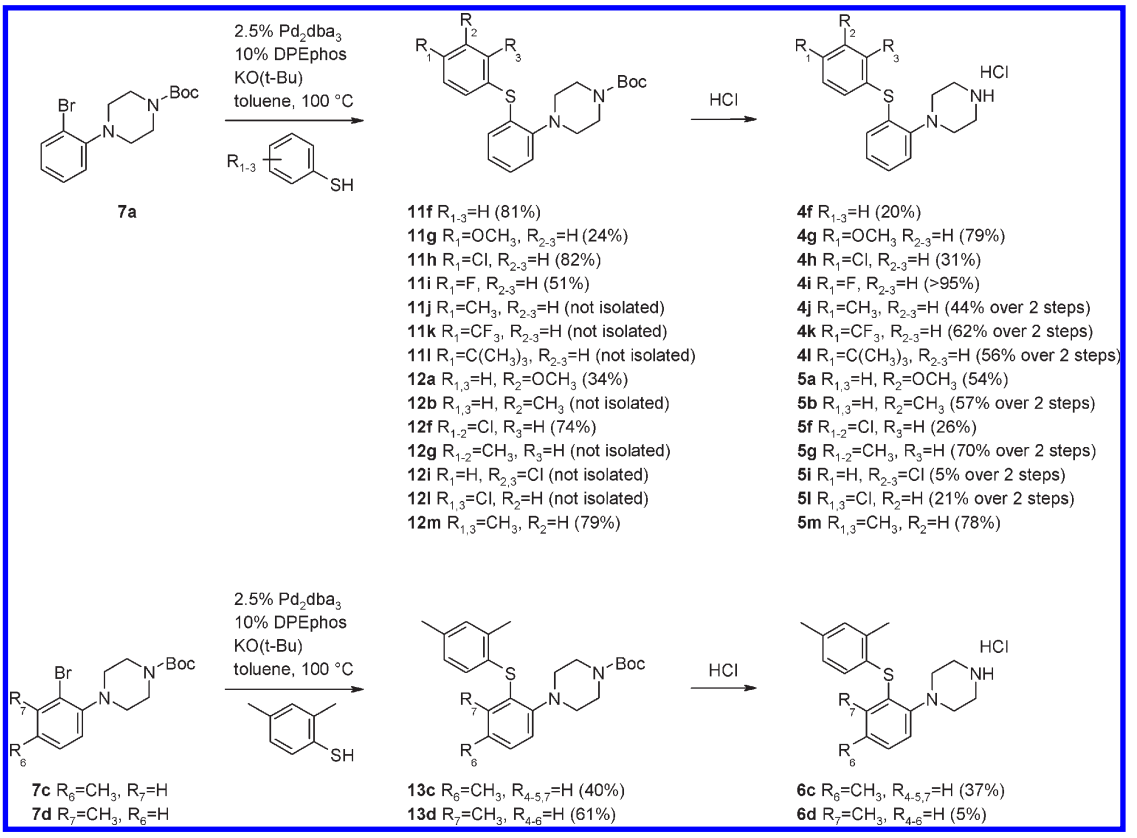
Scheme 1. Synthesis of Target Compounds by Method A



occupancy in experimental animals and humans and the efficacy at 5-HT_3 and 5-HT_{1A} receptors is less well described. However, receptor occupancies of >50% have been reported for the antiemetic activity of 5-HT_3 receptor antagonists including ondansetron (**3**),[36] whereas much lower receptor occupancies have been estimated to be efficacious for buspirone (**2**) at 5-HT_{1A} receptors.[37−39] Hence, compounds were selected that displayed relative affinities/potencies (K_i/IC_{50}) at h5-HT_{3A} and h5-HT_{1A} receptors and rSERT corresponding to rSERT/h5-HT_{3A}/h5-HT_{1A} ≈ 1:(1−5):(5−15). From a hypothesis that the in vitro profile alone would reflect the relative in vivo occupancies, these ratios would correspond to in vivo occupancy levels of 45−80% at 5-HT_3 receptors and 20−45% at 5-HT_{1A} receptors for a compound with an in vivo occupancy of 80% at SERT (Figure 1). Furthermore, the compounds were only considered if the 5-HT_{1A} receptor affinity was <100 nM in order to limit the amount of compounds for broader in vitro and in vivo profiling.

We first focused on the R_1 substituent in the phenylsulfanyl ring and the R_5 substituent in the central benzene ring of lead compound **4b** (Table 1). A number of analogues of **4b** were available at the onset of the project. In contrast to the methoxy in **4b**, these analogues were furnished with either electron-withdrawing substituents (R_1 = F or CF_3) or substituents with limited electronic effects (R_1 = Cl, CH_3, and *tert*-butyl). The series of compounds was extended by synthesizing analogues carrying a hydrogen atom at R_5 instead of the methyl substituent of **4b**. These latter compounds demonstrated higher affinities at the 5-HT_{3A} and 5-HT_{1A} receptors in comparison with compounds furnished with a methyl substituent at this position, i.e., **4a**−**e** compared with **4f**−**j**. All compounds with relatively small substituents at R_1 and either a hydrogen atom or a methyl substituent at R_5 (**4b**,**c**,**e**,**g**,**h**,**j**,**k**) inhibited rSERT in the same range, whereas compounds with a fluoro atom (**4d** and **4i**) or a hydrogen atom (**4a** and **4f**) exhibited weaker rSERT inhibition. The compound **4l** with the large *tert*-butyl substituent was a significant weaker inhibitor of rSERT, demonstrating that small R_1 substituents are preferred for potent rSERT inhibition. It was also shown that rSERT inhibition seemed to be less affected by the electronic effects of the R_1 substituent as long as the substituent was above a certain size. The compounds with an electron-donating methoxy substituent (**4b** and **4g**), a more or less neutral methyl or chloro substituent (**4e**, **4j**, **4c**, and **4h**), or an electron-withdrawing trifluoromethyl substituent (**4k**) were more or less equipotent rSERT inhibitors. From this initial series, compounds **4h** and **4j** were selected for further in vitro evaluation (Tables 3 and 4), whereas compounds **4a**−**e**,**g**,**k**,**l** were discarded because of their relative low h5-HT_{1A} receptor affinities and compounds **4f** and **4i** were discarded because of their low rSERT inhibition and suboptimal ratio between rSERT inhibition and h5-HT_{1A} receptor affinity.

We extended the series of compounds through the synthesis of analogues carrying a hydrogen atom at R_5 and methoxy, chloro, and/or methyl groups at other positions of the phenylsulfanyl ring. Thus, the effects of moving the substituent from R_1 to either R_2 or R_3 and of having two substituents (two identical or two different substituents) in the phenylsulfanyl ring were investigated (Table 2). In general, all of these

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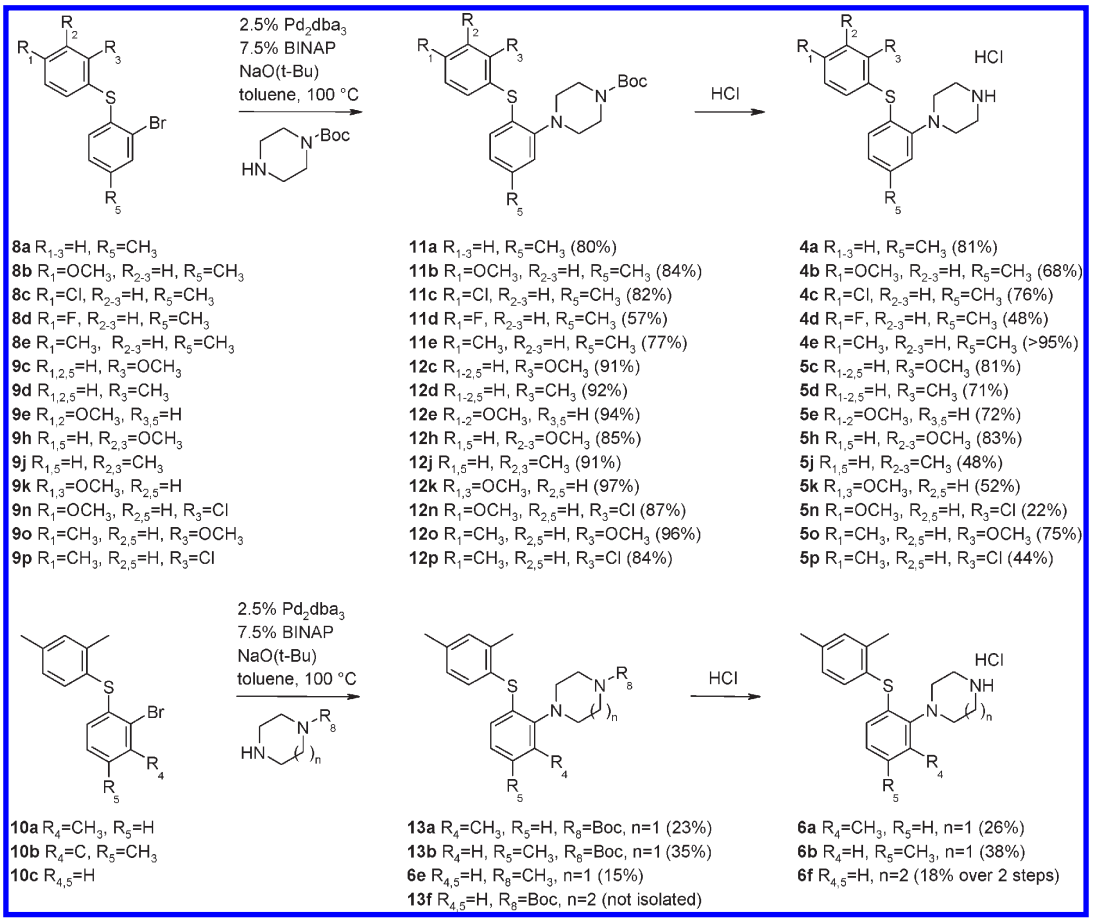
Scheme 2. Synthesis of Target Compounds by Method B



compounds (**5a**−**p**) turned out to have relative high affinities for h5-HT_{3A} receptors (K_i from 7.3 to 110 nM). These compounds also showed moderate to high affinities at the h5-HT_{1A} receptor (K_i from 14 to 330 nM) and moderate to high activity at rSERT (IC_{50} from 4.3 to 330 nM). Compounds with a methoxy or a methyl substituent at R_2 or R_3 (**5a**−**d**) were weaker rSERT inhibitors than the corresponding R_1 analogues (**4g** and **4j**). Compound **5d**, with a monomethyl substituent at R_3, showed high affinity for h5-HT_{1A} receptors, and the corresponding R_1 and R_2 analogues **4j** and **5b** showed weaker affinities for h5-HT_{1A} receptors compared with **5d** by factors of 3 and 7, respectively.

Six compounds were synthesized with two identical adjacent substituents, i.e., compounds with the same substituent at R_1 and R_2 (**5e,f,g**) or at R_2 and R_3 (**5h,i,j**). These compounds were all relatively potent rSERT inhibitors with favorable ratios between the three targets. However, only compounds **5g**, **5i**, and **5j** were selected for further in vitro investigations (Tables 3 and 4). Three compounds with identical substituents at R_1 and R_3 were also synthesized (**5k,l,m**), and all these compounds had the desired h5-HT_{3A}/rSERT ratio. Whereas compound **5k** showed weak h5-HT_{1A} receptor affinity, compounds **5l** and **5m** were selected for further in vitro profiling (Tables 3 and 4). Finally, three analogues with different substituents at R_1 and R_3 were synthesized (**5n,o,p**). Only compound **5p** was selected for further in vitro profiling (Tables 3 and 4), since compounds **5n** and **5o** showed low 5-HT_{1A} receptor affinities, i.e., compound **5n** in relation to inhibition of SERT and **5o** with K_i > 100 nM.

Compounds **4h**, **4j**, **5g**, **5i**, **5j**, **5l**, **5m**, and **5p** were tested for rNET and rDAT inhibition. The ratios between rDAT and rSERT and between rNET and rSERT inhibition are shown in Table 3. The rNET/rSERT ratios ranged from 7 to 150, whereas the rDAT/rSERT ratios ranged from 1 to 170. Compound **5m** exhibited the highest ratio (170) between rDAT and rSERT inhibition. Compound **5g** showed the highest ratio (150) between rNET and rSERT inhibition, and a group of compounds including **5m** showed rSERT selectivity with ratios ranging from 20 to 35. Furthermore, drug metabolism and pharmacokinetic (DMPK) properties of the compounds in the series were optimized by monitoring their in vitro metabolic stability in rat and human liver microsomes and by assessing the inhibitory potential of the compounds toward recombinant (cDNA-expressed) human CYP450 enzymes. As shown in Table 4, the compounds generally showed good stability in human liver microsomes; i.e., the intrinsic clearances (CL_{int}) of all but one (**5i**) were below that for the human liver blood flow (LBF). Conversely, in rat liver microsomes the CL_{int} were all above the rat LBF of 20 mL/min, indicating high clearance in vivo. This was confirmed in vivo for all compounds tested in the project, e.g., for compounds **4h** and **5m** the hepatic clearances and oral bioavailabilities in rats were found to be 5.8 and 7.1 (L/h)/kg and 26% and 16%, respectively. Furthermore, inhibition of CYP1A2,

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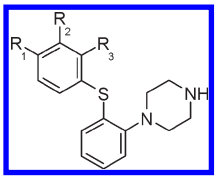
Table 2. Structure−Activity Relationship of Compounds 5a−p at Human (h) 5-HT$_{3A}$ and h5-HT$_{1A}$ Receptors and the Rat (r) Serotonin Transporter (rSERT)



					K_i (nM)[a]		IC_{50} (nM)[a]
compd	synthesis method	R_1	R_2	R_3	h5-HT$_{3A}$[b]	h5-HT$_{1A}$[c]	rSERT[d]
5a	A	H	OCH_3	H	62	140	53
5b	A	H	CH_3	H	64	100	54
5c	B	H	H	OCH_3	52	59	330
5d	B	H	H	CH_3	32	14	51
5e	B	OCH_3	OCH_3	H	86	160	15
5f	A	Cl	Cl	H	110	68	4.7
5g	A	CH_3	CH_3	H	33	53	5.8
5h	B	H	OCH_3	OCH_3	80	68	37
5i	A	H	Cl	Cl	67	75	17
5j	B	H	CH_3	CH_3	29	31	4.4
5k	B	OCH_3	H	OCH_3	32	330	7.6
5l	A	Cl	H	Cl	26	78	5.2
5m	A	CH_3	H	CH_3	23/3.7[e]	39/15[f]	5.3/5.4[g]
5n	B	OCH_3	H	Cl	12	94	4.3
5o	B	CH_3	H	OCH_3	17	210	11
5p	B	CH_3	H	Cl	7.3	78	9.7

[a] Data are the mean of a minimum of two determinations covering 6 decades for each compound. K_i values were calculated from the Cheng−Prusoff equation ($K_i = IC_{50}/(1 + [L]/K_d)$). [b] [^{3}H]Granisetron binding to cloned h5-HT$_{3A}$ receptors. [c] [^{3}H]5-Carboxamidotryptamine binding to cloned 5-HT$_{1A}$ receptors. [d] [^{3}H]5-HT uptake from rat synaptosomes. [e] External data obtained from Cerep, catalogue number 808-3h. [f] External data obtained from MDS Pharmaservices, catalogue number 271110. [g] [^{3}H]5-HT uptake from CHO cells expressing hSERT. For compound **5m**, in-house and external data are shown for h5-HT$_{3A}$ and h5-HT$_{1A}$ receptors, and for SERT both rat and human data are shown.

CYP2C9, and CYP3A4 was found to be in the high micromolar range for all compounds, while inhibition toward CYP2D6 ranged from 0.4 μM for compound **4h** to 9.8 μM for compound **5m**. On the basis of the favorable in vitro and DMPK profile, compound **5m** was selected for further SAR exploration, as well as for broader in vitro and in vivo profiling.

The SAR of **5m** was further explored by a "methyl walk" around the entire molecular framework (Table 5). In addition, the piperazine ring was replaced with a homopiperazine (**6f**) and the sulfur atom with an oxygen atom (**6g**). In comparison to **5m**, all of these compounds showed weaker rSERT inhibition, and only compound **6g** had acceptable h5-HT$_{1A}$ receptor affinity. They also had relatively large variations in their affinity for h5-HT$_{3A}$ receptors, with K_i ranging from 45 to 670 nM. Compound **6c** ($R_6 = CH_3$) and homopiperazine **6f** showed some selectivity for h5-HT$_{3A}$ receptors, whereas the oxygen analogue **6g**, in agreement with literature data, had some selectivity for h5-HT$_{1A}$ receptors.[25]

Further Pharmacological and Pharmacokinetic Characterization of 5m. Compound **5m** displayed high binding affinity for cloned homomeric h5-HT$_{3A}$ receptors [K_i = 23 nM (in-house) and K_i = 3.7 nM (Cerep)]. In agreement with this, **5m** was shown to be a potent functional antagonist (IC_{50} = 12 nM, tested against 10 μM 5-HT) when tested in an electrophysiological setup using *Xenopus* oocytes expressing cloned homomeric h5-HT$_{3A}$ receptors (Figure 2). Conversely, at higher concentrations, **5m** showed agonistic properties (EC_{50} = 2100 nM), reaching a maximal response of 64% relative to 5-HT (Figure 3). The agonistic response could be blocked by the 5-HT$_3$ receptor antagonist ondansetron (data not shown). At r5-HT$_{3A}$ receptors, no agonistic response was found over a large concentration range (0.1−100000 nM), but in agreement with the findings at h5-HT$_{3A}$ receptors, **5m** was found to be a potent antagonist at r5-HT$_{3A}$ receptors (IC_{50}= 0.18 nM) (Figure 2). Compound **5m** displayed high binding affinity for cloned h5-HT$_{1A}$ receptors in two different assays (K_i = 39 nM (in-house) and K_i = 15 nM (MDS Pharmaservice), whereas relative low affinity binding was found at r5-HT$_{1A}$ receptors (K_i = 230 nM). In a [^{35}S]guanosine 5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) binding assay with h5-HT$_{1A}$ receptors, **5m** was found to be an agonist (EC_{50} = 200 nM, efficacy = 96%, Figure 4). In vitro characterization showed that **5m** was a potent rSERT inhibitor (IC_{50} = 5.3 nM) when assessed in rat brain synaptosomes, and a similar potency was found on hSERT when expressed in Chinese hamster ovary (CHO) cells (IC_{50} = 5.4 nM). Comparably, binding affinity at hSERT was found to be 1.6 nM. Weak potencies (IC_{50} > 100 nM) of **5m** were found at rNET and rDAT (Table 3). At 1 μM, **5m** was tested against a panel of more than 75 ion channels,

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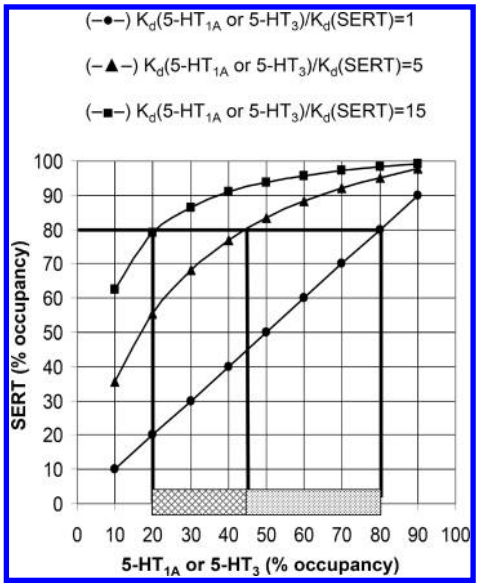


Figure 1. Calculated relationships between the occupancy levels at 5-HT_{1A} or 5-HT_3 receptors relative to serotonin (5-HT) transporter (SERT) occupancy. The line with circles (−●−) shows the relationship between the occupancies if the ratio between the SERT and 5-HT_{1A} or 5-HT_3 is 1. The line with triangles (−▲−) shows the relationship when the ratio is 5, and the line with squares (−■−) shows the relationship when the ratio is 15; i.e., a compound with occupancy of 80% at SERT would give occupancy of 45% at 5-HT_{1A} receptors if the affinity at 5-HT_{1A} receptors is 5-fold lower than at SERT, whereas only 20% occupancy would be obtained if the 5-HT_{1A} receptors affinity is 15-fold lower than at SERT. The two hatched areas depict estimated clinically relevant occupancy levels at 5-HT_{1A} and 5-HT_3 receptors based on these considerations.

Table 3. Structure−Activity Relationship of Key Compounds Selected from Tables *1* and *2* at Rat Serotonin Transporter (rSERT), Rat Dopamine Transporter (rDAT), and Rat Norepinephrine Transporter (rNET), as well as the Calculated Ratios rDAT/rSERT and rNET/rSERT

	IC_{50} (nM)[a]			ratio	
compd	rSERT[b]	rDAT[b]	rNET[b]	rDAT/rSERT	rNET/rSERT
4h	13	37/45[c]	160/190[c]	2.8/3.5[d]	12/15[d]
4j	18	110	460	6.1	25
5g	5.8	120[c]	880[c]	21	150
5i	17	21[c]	120[c]	1	7
5j	4.4	170[c]	90[c]	38	20
5l	5.2	250[c]	120[c]	48	23
5m	5.3	890	140	170	26
5p	9.7	320[c]	340[c]	33	35

[a] Data are the mean of a minimum of two values. IC_{50} values were determined using drug concentrations covering at least 3 decades. [b] [^{3}H]5-HT, [^{3}H]DA, and [^{3}H]NE uptake from rat synaptosomes. [c] Cerep catalogue numbers 711 and 712 for rNET and rDAT, respectively. [d] For compound **4h**, ratios from in-house and external data are shown.

G-protein-coupled receptors, enzymes, and transporters (Table 6). Modest binding affinities were found at histamine hH_2, melanocortin hMC_4, noradrenergic hβ_2, serotonergic h5-HT_{2C}, h5-HT_{5A}, and h5-HT_6 receptors, whereas more potent

Table 4. In Vitro Metabolic Stability in Rat and Human Liver Microsomes and Inhibition of Recombinant (cDNA-expressed) Human CYP Enzymes

	Cl_{int} (L/min)	Cl_{int} (mL/min)	IC_{50} (μM)			
compd	human	rat	CYP1A2	CYP2C9	CYP2D6	CYP3A4
4h	0.6	70	53	31	0.4	6.8
4j	1.1	260	40	40	2.7	16
5g	0.5	290	26	34	2.8	11
5i	1.9	260	32	40	0.5	6.1
5j	0.7	120	24	40	3.3	8.7
5l	0.5	38	27	34	0.9	4.4
5m	0.5	55	40	39	9.8	10
5p	0.6	120	34	40	1.5	5.5
LBF[a]	1.4	20				

[a] LBF, liver blood flow.

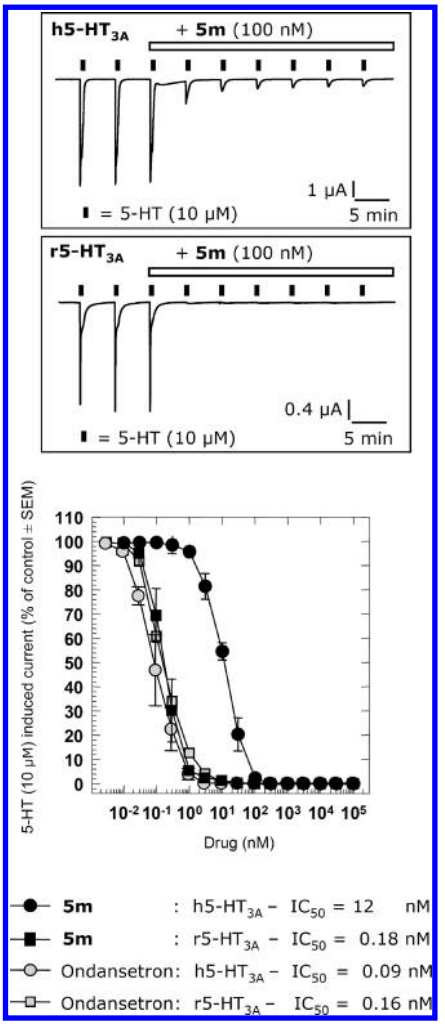


Figure 2. Compound **5m** and ondansetron (**3**) were tested for antagonistic effects on human (h) and rat (r) 5-HT_{3A} receptors expressed in oocytes. Compound **5m** inhibited the h5-HT_{3A} and r5-HT_{3A} receptors with IC_{50} equal to 12 and 0.18 nM, respectively. Ondansetron (**3**) showed similar inhibition of both h5-HT_{3A} and r5-HT_{3A} receptors with $IC_{50} \approx 0.1$ nM.

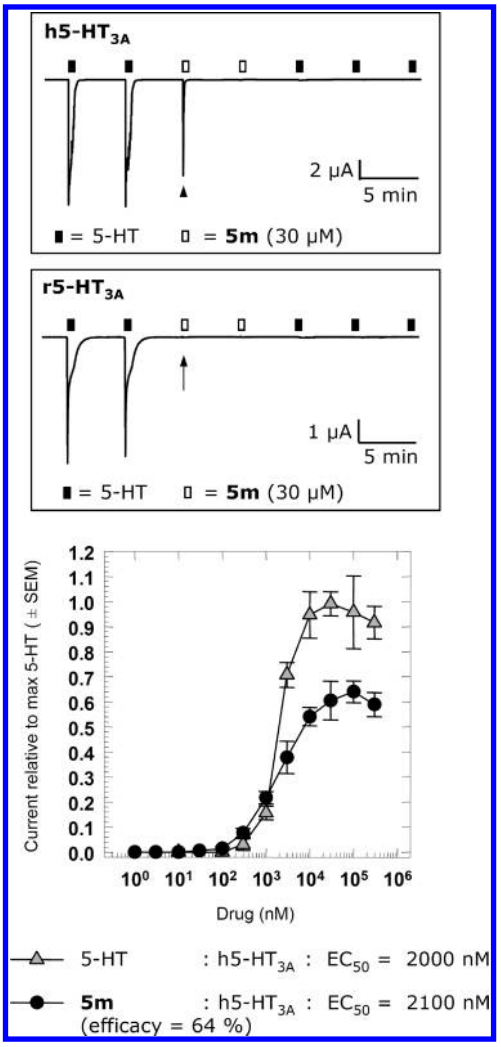


Figure 3. Compound **5m** displayed an agonistic response on human (h) 5-HT_{3A} receptors (arrowhead) but not on rat (r) 5-HT_{3A} receptors (arrow). At h5-HT_{3A} receptors, **5m** had an EC_{50} of 2100 nM, reaching a maximal response of 64% compared with the maximal response with 5-HT. Note that after only one application of **5m** for 30 s, the subsequent application of either **5m** or 5-HT (10 μM) did not induce any response at all, indicating that **5m** induces functional antagonism. That means that it is only possible to test one concentration of **5m** on each oocyte, so the agonism dose-reponse curves for **5m** has been obtained from a test of each single concentration of **5m** using three to six separate oocytes.

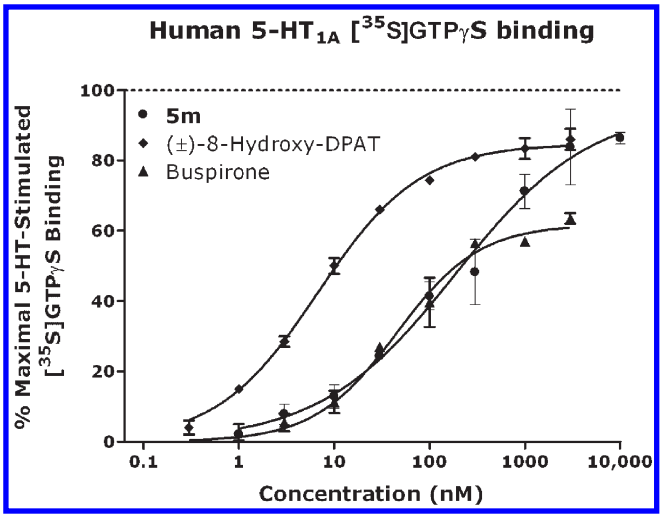


Figure 4. Dose relationship depicting potency and intrinsic activity (IA) of **5m**, buspirone, and 5-OH DPAT in a [^{35}S]guanosine 5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) binding assay in CHO membranes expressing h5-HT_{1A} receptors. IA_{max} was defined by 5-HT binding. Raw data were obtained by MDS Pharmaservices and were then analyzed by sigmoidal dose response curve-fittings using GraphPad Prism 4 to generate EC_{50} and intrinsic activity (IA) results. Data shown in the graph were averaged from $n = 3$.

binding (K_i < 180 nM) was found at noradrenergic hβ_1 receptors (K_i = 46 nM) and serotonergic h5-HT_{1B} (K_i = 33 nM) and h5-HT_7 (K_i = 19 nM) receptors (Table 6). At h5-HT_{1B} receptors, **5m** proved to be a partial agonist (EC_{50} = 120 nM, efficacy = 55%) when tested in a [^{35}S]GTPγS binding assay, whereas **5m** was shown to be an antagonist (K_i = 450 nM) at h5-HT_7 receptors when tested in a cAMP dependent fluorescence based assay using cloned h5-HT_7 receptors expressed in HEK-CNG cells. The unique in vitro pharmacological properties of **5m** (Table 7) translate into an in vivo profile that differs from those of currently used antidepressants. Compound **5m** (2.5, 5, or 10 mg/kg sc) increased the extracellular levels of 5-HT in the ventral hippocampus in conscious rats (Figure 5). In a similar experiment administration of the SSRI escitalopram enhanced hippocampal 5-HT levels by 700% at the dose yielding maximal response (data not shown). Three days of treatment with **5m** (5 or 10 (mg/kg)/day) also resulted in significantly higher basal levels of 5-HT in the medial prefrontal cortex (mPFC) compared to animals treated with vehicle. The occupancy of SERT by **5m** after 3 days of treatment with 5 or 10 (mg/kg)/day was 43% and 57%, respectively (Figure 6). Furthermore, **5m** showed a robust antidepressant- and anxiolytic-like profile in multiple preclinical assays, such as the mouse forced-swim and tail-suspension tests and the rat social interaction and conditioned-fear paradigms.[40]

The prediction of the hepatic clearance of **5m** in humans was done by in vitro/in vivo extrapolation from the predicted hepatic clearance in rats and humans and the observed systemic in vivo clearance in rats. According to the well-stirred model, the hepatic clearance in each species (CL_{pred}) was predicted from hepatic microsomal intrinsic clearance (CL_{int}) according to

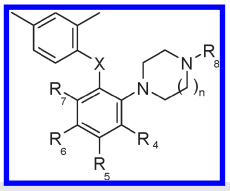
$$CL_{pred} = \frac{(LBF)(CL_{int})(f_u)}{LBF + (CL_{int})(f_u)}$$

where LBF is liver blood flow and f_u is the unbound fraction in plasma. Human clearance was then predicted from the observed in vivo rat clearance and the clearances that are predicted based on in vitro clearances from each species ($CL_{pred,human}$ and $CL_{pred,rat}$) according to

$$CL_{human} = CL_{rat}\frac{CL_{pred,human}}{CL_{pred,rat}}$$

The plasma protein binding of **5m** was determined to 98.3% and 99.2% for human and rat, respectively, resulting in a predicted low human hepatic clearance of 35 L/h, which turned out to be off by only a few liters when measured in

Table 5. Structure−Activity Relationship of Compounds 6a−g Compared with 5m at Human (h) 5-HT$_{3A}$ and h5-HT$_{1A}$ Receptors and the Rat Serotonin Transporter (rSERT)



									K_i (nM)[a]		IC_{50} (nM)[a]
compd	synthesis method	R_4	R_5	R_6	R_7	R_8	n	X	h5-HT$_{3A}$[b]	h5-HT$_{1A}$[c]	rSERT[d]
6a	B	CH_3	H	H	H	H	1	S	160	420	44
6b	B	H	CH_3	H	H	H	1	S	150	2700	25
6c	A	H	H	CH_3	H	H	1	S	45	1400	200
6d	A	H	H	H	CH_3	H	1	S	670	540	540
6e	B	H	H	H	H	CH_3	1	S	130	120	680
6f	B	H	H	H	H	H	2	S	27	310	130
6g	C	H	H	H	H	H	1	O	270	65	250
5m	A	H	H	H	H	H	1	S	23/3.7[e]	40/15[f]	5.3/5.4[g]

[a] Data are the mean of a minimum of two determinations covering 6 decades for each compound. K_i values were calculated from the Cheng−Prusoff equation ($K_i = IC_{50}/(1 + [L]/K_d)$). [b] [^{3}H]Granisetron binding to cloned 5-HT$_{3A}$ receptors. [c] [^{3}H]5-Carboxamidotryptamine binding to cloned 5-HT$_{1A}$ receptors. [d] [^{3}H]5-HT uptake from rat synaptosomes. [e] External data obtained from Cerep, catalogue number 808-3h. [f] External data obtained from MDS Pharmaservices, catalogue number 271110. [g] [^{3}H]5-HT uptake from CHO cells expressing hSERT. For compound **5m**, in-house and external data are shown for h5-HT$_{3A}$ and h5-HT$_{1A}$ receptors, and for SERT both rat and human data are shown.

Table 6. Broad Screening of 5m at Cerep, MDS Pharmaservices, or H. Lundbeck A/S[a]

	Targets
adenosine: hA$_1$, hA$_{2A}$, hA$_3$	angiotensin II: hAT$_1$, hAT$_2$
benzodiazepine: BZD (central); BZD (peripheral)	bombesin: BB (nonselective)
bradykinin: hB$_2$	calcitonin: hCGRP
cannabinoid: hCB$_1$	cholecystokinin: hCCK$_1$, hCCK$_2$
dopamine: hD$_1$, hD$_2$, hD$_3$, hD$_{4.4}$, hD$_5$	endothelin: hET$_A$, hET$_B$,
γ-aminobutyric acid (nonselective)	galanin: hGAL1, hGAL2
growth factors: platelet-derived growth factor, h interleukin-8B, tumor necrosis factor-α, chemokine receptor 1	glutamate (nonselective): phenylcyclidine binding site
histamine: hH$_1$, hH$_2$ (K_i = 180 nM)	melanocortin: hMC$_4$ (% inh = 51)
melatonin: hML$_1$	muscarinic: hM$_1$, hM$_2$, hM$_3$, hM$_4$, hM$_5$
neurokinin: hNK$_1$, hNK$_2$, hNK$_3$	noradrenergic (nonselective): α_1 (nonselective), α_2 (nonselective), β_1 (K_i = 46 nM), β_2 (K_i = 560 nM)
neuropeptide Y: hY$_1$, hY$_2$	neurotensin: hNT$_1$
opiate: hδ, κ, hμ	orphanin: hORL$_1$
pituitary adenylate cyclase activating polypeptide: PAC1	prostaglandin: human thromboxane A/PGH$_2$, hPGI$_2$
purinic: P2X, P2Y	serotonergic: h5-HT$_{1A}$ (% inh = 98),[b] h5-HT$_{1B}$ (% inh = 100),[b] h5-HT$_{2A}$, h5-HT$_{2C}$ (K_i = 180 nM), h5-HT$_{3A}$ (% inh 100),[b] h5-HT$_{5A}$ (K_i = 220 nM), h5-HT$_6$ (K_i = 330 nM), h5-HT$_7$ (K_i = 19 nM)
σ (nonselective)	somatostatin (nonselective): sst
vasoactive intestinal peptide: hVIP$_1$ (VPAC$_1$)	vasopression: hV$_{1a}$
ion channels: Ca^{2+} (L, verapamil site), K^+v channel, SK^+_{Ca} channel, Na^+ channel (site 2), Cl^- channel	transporters: hNE transporter (% inh = 88),[b] hDA transporter

[a] For targets where % inhibition (inh) of binding at 1000 nM was greater than 50%, K_i values or % inhibition are specified. Data were generated in a high-throughput profile at Cerep 2002. Follow-up was made at Cerep, MDS Pharmaservices or at H. Lundbeck A/S. Data are the mean of a minimum of two determinations covering 6 decades for each compound. K_i values were calculated from the Cheng−Prusoff equation ($K_i = IC_{50}/(1 + [L]/K_d)$). [b] IC_{50}/K_i values are reported elsewhere in manuscript.

human healthy volunteers (data not shown). Thus, compound **5m** was indeed predicted to display promising pharmacokinetic characteristics and low CYP450 enzyme inhibitory potential in humans.

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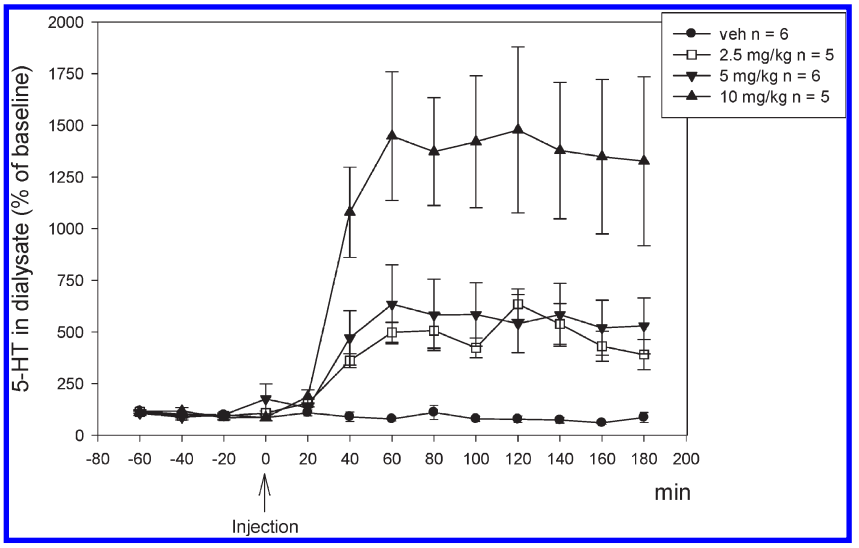


Figure 5. Effects of administration of **5m** (2.5, 5, and 10 mg/kg) or vehicle subcutaneous on extracellular 5-HT levels in the rat ventral hippocampus. Data are expressed as the mean ± SEM. There was a dose and time-dependent increase in extracellular 5-HT following drug administration [$F(27,159) = 7.081$; $P < 0.001$]. Extracellular concentrations of 5-HT were significantly increased relative to baseline from $t = 60$ until $t = 120$ min after administration of 2.5 mg/kg **5m** and from $t = 40$ until $t = 180$ min after 5 or 10 mg/kg **5m** ($p < 0.05$).

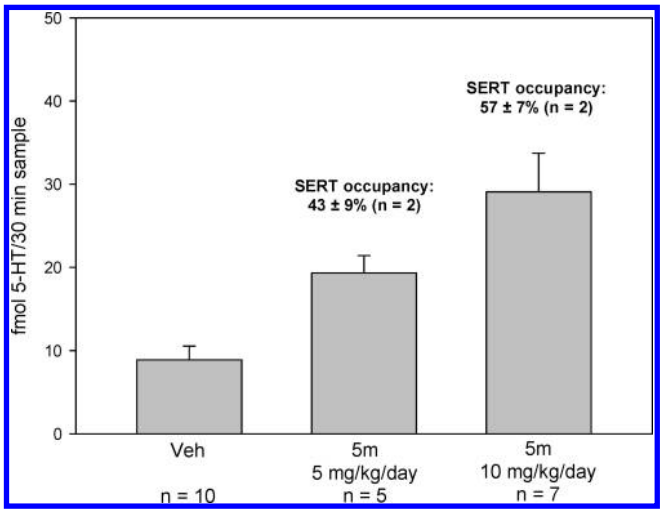
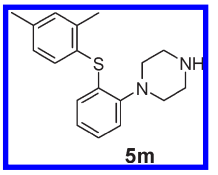


Figure 6. Effects of 3-day treatment with vehicle or **5m** (5 or 10 mg/kg sc) on extracellular 5-HT levels in the rat medial prefrontal cortex (mPFC) and SERT occupancy. Data are expressed as the mean ± SEM. Three days of treatment with **5m** (5 or 10 (mg/kg)/day) resulted in significantly higher basal levels of 5-HT in the mPFC compared to animals treated with vehicle ($p < 0.05$).

DISCUSSION AND CONCLUSION

A novel series of compounds with combined effects on h5-HT_{3A} and h5-HT_{1A} receptors and on the rSERT was discovered. Compound **5m** was identified as the lead compound from this series, displaying the desired relative affinities/potencies (K_i/IC_{50}) between 5-HT_{3A}, 5-HT_{1A}, and SERT, as well as appropriate pharmacokinetic properties for further development. Compound **5m** met the criteria set for efficacy at these targets, displaying antagonistic properties at h5-HT_{3A} receptors, agonistic properties at h5-HT_{1A} receptors, and inhibition of hSERT. Whereas **5m** showed comparable potencies at both rat and human SERT, species differences were found at 5-HT_{1A} and 5-HT_{3A} receptors. Hence, rat models should be used with caution when predicting the clinical potential of **5m**. Additional profiling revealed that compound **5m** exerted high affinity (K_i < 100 nM) at noradrenergic hβ_1 (K_i = 46 nM), serotonergic h5-HT_{1B} (K_i = 33 nM), and h5-HT_7 (K_i = 19 nM) receptors. In vitro functional characterization at these receptors showed that **5m** was a partial agonist at h5-HT_{1B} receptors and an antagonist at h5-HT_7 receptors. Taken together, compound **5m** exerts a multimodal serotonergic action, by inhibiting SERT and interacting with h5-HT_{1A}, h5-HT_{1B}, h5-HT_3, and h5-HT_7 receptors. Indeed, the action of an efficacious 5-HT_{1A} receptor agonist is expected to rapidly desensitize the somatodendritic 5-HT_{1A} autoreceptor[41] and simultaneously activate postsynaptic 5-HT_{1A} receptors.[42,17,18] Moreover, the 5-HT_3 receptor antagonistic effect of **5m** may counteract the inhibitory tonus of the 5-HT_3 receptor on the noradrenergic and cholinergic systems, leading to a facilitating effect on these systems,[19,20] both of which are known to be important in the pathology of mood disorders.[43,44] Additionally, we have shown that the 5-HT_3 receptor antagonist ondansetron potentiates citalopram-induced increases in 5-HT levels in the rat brain,[45]

Table 7. Primary Human (h) in Vitro Binding Profile of 5m



target	K_i (nM)
h5-HT_{1A}	15
h5-HT_{1B}	33
h5-HT_{3A}	3.7
h5-HT_7	19
hSERT	1.6

demonstrating that 5-HT$_3$ receptor blockade also facilitates 5-HT release. While the affinity of **5m** for the noradrenergic hβ_1 receptor did not translate to an obvious pharmacology in vivo, the h5-HT$_{1B}$ and h5-HT$_7$ receptor affinity may contribute to the in vivo pharmacological profile. The 5-HT$_{1B}$ receptors are present on 5-HT nerve terminals, and a number of studies have shown that stimulating 5-HT$_{1B}$ receptors leads to a decrease in 5-HT output. Conversely, the combination of SERT blockade and 5-HT$_{1B}$ receptor antagonism has been shown to augment 5-HT levels in microdialysis studies.[46] We have demonstrated that **5m** exerts a partial agonistic profile at the h5-HT$_{1B}$ receptors, which could translate to an inhibitory control of 5-HT$_{1B}$ receptors in vivo, thus further substantiating the potential of **5m** in a clinical setting. Similarly, 5-HT$_7$ receptor blockade has been shown to be efficacious in animal models of depression, e.g., the mouse forced-swim test and tail-suspension test.[47] The combination of an SSRI with 5-HT$_7$ receptor blockade synergistically augments the levels of 5-HT in the prefrontal cortex,[48] suggesting that 5-HT$_7$ receptor antagonism alone or in combination with SERT blockade has antidepressant potential.

In this study we demonstrate that **5m** affects 5-HT levels in both the ventral hippocampus and mPFC, two important brain regions involved in regulation of mood.[49,50] After acute administration in rat microdialysis experiments, compound **5m** induced a robust increase in extracellular 5-HT levels in the ventral hippocampus. This increase is higher than those obtained after acute administration of SSRIs[51] and SNRIs[52,53] at doses that maximally block SERT,[52] suggesting that the combined pharmacological effects of **5m** in rats on SERT and 5-HT$_{1B}$, 5-HT$_3$, and 5-HT$_7$ receptors lead to an augmentation of the extracellular 5-HT levels when compared to that of SSRIs or SNRIs. As mentioned above, activation by 5-HT$_{1A}$ receptor agonists leads to rapid desensitization of somatodendritic 5-HT$_{1A}$ receptors,[41] suggesting an additional beneficial effect in humans, which might be difficult to model in rats because the r5-HT$_{1A}$ receptor affinity is low. Thus, the effects of **5m** on the 5-HT receptors may cancel out inhibitory feedback effects exerted on acute SERT inhibition.[54] In the rat microdialysis experiments, 3 days of treatment with **5m** resulted in significant increases in extracellular 5-HT levels at low SERT occupancy, again suggesting a multimodal mode of action of **5m**. This is in contrast to treatment with SSRIs, where chronic treatment is generally required to overcome the inhibitory feedback mechanisms and to produce a positive 5-HT response in preclinical models.[55] In conclusion, these characteristics indicate that **5m** is a novel multimodal serotonergic compound that has the potential to benefit symptomatologies associated with mood disorders. Compound **5m** (Lu AA21004) is currently in clinical development for MDD.

EXPERIMENTAL SECTION

Chemistry. Reagents and solvents were obtained from commercial sources and used as received. Thin layer chromatography was carried out on Merck silica gel 60 F254. Flash chromatography was performed using Merck silica gel 60 (40−63 μm). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature on a Bruker Avance AV-500 at 500.13 and 125.77 MHz, respectively. Chemical shifts (δ) are reported in parts per million relative to tetramethylsilane (TMS) or residual solvent, and coupling constants (J) are given in hertz. High-resolution mass spectrometry (HRMS) was recorded on a Bruker Daltonics MicrOTOF operating with external calibration with lithium formate (for ESI) and PEG200/PEG400/PEG600 (1:1:1) [for atmospheric pressure photo ionization (APPI)]. Liquid chromatography/MS (LC/MS), solvent system A was water/trifluoroacetic acid (TFA) (100:0.05) and B was acetonitrile/water/TFA (95:5:0.035). Instruments were API 150EX and LC system Shimadzu LC10ADvp/SLC-10Avp with Sedere Sedex75 ELS and column operating at 60 °C. LC/MS method 1 involved the following: column 30 mm × 4.6 mm Waters Symmetry C18 with 3.5 μm particles operating at room temperature; linear gradient elution with 10% B to 100% B in 2.4 min at a flow rate of 3.3 mL/min. LC/MS method 2 involved the following: as above but instead with a 30 mm × 4.6 mm Waters Atlantis column with 3 μm particles operating at 40 °C; linear gradient elution with 2% B to 100% B in 2.4 min at a flow rate of 3.3 mL/min. LC/MS method 3 involved the following: solvent system A consisting of water/ammonia (99.9:0.1) and B consisting of acetonitrile/water/ammonia (94.9:5:0.1); instruments were Sciex API300 and Waters Acquity UPLC with diode array detector (DAD) and evaporative light scattering (ELS) (Waters); column 50 mm × 2.1 mm Waters BEH with 1.7 μm particles operatomg at 60 °C. Linear gradient elution with 10% B to 100% B in 1 min at a flow rate of 1.2 mL/min. All pharmacologically characterized compounds were >95% pure as determined by LC/MS, with the exception of **6c** and **6d**, which had ultraviolet (UV) purities of 94% and 93%, respectively. These compounds were also characterized by ^{1}H NMR and ^{13}C NMR as well as either elemental analysis or HRMS. Synthesis intermediates were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS data for new compounds, except for synthesis intermediates **13b**, **13c**, and **13d** for which no ^{13}C NMR data are reported due to rotamers.

General Procedure 1 for the Formation of Diaryl Sulfides[31]. Potassium *tert*-butoxide (1.1 equiv) and a solution of an aryl bromide/iodide (1.0 equiv, if this was a solid) were added to Pd_2dba_3 (2.5 mol %) and DPEphos (10 mol %). Subsequent toluene (the amount required to make a ∼10% w/w solution of the aryl bromide/iodide), the aryl bromide/iodide (1.0 equiv, if this was a liquid), and the thiophenol (1.0 equiv) were added. The flask was immersed in an oil bath preheated to 100 °C. The mixture was stirred at this temperature until the reaction was complete (typically <2 h for aryl iodides and overnight for aryl bromides). The crude mixture was loaded onto a silica gel column and eluted with heptane/ethyl acetate to give the products in >95% purity as determined by ^{1}H NMR.

General Procedure 2 for the Formation of Boc-Protected Arylpiperazines from Aryl Bromides[56]. Aryl bromide (1.0 equiv, if this was a solid), Boc-piperazine (1.2 equiv), and sodium *tert*-butoxide (1.4 equiv) were added to Pd_2dba_3 (2.5 mol %) and racemic BINAP (7.5 mol %). Subsequently toluene (the amount required to make a ∼10% w/w solution of the aryl bromide) and the aryl bromide (1.0 equiv, if this was a liquid) were added. The flask was immersed in an oil bath preheated to 100 °C. The mixture was stirred at this temperature overnight. The crude mixture was loaded onto a silica gel column and eluted with heptane/ethyl acetate to give the products in >95% purity as determined by ^{1}H NMR.

General Procedure 3 for the Cleavage of Boc Groups. The substrate was dissolved in methanol (the amount required to make a solution of approximately 20% w/w of the substrate). An HCl solution (2 M) in Et_2O (approximately 3 equiv) was added to the mixture, and the resulting mixture was stirred at room temperature overnight. If required, the mixture was cooled in an ice/water bath and diluted with Et_2O to precipitate the product as the hydrochloride salt. No attempts were made to isolate additional product from the filtrate.

The synthesis procedure and compound characterization data for the final compounds in Table 3 are summarized below. The synthesis and characterization data for the remaining final compounds and all intermediates are in the Supporting Information.

Compounds Prepared According to Method A (Scheme 1). These compounds were prepared by initial formation of 2-bromophenylpiperazines accordoing to general method 2 followed by formation of the thioether linkage according to general procedure 1. Deprotection and salt formation were done according to general method 3.

*1-[2-(4-Chlorophenylsulfanyl)phenyl]piperazine Hydrochloride (**4h**).* **4h** was prepared according to general procedure 3 starting from intermediate **11h** in a yield of 31%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.20 (s, 2H), 7.48 (d, J = 6.4, 2H), 7.42−7.34 (m, 2H), 7.27 (t, J = 7.2, 1H), 7.20 (d, J = 7.7, 1H), 7.06 (d, J = 7.0, 1H), 6.92 (d, J = 7.7, 1H), 3.22−3.08 (m, 8H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 149.73, 134.19 (2C), 133.10, 132.69, 131.70, 130.14, 130.01 (2C), 128.27, 125.58, 121.10, 48.54 (2C), 43.62 (2C). HRMS calcd for $C_{16}H_{17}ClN_2S$ + H, 305.0874; found, 305.0876. LC/MS (method 1): t_R = 0.98 min, UV purity 97%, ELS purity 100%. Anal. ($C_{16}H_{17}ClN_2S\cdot$HCl) C, H, N.

*1-(2-p-Tolylsulfanylphenyl)piperazine Hydrochloride (**4j**).* **4j** was prepared according to general procedure 3 from impure 4-[2-(4-methylphenylsulfanyl)phenyl]piperazine-1-carboxylic acid *tert*-butyl ester (**11j**) in a yield of 60%. **11j** was prepared starting from **7a** and 4-methylthiophenol according general procedure 1. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (s, 2H), 7.35 (d, J = 8.0, 2H), 7.28 (d, J = 8.0, 2H), 7.22−7.13 (m, 2H), 7.05−6.96 (m, 1H), 6.69 (d, J = 7.6, 1H), 3.18 (broad s, 8H), 2.34 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 148.51, 138.82, 134.33 (2C), 134.13, 130.91 (2C), 128.69, 127.86, 126.94, 125.44, 120.69, 48.53 (2C), 43.67 (2C), 21.14. HRMS calcd for $C_{17}H_{20}N_2S$ + H, 285.1420; found, 285.1422. LC/MS (method 1): t_R = 0.96 min, UV purity 98%, ELS purity 100%. Anal. ($C_{17}H_{20}N_2S\cdot$HBr) C, H, N for a batch of the hydrobromide salt.

*1-[2-(3,4-Dimethylphenylsulfanyl)phenyl]piperazine Hydrochloride (**5g**).* **5g** was prepared according to general procedure 3 from impure 4-[2-(3,4-dimethylphenylsulfanyl)phenyl]piperazine-1-carboxylic acid *tert*-butyl ester (**12g**) in a yield of 70% over two steps. **12g** was prepared starting from **7a** and 3,4-dimethylthiophenol according general procedure 1. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.38 (s, 2H), 7.30−7.10 (m, 5H), 7.05−6.94 (m, 1H), 6.66 (d, J = 7.8, 1H), 3.18 (broad s, 8H), 2.25 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.57, 143.74, 143.01, 140.66, 139.74, 137.36, 136.54, 133.80, 132.80, 131.96, 130.65, 125.84, 53.75 (2C), 48.87 (2C), 24.85, 24.75. HRMS calcd for $C_{18}H_{22}N_2S$ + H, 299.1576; found, 299.1586. LC/MS (method 1): t_R = 1.03 min, UV purity 97%, ELS purity 100%.

*1-[2-(2,3-Dichlorophenylsulfanyl)phenyl]piperazine Hydrochloride (**5i**).* **5i** was prepared according to general procedure 3 starting from impure 4-[2-(2,3-dichlorophenylsulfanyl)phenyl]piperazine-1-carboxylic acid *tert*-butyl ester (**12i**) in a yield of 6%. **12i** was prepared from **7a** and 2,3-dichlorothiophenol according general procedure 1. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.98 (s, 2H), 7.59 (dd, J = 8.0, 1.1, 1H), 7.40 (dd, J = 10.9, 4.3, 1H), 7.34−7.24 (m, 2H), 7.19−7.07 (m, 2H), 7.03−6.97 (m, 1H), 3.22−3.13 (m, 4H), 3.04 (s, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 150.34, 135.99, 131.80, 130.41, 129.20, 129.01, 128.38, 128.12, 127.25, 124.81, 120.70, 112.10, 47.62 (2C), 42.72 (2C). HRMS calcd for $C_{16}H_{16}Cl_2N_2S$ + H, 339.0484; found, 339.0486. LC/MS (method 1): t_R = 1.03 min, UV purity 99%, ELS purity 100%.

*1-[2-(2,4-Dichlorophenylsulfanyl)phenyl]piperazine Hydrochloride (**5l**).* **5l** was prepared according to general procedure 3 starting from impure 4-[2-(2,4-dichlorophenylsulfanyl)phenyl]piperazine-1-carboxylic acid *tert*-butyl ester (**12l**) in a yield of 6%. **12l** was prepared from **7a** and 2,4-dichlorothiophenol according general procedure 1. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.66 (s, 2H), 8.33 (s, 1H), 8.00−7.85 (m, 2H), 7.79 (d, J = 7.9, 1H), 7.74−7.63 (m, 2H), 7.53 (d, J = 7.8, 1H), 3.72 (s, 4H), 3.62 (s, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 150.69, 135.51, 133.93, 133.22, 132.68, 131.55, 129.90, 129.39, 128.95, 128.69, 125.79, 121.56, 48.52 (2C), 43.66 (2C). HRMS calcd for $C_{16}H_{16}Cl_2N_2S$ + H, 339.0484; found, 339.0492. LC/MS (method 1): t_R = 1.07 min, UV purity 96%, ELS purity 100%.

*1-[2-(2,4-Dimethylphenylsulfanyl)phenyl]piperazine Hydrochloride (**5m**).* **5m** was prepared according to general procedure 3 starting from intermediate **12m** in a yield of 78%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.39 (s, 2H), 7.33 (d, J = 7.7, 1H), 7.24 (s, 1H), 7.17−7.07 (m, 3H), 6.96 (dd, J = 7.6, 6.0, 1H), 6.41 (d, J = 7.8, 1H), 3.21 (broad s, 8H), 2.31 (s, 3H), 2.24 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 148.22, 142.04, 139.68, 136.11, 133.74, 132.14, 128.46, 127.19, 126.40, 126.13, 125.46, 120.64, 48.47 (2C), 43.67 (2C), 21.10, 20.47. HRMS calcd for $C_{18}H_{22}N_2S$ + H, 299.1576; found, 299.1584. LC/MS (method 1): t_R = 1.02 min, UV purity 97%, ELS purity 100%. Anal. ($C_{18}H_{22}N_2S\cdot$HCl) C, H, N.

Compounds Prepared According to Method B (Scheme 2). These compounds were prepared by initial formation of brominated diarylsulfides according to general procedure 1 followed by aryl amination under the conditions described for general procedure 2. Deprotection and salt formation were done according to general method 3.

*1-[2-(2,3-Dimethylphenylsulfanyl)phenyl]piperazine Hydrochloride (**5j**).* **5j** was prepared according to general procedure 3 starting from intermediate **12j** in a yield of 48%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.50 (s, 2H), 7.29−7.25 (m, 2H), 7.15 (t, J = 6.4, 3H), 6.97 (dt, J = 8.3, 4.3, 1H), 6.48 (d, J = 7.8, 1H), 3.25−3.17 (m, 8H), 2.31 (s, 3H), 2.25 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 148.42, 140.37, 138.43, 133.54, 133.31, 131.23, 131.10, 127.03, 126.97, 126.63, 125.46, 120.72, 48.48 (2C), 43.61 (2C), 20.92, 17.16. HRMS calcd for $C_{18}H_{22}N_2S$ + H, 299.1576; found, 299.1582. LC/MS (method 1): t_R = 1.05 min, UV purity 98%, ELS purity 100%.

*1-[2-(2-Chloro-4-methylphenylsulfanyl)phenyl]piperazine Hydrochloride (**5p**).* **5p** was prepared according to general procedure 3 starting from intermediate **12p** in a yield of 44%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.47 (s, 2H), 7.48 (s, 1H), 7.35−7.25 (m, 4H), 7.04 (t, J = 7.5, 1H), 6.68 (d, J = 7.8, 1H), 3.24−3.07 (m, 8H), 2.34 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 149.33, 140.95, 136.48, 135.19, 131.47, 131.04, 129.49, 128.72, 128.27, 127.85, 125.60, 121.07, 48.44 (2C), 43.58 (2C), 20.74. HRMS calcd for $C_{17}H_{19}ClN_2S$ + H, 319.1030; found, 319.1032. LC/MS (method 1): t_R = 1.04 min, UV purity 98%, ELS purity 100%.

Compound Prepared According to Method C. Final compound **6g** was prepared by aromatic nucleophilic substitution of 2-fluoronitrobenzene with 2,4-dimethylphenol leading to 2,4-dimethyl-1-(2-nitrophenoxy)benzene (**14**) that was reduced to 2-(2,4-dimethylphenoxy)phenylamine (**15**), which was reacted further as described below.

*1-[2-(2,4-Dimethylphenoxy)phenyl]piperazine Hydrochloride (**6g**).* Compound **15** (2.13 g) and bis(2-bromoethyl)amine hydrobromide[S7] (3.89 g) were suspended in chlorobenzene (50 mL). The mixture was boiled under reflux for 4 h. The solvent was evaporated off, and the residual oil was partitioned between water (100 mL) and ethyl acetate (50 mL). The aqueous layer was basified and extracted with ethyl acetate (2 × 50 mL). The combined organic layers were washed with brine (100 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo to afford an oil. This material was purified by column flash chromatography (eluent EtOAc/MeOH 9:1 → EtOAc/MeOH/Et_3N 9:1:1) to afford the crude title compound. This material was partitioned between water/brine and methylene chloride. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to afford the free base. This material was dissolved in ethyl acetate and treated with 2 M HCl in Et_2O to precipitate the title compound (0.83 g, 26%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.54 (s, 2H), 7.13−7.08 (m, 2H), 7.06 (t, J = 7.4, 1H), 7.00−6.92 (m, 2H), 6.69−6.60 (m, 2H), 3.31 (s, 4H), 3.08 (s, 4H), 2.24 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 150.17, 147.60, 139.38, 130.82, 130.23, 126.23, 126.01, 122.14, 122.01, 118.10, 116.47, 116.07, 45.41 (2C), 41.24 (2C), 18.66, 14.25. HRMS calcd for $C_{18}H_{22}N_2O$ + H, 283.1805; found, 283.1818. LC/MS (method 1): t_R = 0.98 min, UV purity 100%, ELS purity 98%.

In Vitro Pharmacology. The assays used for the SAR studies are described below. Details and all other pharmacology are described in Supporting Information. In general for binding affinity assays, data points were expressed as percent of the specific binding, and the IC_{50} values were determined by nonlinear regression analysis using a sigmoidal variable slope curve fitting. The dissociation constant (K_i) was calculated from the Cheng−Prusoff equation ($K_i = IC_{50}/(1 + ([L]/K_d))$), where the concentration of free radioligand L is approximated to the concentration of added radioligand in the assay, and K_d equals the affinity of the radioligand to the receptor. In general IC_{50} values are based on 6−8 different concentrations covering 4−6 decades.

h5-HT_{3A} Binding Affinity Assay. [^{3}H]Granisetron (1 nM) (Perkin-Elmer) binding to h5-HT_{3A} receptors was evaluated in membranes from a HEK-293 cell line stably expressing the hHT_{3A} receptor. Nonspecific binding was measured in the presence of 10 μM bemesetron. After incubation, the membrane preparations were rapidly filtered under a vacuum using glass-fiber filters. Compounds were tested at least three times over a 6 log concentration range. IC_{50} values were determined by nonlinear regression analysis using Hill equation curve fitting, and K_i values were calculated based on the Cheng−Prusoff equation.

h5-HT_{1A} Binding Affinity Assay. [^{3}H]5-Carboxamidotryptamine (0.15 nM) (Perkin-Elmer) binding to cloned human 5-HT_{1A} receptors in CHO cells or HeLa was assayed in a membrane-based scintillation proximity assay. The level of nonspecific binding was obtained in the presence of 10 μM 5-HT or metergoline. Compounds were tested at least three times over a 6 log concentration range. IC_{50} values were determined by nonlinear regression analysis using Hill equation curve fitting, and K_i values were calculated based on the Cheng−Prusoff equation.

Inhibition of [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT Uptake by Rat Synaptosomes. NET, DAT, and SERT transporter inhibition (IC_{50}) in rat synaptosomes was measured using [^{3}H]NE, [^{3}H]DA, and [^{3}H]5-HT, respectively. After incubation, the membrane preparations were rapidly filtered under a vacuum using glass-fiber filters. The filters were washed with ice-cold buffer using a harvester. Bound radioactivity was measured by scintillation counting using a scintillation cocktail. Compounds were tested at least twice over a 6 log concentration range. IC_{50} values were determined by nonlinear regression analysis using Hill equation curve fitting.

Cytochrome P450 Enzyme Inhibition. CYP enzyme inhibition was performed using recombinant enzymes (Supersome, Gentest), expressing individual CYP enzymes (CYP1A2, CYP2C9, CYP2D6 and CYP3A4). The inhibition of CYP enzymes by the test compounds was assessed by incubating each enzyme with its substrate (7-ethoxy-3-cyanocoumarin was used for CYP1A2 (10 μM); dibenzylfuorescein (2 μM) was used for CYP2C9; resorufin benzyl ether (100 μM) was used for CYP3A4; and 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (3 μM) was used for CYP2D6) alone or in the presence of the test compounds at eight different concentrations (40−0.02 μM). All incubations (28 min for CYP1A2, 30 min for CYP3A4, and 45 min for CYP2C9 and CYP2D6) were performed in phosphate buffer with an NADPH regenerating system containing $NADP^+$, glucose 6-phosphate dehydrogenase, glucose 6-phosphate, sodium citrate and $MgCl_2$. At the end of the incubation period, the amount of metabolite formed was measured using a fluorescence spectrophotometer. The excitation and emission wavelengths in nanometers are 405 (ex) and 465 (em) for CYP1A2 and CYP2D6, 485 (ex) and 535 (em) for CYP2C9, and 530 (ex) and 590 (em) for CYP3A4. Inhibitions were expressed relative to controls (without inhibitors), and IC_{50} values were calculated using Microsoft Excel software.

Microsomal Stability Determination. The microsomal intrinsic clearance was determined by assessing the elimination of test compound over the incubation time. The test compounds were incubated at 1 μM with human and rat microsomes (BD Biosciences) for 60 min, using NADPH as cofactor. An NADPH regenerating system containing $NADP^+$, glucose 6-phosphate dehydrogenase, glucose 6-phosphate, sodium citrate, and $MgCl_2$ (cofactor mix) was used as a source of NADPH. Human and rat microsomes were thawed at room temperature, and cofactor mix was added. The mixture was stirred on a vortex and put in a water bath at 37 °C for 10 min. The reaction was initiated by adding test compound (final concentration 1 μM, 0.5 mg of protein/mL). Separate incubations were made for each test compound and time point. The samples were incubated for 0, 5, 15, 30, and 60 min, and the reactions were stopped by adding 100 μL of acetonitrile and transferred to a 96-well stop plate with seals. The stop plates were centrifuged for 10 min at 3300 rpm and 4 °C before being analyzed by liquid chromatography coupled to a tandem mass spectrometer (LC−MS/MS, Waters QuattroMicro, Manchester, U.K.).

Plasma Binding by Equilibrium Dialysis Measurements. The method was a modification of that reported by Kalvass et al.[58] Briefly, a 96-well equilibrium dialysis apparatus was used to determine the free fraction in the plasma (HT Dialysis LLC, Gales Ferry, CT, U.S.). Membranes (3 kDa cutoff) were conditioned in deionized water for 40 min, followed by conditioning in 80:20 deionized water/ethanol for 20 min, and then rinsed in deionized water before use. Plasma was diluted 1:1 with PBS. Diluted plasma was spiked with the 1 μM test compound and loaded into the 96-well equilibrium dialysis plate. Dialysis versus PBS (100 uL) was carried out for 5 h in a temperature controlled incubator at 37 °C, before being analyzed by liquid chromatography coupled to a tandem mass spectrometer (LC−MS/MS, Waters QuattroMicro, Manchester, U.K.).

In Vivo Pharmacokinetics in Rats. In vivo pharmacokinetics was studied in rats following single intravenous (1 mg/kg) or oral (2 mg/kg) administration of compounds. Serial blood samples were collected at various time points up to 6 h after dosing. In vivo pharmacokinetic parameters were obtained following compartmental modeling. Bioanalysis of samples were analyzed by liquid chromatography coupled to a tandem mass spectrometer (LC−MS/MS, Waters QuattroMicro, Manchester, U.K.).

Microdialysis in Rats. Rats (300−350 g; Harlan, Horst, The Netherlands) were used for the experiments. For acute administration **5m** was dissolved in 10% hydroxypropyl-β-cyclodextrin and administered subcutaneously (sc) in a volume of 1 mL/kg. For 3-day treatment with **5m** minipumps were used to deliver the solutions. Under isoflurane anesthesia osmotic minipumps (2ML2, Alzet, U.S.) were implanted in a subcutaneous pocket created on the right side parallel to the spine of the animal. By stereotaxic surgery I-shaped microdialysis probes (Hospal AN 69 membrane, 4 mm exposed surface; BrainLink) were inserted into the ventral hippocampus or medial prefrontal cortex under isoflurane anesthesia. Microdialysis experiments were performed 24−48 h after surgery by perfusing the probes with artificial CSF (147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$) at a flow rate of 1.5 μL/min. Microdialysis samples were collected at 20 or 30 min intervals into minivials. After the experiment the rats were sacrificed and the position of each probe was histologically verified by making coronal sections of the brain.

Serotonin Analysis. Aliquots were injected onto a HPLC column. Chromatographic separation was performed using an isocratic mobile phase. Mobile phase was run through the system at a flow rate of 0.4 mL/min, and 5-HT was detected electrochemically at +500 mV. For acute experiments four consecutive pretreatment samples were taken as baseline and their mean concentration was set to 100%. Drug effects were expressed as percentages of basal level (mean ± SEM) within the same animal. Moreover, differences between basal outputs of 5-HT (fmol/30 min sample) after treatment with **5m** or vehicle for 3 days were compared.

SERT Occupancy in Rats. After the microdialysis experiments rats were sacrificed and the brains were removed and stored at −80 °C until cutting. Sections of 20 μm containing the striatum were cut and air-dried before the binding experiment. For SERT occupancy 0.5 nM [^{3}H]DASB was used as radioligand. The control for nonspecific binding was 1 μM escitalopram. The binding buffer consisted of 50 mM Tris, 150 mM NaCl, and 5 mM KCl, pH 7.4, and the incubation time was 90 min. Slides were washed in the respective buffers at 4 °C three times for 5 min, then briefly dipped in distilled water and air-dried. The slides were analyzed in a Biospace β-imager 2000 for at least 6 h.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures for synthesis of intermediates and target compounds; combustion analysis and HRMS data; descriptions for in vitro and in vivo assays; pK_i ± SEM values for h5-HT$_{3A}$ and h5-HT$_{1A}$ receptors; and pIC_{50} ± SD/SEM values for rSERT, rNET, and rDAT. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AcCh, acetylcholine; APPI, atmospheric pressure photoionization; Boc, *tert*-butyloxycarbonyl; CHO, Chinese hamster ovary; CYP450, cytochrome P450; DA, dopamine; DAD, diode array detector; DAT, dopamine transporter; DML, designed multiple ligand; ELS, evaporative light scattering; GABA, γ-aminobutyric acid; GAD, generalized anxiety disorder; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HEK, human embryonic kidney; HRMS, high-resolution mass spectrometry; MAOI, monoamine oxidase inhibitor; MDD, major depressive disorder; mPFC, medial prefrontal cortex; NE, norepinephrine; NET, norepinephrine transporter; SAR, structure−activity relationship; SD, standard deviation; SEM, standard error of the mean; SERT, serotonin transporter; SNRI, serotonin and norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TFA, trifluoroacetic acid

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