Bioorganic & Medicinal Chemistry 17 (2009) 337–343

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Stereoselective inhibition of serotonin re-uptake and phosphodiesterase by dual inhibitors as potential agents for depression

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ARTICLE INFO

Article history:
Received 1 September 2008
Revised 24 October 2008
Accepted 29 October 2008
Available online 5 November 2008

Keywords:
Dual selective serotonin re-uptake inhibitor/PDE4 inhibitor
Depression
Transporters
Fluoxetine

ABSTRACT

Multi-target compounds where more than one functional activity is incorporated into the same molecule may have advantages in treating disease states. Selective serotonin re-uptake inhibitors (SSRIs)[a] (i.e., (*R*)- and (*S*)-norfluoxetine) were chemically linked to a PDE4 inhibitor via a five carbon bridge. The new dual PDE4 inhibitor/SSRIs (i.e., (***R***)-**8** and (***S***)-**8**) showed moderately potent but highly selective serotonin re-uptake inhibition (IC_{50} values of 173 and 42 nM, respectively) in vitro. The dual PDE4 inhibitor/SSRIs (***R***)-**8** and (***S***)-**8** also inhibited PDE4D2 (i.e., K_i values of 106 and 253 nM, respectively). Due to the synergistic functional activity, PDE4 inhibitor/SSRIs may be effective in treating diseases such as depression.
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1. Introduction

The human serotonin transporter (hSERT) is a plasma membrane protein responsible for the re-uptake of 5-hydroxytryptamine (5-HT). Transport of 5-HT is sensitive to nanomolar concentrations of SERT re-uptake inhibitors including selective serotonin re-uptake inhibitors (SSRIs).[1] A central hypothesis of antidepressant action is that SSRI re-uptake inhibitor functional activity is due to an elevation of excitatory neurotransmitters such as 5-HT at post-synaptic sites of the brain. Fluoxetine is an SSRI that enhances 5-HT neurotransmission in vitro and in vivo by decreasing 5-HT clearance via uptake inhibition.[2] When animals are treated repeatedly with SSRIs such as fluoxetine, the SERT is down-regulated.[3] The extent of SSRI re-uptake inhibition after repeated treatment is greater than that seen after acute drug treatment due to the increase in 5-HT concentrations.[3,4] Fluoxetine is selective in that it does not potently bind to the human dopamine (hDAT) or human norepinephrine (hNET) transporters and selective re-uptake inhibition of the hSERT is associated with antidepressant efficacy.[5]

Cyclic nucleotide phosphodiesterases (PDEs) comprise a diverse group of enzymes that are important regulators of signal transduction. PDEs are classified into 11 families based on sequence homology, substrates and regulation by modulators. Enzymes in the PDE4 family are particularly important in neuropsychopharmacology.[6] PDE4 hydrolyzes cyclic AMP formed by stimulation of beta adrenergic receptor-linked adenylyl cyclase in brain cortical slices.[7] Rolipram or other selective inhibitors of PDE4 has antidepressant activity in both preclinical[8] and clinical tests[9] and produces memory-enhancing effects in animal models.[10,11] When animals are repeatedly treated with SSRIs, PDE4 is up-regulated.[12] This may be a consequence of 5-HT receptor-mediated cAMP signaling. Increased cAMP augments the expression of a number of PDE4 variants in neurons.[6] Thus, one adaptation of PDE4 that occurs in response to repeated treatment with SSRIs increases 5-HT receptor-mediated cAMP signaling. With repeated treatment, this effect will be blunted as tolerance develops because PDE4 is up-regulated and cAMP hydrolysis is increased.

A dual PDE4 inhibitor/SSRI offers advantages beyond simple additive effects of administration of the individual agents including providing greater symptomatic efficacy and better utility. The 'message-address' concept of a dual agent could afford proximal inhibition of PDE4 thus keeping ample cAMP concentrations present near the activated transporter for greater functional selectivity. Dual PDE4 inhibitor/SSRIs can affect local control in a stimulus-selective manner because of the compartmentalization of the

Abbreviations: SSRIs, selective serotonin re-uptake inhibitors; 5-HT, 5-hydroxytryptamine; hDAT, human dopamine; hNET, human norepinephrine; hSERT, human serotonin transporters; PDEs, phosphodiesterases.

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0968-0896/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmc.2008.10.065

PDE enzymes. The greater PDE4 inhibitor potency of the dual agent will allow lower doses to be used and decrease side effects. Because CNS disorders are recognized as poly-etiological in nature, drugs that modulate multiple targets will contribute to the multi-factorial processes in disease treatment. Reports of the use of multi-functional or multi-modal drugs for CNS targets have increased. For example, single molecular entities for treatment of cognition impairment, motor dysfunction, depression and neurodegeneration that combine one or more of the following properties: (a) cholinesterase inhibition, (b) inhibition or activation of acetylcholine receptors, (c) anti-inflammatory activity, (d) monoamine oxidase inhibition, nitric oxide production, (e) neuroprotection, (f) anti-apoptotic activity and (g) activation of mitochondrial-dependent cell-survival genes and proteins has been reported.[12] New antidepressants with dual serotonin transporter and 5-HT1A receptor affinity have been synthesized.[13] A dual inhibitor of acetylcholinesterase and the serotonin transporter for use in Alzheimer's Disease was reported.[14]

When animals are treated repeatedly with SSRIs such as fluoxetine, PDE4 is up-regulated[15] but dual PDE4 inhibitors/SSRIs offer the advantage of blocking the effect of up-regulation of PDE4. While PDE4 expression will still increase, its hydrolytic activity will be blocked and thus, the overall increase in serotonin receptor-mediated cAMP signaling will be preserved with repeated SSRI treatment. A chemical biology strategy for synthesis of dual function compounds that selectively inhibit PDE4 and 5-HT re-uptake was devised. Compounds with SSRI potency and PDE4 inhibition potency were chemically synthesized and combined by a five carbon linker. Dual inhibitor diastereomers (**8**) were found to be potent re-uptake inhibitors of the SERT and inhibitors of PDE4D2. The successful synthesis of dual SSRI/PDE4 inhibitors based on fluoxetine and phthalazinones and their biological evaluation as inhibitors of biogenic amine transporters and PDE4D2 was done and is reported herein.

2. Results and discussion

2.1. Chemistry

The chemical synthesis of dual SSRI/PDE4 inhibitors consisted of coupling norfluoxetine through a five carbon linker with a known PDE4 inhibitor (i.e., a phthalazinone) to afford the target compound. The enantioselective synthesis of the norfluoxetine portion of the dual inhibitor was obtained by combining the requisite (*R*)- or (*S*)-1-chloro-3-(hydroxy)-3-phenyl-propane with the appropriate Mitsunobu reagent (i.e., made by combining triphenylphosphine, DIAD and trifluoromethyl-*p*-cresol[16]) followed by introduction of the nitrogen atom with potassium phthalimide, NaI and heat (Scheme 1). (*R*)- and (*S*)-norfluoxetine **(*R*)-3** and **(*S*)-3**, respectively, were synthesized from the phthalimides **2** by reduction with hydrazine. The enantioselectivity of the norfluoxetine synthesis of **(*R*)-3** and **(*S*)-3** was confirmed by synthesizing diastereomeric ureas (i.e., *N*′-(*S*)-[1-(1-naphthyl)ethyl]-*N*-(*S*)-3-[4-(triflouromethyl)phenoxy]-3-phenylpropylureas, compounds **(*R*)-4** and **(*S*)-4**) (Scheme 1) and quantifying the benzylic methyl group's center of chirality by NMR. Based on the NMR data it was calculated the enantiopurity of **(*R*)-3** and **(*S*)-3** was >98% and 99%, respectively. The PDE4 inhibitor portion of the dual inhibitor (i.e., compound **6**) (Scheme 2) was synthesized by a previously described route.[17,18] The final target compounds **(*R*)-8** and **(*S*)-8** were prepared in a two step sequence involving: (1) attachment of a five carbon linker by treating the phthalazinone **6** with NaH and 1,5-dibromopentane to afford **7** and (2) linking **7** to **(*R*)-3** and **(*S*)-3** with the aid of CsOH (to diminish formation of the tertiary amine) to afford **(*R*)-8** and **(*S*)-8**, respectively, (Scheme 2). Preliminary studies showed that a five carbon linker was optimal because a three carbon linker afforded a molecule with less potency against the hSERT (data not shown).

2.2. Pharmacology

Binding affinity of each compound (i.e., K_i value) was calculated from the IC_{50} value and measured for each compound by assessing the potency of inhibition of binding [^{125}I] RTI-55 to the hDAT, hSERT and hNET in cells expressing the respective recombinant human transporter.[19,20] In vitro functional potency was measured by determining the re-uptake inhibition (i.e., IC_{50} value) of [^{3}H]-DA, [^{3}H]-5-HT or [^{3}H]-NE by the recombinant hDAT, hSERT or hNET expressed in transfected HEK-293 cells.

Selective inhibitors of PDE4 have produced antidepressant effects in preclinical tests of depression.[9] PDE4 inhibitors antagonize the behavioral and physiological effects of reserpine,[21,22] decrease the time of immobility in the forced-swim test in rats and mice[23,24] and decrease the response rate and increase reinforcement rate in rats under a differential-reinforcement-of-low-rate (DRL) schedule.[25] A number of clinical reports support the conclusion that PDE4 inhibitors have antidepressant activity.[26–28]

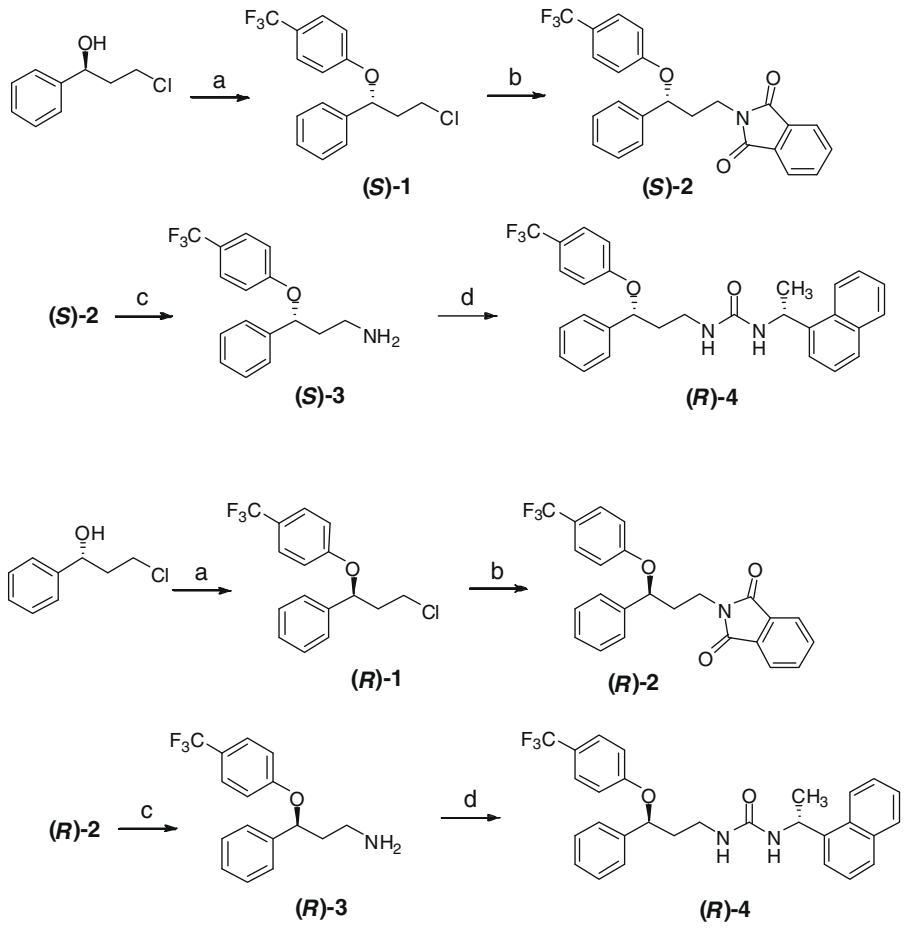
The pattern of effects of PDE4 inhibitors on behavior is distinct from that of selective inhibitors of other PDE families.[24] For example, Ca^{2+}/calmodulin activated PDE1 inhibitors do not improve memory, catecholamine-generated cGMP-activated PDE2 inhibitors have low effects on cAMP, and PDE3 is a cGMP-inhibited PDE but is primarily in adipocytes, platelets and cardiomyocytes and inhibitors are associated with side effects, PDE5 is not present in neurons, and PDE6 is not in the brain.[24] Generalized increases in cAMP do not adequately explain the behavioral effects of PDE4 inhibitors. Thus, changes in cAMP signaling in particular pathways (e.g., beta adrenergic or NMDA receptor-mediated signaling) result in the pattern of behavioral changes observed.[7] There are four PDE4 genes (i.e., *PDE4A, PDE4B, PDE4C* and *PDE4D*). The enzyme PDE4C is mainly in the periphery and was not studied herein.[21,29] PDE4A, PDE4B and PDE4D are present in the brain and their distribution suggests distinct roles in neuropsychopharmacology. Repeated treatment of animals with the antidepressant desipramine that indirectly stimulates beta receptors alters PDE4A and PDE4B expression.[15] However, because the PDE4 inhibitor rolipram only produced antidepressant-like effects in PDE4D knockout mice[21,30] and because PDE4D may play a role in pharmacotherapy of depression, only the inhibition of PDE4D2 was studied using a rapid fluorescence-based assay (Table 1).

2.3. Selective serotonin re-uptake inhibitors

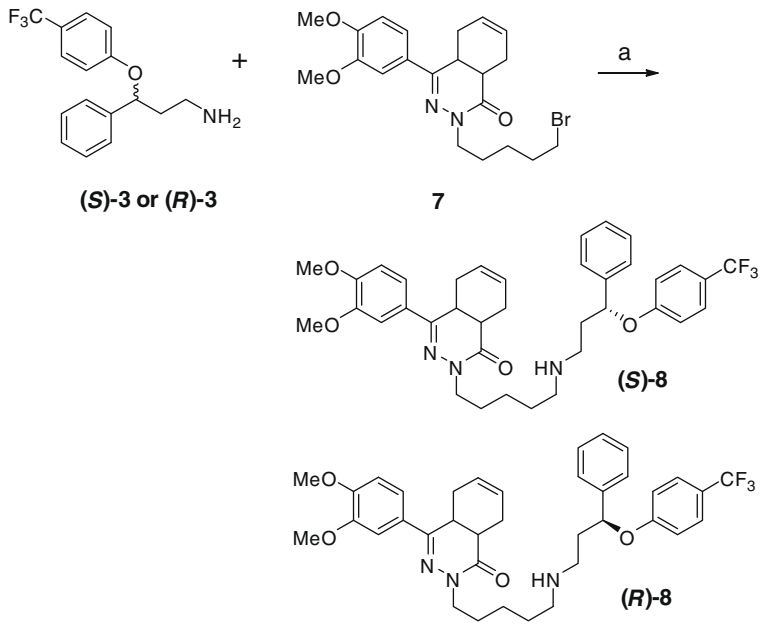
Norfluoxetine **(*S*)-3** and **(*R*)-3** were relatively potent inhibitors of serotonin re-uptake (i.e., K_i values of 4.6 and 13 nM, respectively) (Table 1) compared to the positive control (−)-cocaine. Compounds **(*S*)-3** and **(*R*)-3** were also moderately potent inhibitors of radiolabeled RTI-55 binding to the hSERT (i.e., K_i values of 50 and 26 nM, respectively) and re-uptake (i.e., K_i values of 4.6 and 13 nM, respectively) (Table 1). Compounds **(*S*)-3** and **(*R*)-3** were less potent than fluoxetine in binding and re-uptake inhibition of the hSERT but like fluoxetine, norfluoxetine (i.e., **(*R*)-3** and **(*S*)-3**) were not effective at inhibiting [^{125}I]RTI-55 binding to the hDAT or hNET. Similar to fluoxetine, for **(*R*)-3** and **(*S*)-3**, the selectivity for either binding or re-uptake efficacy for hSERT was very great. Norfluoxetine **(*S*)-3** and **(*R*)-3** showed no detectable inhibition of PDE4D2 (Table 2).

2.4. PDE4 Inhibition

Phthalazinone **6** is a known PDE4 inhibitor and we confirmed this observation (Table 2). It has been previously reported that *N*-substitution in the phthalazinone series (i.e., *cis*-(±)-4a,5,8,8a-



Scheme 1. Synthetic scheme for asymmetric synthesis of norfluoxetine. (a) Triphenylphosphine, DIAD, α,α,α-trifluoromethyl-*p*-cresol; (b) potassium phthalimide, NaI, DMSO, heat; (c) $H_2NNH_2{\cdot}H_2O$, DCM/MeOH (1:1); (d) (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate, toluene.



Scheme 2. Synthetic scheme for synthesis of dual PDE4 inhibitor/SSRIs. (a) NaH, DMF.

tetra- and *cis*-(±)-4a,5,6,7,8,8a-hexahydro-2*H*-phthalazin-1-ones) increased PDE4 inhibition potency.[17] We hypothesized that *N*-substituted derivatives distal to the fused ring system of **6** would increase PDE4 inhibitory potency and this is what was observed. As shown in Table 2, the unmodified racemic phthalazinone **6** was an inhibitor of PDE4D2 (i.e., K_i value 213 nM). Even though it was

Table 1
Inhibition of [^{125}I]RTI-55 binding and [^{3}H]neurotransmitter re-uptake in HEK-hDAT, -hSERT and -hNET cells[a]

Compound	Description	Binding (K_i, nM)			Re-uptake (IC_{50}, nM)		
		hDAT	hSERT	hNET	hDAT	hSERT	hNET
Cocaine		371 ± 81	276 ± 87	1115 ± 198	303 ± 74	416 ± 135	835 ± 229
Fluoxetine		6670 ± 850[b]	1.1 ± 0.5	1560 ± 300	19,500 ± 7600	7.3 ± 2.9	1020 ± 180
(*S*)-**3**	(*S*)-Norfluoxetine	4544 ± 1131	50 ± 21	6186 ± 1993	6488 ± 305	46 ± 34	2806 ± 22
(*R*)-**3**	(*R*)-Norfluoxetine	3661 ± 456	26 ± 3	7490 ± 2299	7133 ± 891	13 ± 3	1995 ± 518
6	PDE4 inhibitor	>100,000	>100,000	>100,000	>100,000	>100,000	>100,000
7	N-modified PDE4 inhibitor	>100,000	>100,000	39,119 ± 4915	>100,000	>100,000	>100,000
(*S*)-**8**	Dual inhibitor	2716 ± 236	300 ± 80	4600 ± 718	5983 ± 2554	42 ± 25	6553 ± 1146
(*R*)-**8**	Dual inhibitor	4492 ± 174	65 ± 13	6567 ± 2578	>7001	173 ± 42	>6811

[a] Values are means ± SEM of at least three independent experiments conducted with duplicate (binding) or triplicate (uptake) determinations, except when two independent experiments exceeded 100 μM.
[b] Data taken from the literature Ref. 19.

Table 2
Inhibition of cAMP hydrolysis by PDE4D2 in the presence of SSRIs or Dual SSRI-PDE4D2 inhibitors[a]

Compound	Description	PDE4D2 Inhibition IC_{50} (nM)
(*S*)-**3**	(*S*)-Norfluoxetine	⩾100 μM
(*R*)-**3**	(*R*)-Norfluoxetine	NI[b]
(*S*)-**8**	Dual inhibitor	253 ± 1
(*R*)-**8**	Dual inhibitor	106 ± 1
6	PDE alone	213 ± 1
7	PDE-C_5-Br	2.3 ± 1

[a] Data are the average of three determinations ± SEM.
[b] NI, no inhibition up to 100 μM concentration.

known that *cis*-(+) **6** is more potent than its (−)-counterpart, the racemic bromopentane derivative **7** was significantly more potent (i.e., K_i value 2.3 nM) and thus, the racemic bromopentane derivative **7** was thus judged to be a good starting point for elaboration of a dual PDE4 inhibitor/SSRI. The PDE4 inhibitors **6** and **7** did not have any detectable potency at binding or re-uptake inhibition of the hDAT, hSERT or hNET (Table 1).

2.5. Dual PDE4 inhibitor/SSRIs

The dual PDE4 inhibitor/SSRIs (i.e., compounds **(*S*)-8** and **(*R*)-8**) were moderately potent inhibitors of binding radiolabeled RTI-55 at the hSERT having K_i values of 300 and 65 nM, respectively. For compounds **(*S*)-8** and **(*R*)-8**, inhibition of neurotransmitter re-uptake was relatively potent and highly selective at the hSERT (i.e., IC_{50} values of 42 and 173 nM, respectively) (Table 1) and **(*S*)-8** and **(*R*)-8** did not potently interact with hDAT or hNET (Table 1).

Compounds **(*R*)-8**, and **(*S*)-8** were examined as inhibitors of cAMP hydrolysis by recombinant human PDE4D2, in vitro. The K_i value for inhibition of human PDE4D2 for **(*S*)-8** and **(*R*)-8** was 253 nM and 106 nM, respectively (Table 2). The decrease in potency for **(*S*)-8** and **(*R*)-8** compared with **7** suggested that the norfluoxetine group substitution makes an impact on inhibition of human PDE4D. However, the inhibition was stereoselective (i.e., **(*R*)-8** > **(*S*)-8**) and that further suggested additional interactions occur at sites distal from the *cis*-fused ring system of the phthalazinone to afford PDE4 inhibition.

3. Conclusions

In summary, the stereoselective synthesis of dual PDE4 inhibitor/SSRIs based on norfluoxetine linked to a phthalazinone PDE4 inhibitor was accomplished and the target compounds were examined in vitro. The results show that ***N***-substitution of the phthalazinone results in a significant increase in PDE4D2 inhibitory potency. However, attachment of norfluoxetine via a five carbon linker to the phthalazinone PDE4 inhibitor decreased PDE4D2 inhibitory potency compared with the compound without the fluoxetine moiety. Additional interactions with PDE4D2 are likely present because the dual PDE4 inhibitor/SSRI with the (*R*)-stereochemistry in the norfluoxetine moiety was considerably more potent than the dual inhibitor with norfluoxetine possessing the (*S*)-stereochemistry. Conversely, the attachment of the phthalazinone moiety to norfluoxetine via a five carbon linker makes a much less pronounced effect on hSERT binding and re-uptake inhibition potency, suggesting the hSERT can accommodate considerable lipophilicity in the region of the aliphatic nitrogen atom. The results show that compounds **(*S*)-8** and **(*R*)-8** retain considerable potency for binding and re-uptake inhibition of the hSERT. Further, the selectivity for hSERT versus hDAT and hNET observed for norfluoxetine is retained in the fluoxetine-based dual PDE4 inhibitor/SSRIs. It is likely that compounds such as **(*R*)-8** possess sufficiently suitable pharmacological and pharmaceutical properties to test in vivo as a new class of antidepressant.

4. Experimental

4.1. Chemistry

4.1.1. General

Chemicals used in this study were of the highest purity available. Commercially available reagents including (*R*)- and (*S*)-α,α,α-trifluoromethyl-*p*-cresol were purchased from Aldrich Chemical Company (Milwaukee, WI) or VWR (San Diego, CA) and were used as received. 2'-Fluo-AHC-cAMP was from Axxora LLC (San Diego, CA). All moisture sensitive reactions were carried out in flame-dried glassware under an argon atmosphere. Tetrahydrofuran (THF) and toluene were freshly distilled from calcium hydride under an argon atmosphere. Methanol (CH_3OH) was passed through a column of neutral alumina and stored over 3 Å molecular sieves prior to use. Fluoxetine was obtained from Sigma Chemical Company (St. Louis, MO). Cocaine was provided by the National Institute on Drug Abuse, NIH (Bethesda, MD). RTI-55 was a kind gift of Dr. Ivy Carroll (RTI, Research Triangle Park, NC). [^{3}H]-DA, [^{3}H]-5-HT, [^{3}H]-NE and [^{125}I]-RTI-55 were purchased from Perkin-Elmer Life Sciences (Boston, MA). The preparation of the hDAT used was described previously.[19] The hSERT cDNA and HEK cells transfected with hNET cDNA was generously supplied by Dr. Randy Blakely (Vanderbilt University, Nashville, TN). The IMAP™ TR-FRET Screening Express with Progressive Binding Kit was obtained from Molecular Devices (Sunnyvale, CA). PDE4D2 was purchased from BPS Biosciences (San Diego, CA). Analytical thin-layer chromatography (TLC) was done on K6F silica gel 60 Å glass-backed plates from Whatman (Clifton, NJ). Compounds were detected using UV absorption at 254 nm and/or stained with I_2

(iodine). Flash chromatography was done on (60 Å) pore silica gel from E. Merck (Darmstadt, Germany). ^{1}H NMR (300 MHz) spectra were determined on a Varian Mercury 300 instrument in the indicated solvent. Low resolution mass spectra (LRMS) were recorded on a Hitachi M8000. High resolution mass spectra (HRMS) were obtained with a Micromass LCT time of flight mass spectrometer at the University of Montana Mass Spectral Facility (Missoula, MT) using ESI.

4.1.2. (*R*)- or (*S*)-1-Chloro-3-(4-trifluormethyl-phenoxy)-3-phenyl-propane (1)

Into a flame dried round bottom flask purged with $Ar_{(g)}$ was placed PPh_3 (368 mg, 1.4 mmol, 1.2 equiv) and 1.5 mL of THF. To this solution was added DIAD (284 mg, 1.4 mmol, 1.2 equiv) and the mixture was stirred at rt for 20 min. The α,α,α-trifluoromethyl-*p*-cresol (190 mg, 1.2 mmol, 1.0 equiv) dissolved in 0.5 mL THF and was added via syringe and the reaction was stirred at rt for 4 h until a light precipitate formed and the solution turned greenish in color. The chiral alcohol (200 mg, 1.2 mmol, 1.0 equiv) dissolved in 0.5 mL THF was then added via syringe and the precipitate and color dissipated within 3 min after addition. The mixture was then stirred at rt overnight and then concentrated to an oil. The crude oil was chromatographed on silica with 5% EtOAc/Hexanes (v:v) as an eluent to afford the pure product as a clear oil (242 mg, 66%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.47 (d, J = 8.7 Hz, 2H), 7.36 (m, 5H), 6.94 (d, J = 8.7 Hz, 2H), 5.46 (dd, J = 3.9, 4.7 Hz, 1H), 3.83 (m, 1H), 3.62 (m, 1H), 2.51 (m, 1H), 2.25 (m, 1H).

4.1.3. (*R*)- or (*S*)-1-Phthalimide-3-(4-trifluormethyl-phenoxy)-3-phenyl-propane (2)

Into a 20 mL vial was placed (*R*)- or (*S*)-1-chloro-3-(4-trifluoromethyl-phenoxy)-3-phenyl-propane (**1**) (468 mg, 1.5 mmol, 1.0 equiv), NaI (10 mg), potassium phthalimide (550 mg, 3.0 mmol, 2.0 equiv) and DMSO (3 mL). The vial was then placed in a 70 °C oil bath and stirred for 13 h. When the reaction was complete as judged by TLC the reaction was cooled to rt and stopped with H_2O. The resulting solution was placed in a separatory funnel and extracted with EtOAc (3× 25 mL). The organic layers were combined and washed with brine, dried over Na_2SO_4, filtered through paper, and then concentrated to give a light yellow solid. The crude material was purified with silica chromatography with 25% EtOAc/Hexanes (v:v) as an eluent to afford pure product as a light yellow solid (461 mg, 73%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.81 (m, 2H), 7.70 (m, 2H), 7.37 (d, J = 8.7 Hz, 2H), 7.31 (m, 5H), 6.81 (d, J = 8.7 Hz, 2H), 5.28 (dd, J = 3.9, 5.5Hz, 1H), 3.95 (m, 2H), 2.43 (m, 1H), 2.22 (m, 1H).

4.1.4. (*R*)- or (*S*)-Norfluoxetine (3)

Into a vial was placed (*R*)- or (*S*)-1-phthalimide-3-(4-trifluoro methyl-phenoxy)-3-phenyl-propane (**2**) (142 mg, 0.3 mmol, 1.0 equiv) and DCM (1 mL). The solution was stirred until all of the phthalimide was dissolved. MeOH (1 mL) followed by hydrazine monohydrate was added to the vial and the reaction was stirred at rt for 8 h while a creamy white precipitate formed. When the reaction was complete it was concentrated in vacuo to a white solid, dissolved in DCM and transferred to a separatory funnel and extracted with H_2O. The solution was re-extracted with DCM (1× 25 mL) and then the aqueous layer was made basic (pH 12) with 10 M NaOH and extracted again with DCM (2× 25 mL). The organic layers were combined and washed with brine, dried over Na_2SO_4, filtered through paper, and concentrated to give a yellow oil (97 mg, 99%). The resulting material was pure enough to use in the following reaction; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.43 (d, J = 8.7 Hz, 2H), 7.29 (m, 5H), 6.90 (d, J = 8.7 Hz, 2H), 5.32 (dd, J = 3.6, 4.5Hz, 1H), 2.89 (t, J = 7.2Hz, 2H), 2.16 (m, 1H), 1.97 (m, 1H), 1.31 (bs, 2H).

4.1.5. *N*′-(*R*)-[1-(1-Naphthyl)ethyl]-*N*-(*S*)-3-[4-(triflouromethyl) phenoxy]-3-phenylpropylurea, (*R*)-4

(*R*)-Norfluoxetine (44 mg, 0.15 mmol, 1.1 equiv) and (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (27 mg, 0.14 mmol, 1.0 equiv) was placed in a vial containing toluene (0.8 mL). The mixture was stirred at room temperature for 13 h and then concentrated in vacuo to afford a light yellow oil. The product was purified with PTLC on silica and eluted with EtOAc/Hexane (1:3, v:v, R_f = 0.3). ^{1}H NMR ($CDCl_3$, 500 MHz) δ *8.22–7.21* (m, 14 H), 6.71 (d, J = 8.7 Hz, 2H), 5.53 (t, J = 6.6 Hz, 1H), 5.01–4.93 (m, 1H), 4.60 (bt, 1H), 3.22 (m, 2H), 2.04–1.96 (m, 2H) 1.54 (d, J = 6.9 Hz, 3H). The enantiopurity of **(*R*)-4** was calculated to be 98% based on the benzylic methyl group at δ 1.54.

4.1.6. *N*′-(*S*)-[1-(1-Naphthyl)ethyl]-*N*-(*S*)-3-[4-(triflouromethyl) phenoxy]-3-phenylpropylurea (*S*)-4

(*S*)-Norfluoxetine (44 mg, 0.15 mmol, 1.1 equiv) and (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (27 mg, 0.14 mmol, 1.0 equiv) was placed in a vial containing toluene (0.75 mL). The mixture was stirred at room temperature for 13 h and then concentrated in vacuo to afford a light yellow oil. The product was purified with PTLC on silica and eluted with EtOAc/Hexane (1:3, v:v, R_f = 0.3). ^{1}H NMR ($CDCl_3$, 500 MHz) δ *8.22–7.21* (m, 14H), 6.71 (d, J = 8.7 Hz, 2H), 5.53 (t, J = 6.6 Hz, 1H), 5.01–4.93 (m, 1H), 4.60 (bt, 1H), 3.22 (m, 2H), 2.04–1.96 (m, 2H) 1.61 (d, J = 6.9 Hz, 3H). The enantiopurity of **(*S*)-4** was calculated to be 99% based on the benzylic methyl group at δ 1.61.

4.1.7. 6-(3,4-Dimethoxy-benzoyl)-cyclohex-3-enecarboxylic acid (5)

A solution of Grignard reagent, 3,4-dimethoxyphenylmagnesium bromide (59 mmol, 0.3 M) in THF was added dropwise to an ice-cooled solution of *cis*-1,2,3,6-tetrahydrophthalic anhydride in THF (120 mL) over a 1 h period. After the addition was complete, the resulting mixture was stirred for another 30 min at 0 °C. The reaction mixture was allowed to warm to rt and stirred overnight. The mixture was stopped with sat. NH_4Cl and the pH adjusted to 2 with concentrated $HCl_{(aq)}$ and extracted with diethyl ether. The organic layer was washed with water and extracted with 1 M NaOH. The combined aqueous extract was acidified with concentrated HCl and extracted with EtOAc (3× 100 mL). The organic layers were combined and dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford an oil. The oil was dissolved CH_2Cl_2 and filtered through silica gel to remove the dicarboxylic acid formed during workup. The product was recrystallized from diethyl ether to afford pure product (1.6 g, 10%); mp = 109 °C; LRMS ESI $[M-H]^-$ calcd for $C_{16}H_{18}O_5$ 289, found *m/z* 289; ^{1}H NMR ($CDCl_3$, 500 MHz): δ *7.60–7.53* (m, 2H), 6.92 (d, J = 8.4Hz, 1H), 5.84–5.82 (m, 1H), 5.71–5.68 (m, 1H), 4.03–4.00 (m, 1H), 3.98 (s, 3H), 3.95 (s, 1H), 3.10–3.07 (m, 1H), 2.90–2.84 (m, 1H), 2.54–2.44 (m, 3H).

4.1.8. 4-(3,4-Dimethoxy-phenyl)-4a,5,8,8a-tetrahydro-2H-phthalazin-1-one (6)

A mixture of 5 (610 mg, 2.1 mmol, 1.0 equiv) and hydrazine hydrate (168 mg, 5.3 mmol, 2.5 equiv) in EtOH (10 mL) was refluxed for 4 h. The mixture was then cooled to rt and concentrated under reduced pressure to afford a white precipitate. The precipitate was dissolved in EtOAc and washed with $Na_2SO_{4(aq)}$, 1 N $HCl_{(aq)}$, and water. The organic layer was then dried over $MgSO_4$, filtered, and concentrated to give a white precipitate. The precipitate was recrystallized in EtOH to afford the product as white crystals (376 mg, 63%); HRMS ESI $[M+H]^+$ calcd for $C_{16}H_{18}N_2O_3$ 287.3385,

found *m/z* 287.3379; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.53 (bs, 1H), 7.46 (d, J = 2.0 Hz, 1H), 7.23 (dd, J = 2.0, 8.4Hz, 1H), 6.87 (d, J = 8.4Hz, 1H), 5.8–5.77 (m, 1H), 5.72–5.7 (m, 1H), 3.94 (s, 3H), 3.93 (s, 3H), 3.40 (dt, J = 5.5, 8.7 Hz, 1H), 3.01–2.97 (m, 1H), 2.85 (t, J = 6.0 Hz, 1H), 2.26–2.19 (m, 3H); HPLC >98% pure (t_R = 4.93, 70(A):30(E); t_R = 5.33, 50(A):50(E)).

4.1.9. 2-(5-Bromo-pentyl)-4-(3,4-dimethoxy-phenyl_-4a,5,8,8a-tetrahydro-2H-phthalazin-1-one (7)

Sodium hydride (60% dispersion in oil, 44 mg, 1.1 mmol, 1.1 equiv) was added to a solution **2** (287 mg, 1.0 mmol, 1.0 equiv). The mixture was stirred at rt for 30 min whereupon it took on a slight yellow color. 1,5-Dibromopentane (600 mg, 2.6 mmol, 2.6 equiv) was added via syringe and the mixture was stirred for 30 min at rt while the yellow color dissipated to an almost clear solution. The reaction was stopped with the addition of water and then transferred to a separatory funnel and extracted with Et_2O (3× 30 mL). The organic fractions were combined and dried over $MgSO_4$, filtered, and concentrated to give an oil. The product was purified on silica and eluted with CH_2Cl_2 to afford a clear oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.47 (d, J = 2.0 Hz, 1H), 7.25 (dd, J = 2.0, 8.5Hz, 1H), 6.87 (d, J = 8.5Hz, 1H), 5.8–5.77 (m, 1H), 5.7–5.67 (m, 1H), 4.01–3.97 (m, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 3.79–3.74 (m, 1H), 2.22–2.18 (m, 2H), 2.1–2.04 (m, 1H), 1.94–1.86 (m, 4H), 1.74–1.69 (m, 4H), 1.63–1.56 (m, 1H), 1.52–1.46 (m, 2H); LRMS ESI $[M+H]^+$ calcd for $C_{21}H_{28}BrN_2O_3$ 436, found *m/z* 435 (Br^{79}), 437 (Br^{81}).

4.1.10. 4-(3,4-Dimethoxy-phenyl)-2-[(3-(4-trifluoromethyl-phenoxy)-3-phenyl]-ethylamino)-pentyl]-4,5,8,8a-tetrahydro-2*H*-phthalazin-1-one (8)

Into a vial was placed (*R*)- or (*S*)-norfluoxetine (30 mg, 0.1 mmol, 1.0 equiv), NaH (5 mg, 0.12 mmol, 1.2 equiv) and DMF (0.3 mL) and the mixture was stirred at rt for 20 min. The alkyl halide 7 (44 mg, 0.1 mmol, 1.0 equiv) dissolved in DMF (0.3 mL) was added via syringe and the mixture was stirred at rt overnight. When the reaction was complete as judged by TLC, it was dissolved in Et_2O transferred to a separatory funnel and extracted with H_2O. Saturated $NaHCO_3$ was added to the solution which was then reextracted with Et_2O (3× 20 mL). The organic layers were combined and washed with H_2O (2× 30 mL) and brine. The organic layer was then dried over $MgSO_4$, filtered and concentrated to afford a clear oil. The product was purified with RP PTLC eluting twice with CH_3NH_2/MeOH (1:1 vol:vol) and four more times with MeOH R_f = 0.1.

4.1.11. *R*-(8) enantiomer

^{1}H NMR ($CDCl_3$, 300 MHz): δ *7.47–7.29* (m, 9 H), 6.90–6.86 (m, 3H), 5.80–5.66 (m, 2H), 5.31–5.27 (m, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.79–3.7 (m, 1H), 3.36–3.28 (m, 1H), 3.02–2.97 (m, 1H), 2.77 (m, 2H), 2.6 (m, 2H), 2.23–2.0 (m, 5H), 1.72–1.3 (m, 7H), 0.87 (m, 2H);LRMS ESI $[M+H]^+$ calcd for $C_{37}H_{42}F_3N_3O_4$ 650, found *m/z* 650, and 364; HRMS ESI $[M+H]^+$ calcd for $C_{37}H_{42}F_3N_3O_4$ 649.3127, found *m/z* 649. 3133, HPLC Supelco Discovery HS F5HPLC column 39693-02, H_2O (0.35% formic acid)/CH_3N/MeOH (1:1:18 vol:vol) t_R = 84.75; H_2O (0.35% formic acid)/CH_3N/MeOH (1:1:8 vol:vol) t_R = 110.17. Purity was >98%.

4.1.12. *S*-(8) enantiomer

^{1}H NMR ($CDCl_3$, 300 MHz): δ *7.47–7.24* (m, 9 H), 6.90–6.86 (m, 3H), 5.81–5.66 (m, 2H), 5.31–5.27 (m, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.79–3.7 (m, 1H), 3.36–3.28 (m, 1H), 3.02–2.97 (m, 1H), 2.77 (m, 2H), 2.6 (m, 2H), 2.23–2.0 (m, 5H), 1.72–1.3 (m, 7H), 0.87 (m, 2H); LRMS ESI $[M+H]^+$ calcd for $C_{37}H_{42}F_3N_3O_4$ 650, found *m/z* 650, and 364; HRMS ESI $[M+H]^+$ calcd for $C_{37}H_{42}F_3N_3O_4$ 649.3127, found *m/z* 649.3121, HPLC Supelco Discovery HS F5 HPLC column 39693–02, H_2O (0.35% formic acid)/CH_3N/MeOH (1:1:18 vol:vol) t_R = 83.68; H_2O (0.35% formic acid)/ CH_3N/ MeOH (1:1:8 vol:vol) t_R = 109.47. Purity was >97%.

4.1.13. Biology

HEK-hDAT, -hSERT, or -hNET cells were grown until confluent as described previously.[19] Cells were scraped from plates and centrifuged for 20 min at 30,000g and the pellet was re-suspended in 0.32 M sucrose with a Polytron at a setting of 1 for 5 s. Assays contained 50 μL of membrane preparation, 25 μL of the test compound and 25 μL of [^{125}I]RTI-55 (40–80 pM final concentration) in a final volume of 250 μL Krebs HEPES buffer (25 mM HEPES, 122 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 μM pargyline, 100 μM tropolone, 2 mg glucose/mL and 0.2 mg ascorbic acid/mL at pH 7.4.). Membranes were pre-incubated with test compounds for 10 min before addition of [^{125}I]RTI-55. Specific binding was determined as the difference in binding observed in the presence and absence of 5 μM mazindol (HEK-hDAT and hNET) or 5 μM imipramine (HEK-hSERT). The incubations were done in the dark and terminated by filtration onto a Whatman GF/C filters using a 96-well Tomtech cell harvester (Tomtech, Orange, CT). Scintillation fluid was added to each filter spot and radioactivity remaining on the filter was determined using a Wallace ß-plate reader (Wallace Labs, Cranbury, NJ). Specific binding was defined as the difference in binding in the presence and absence of 5 μM mazindol (hDAT and hNET) or 5 μM imipramine (hSERT).

4.1.14. Inhibition of substrate uptake

HEK-hDAT, -hSERT or -hNET cells were grown as described above. Cells were scraped from the plates and suspended cells were added to a 96-well plate containing test compounds and Krebs-HEPES buffer in a final assay volume of 0.5 mL. After a 10 min preincubation in a 25 °C water bath, [^{3}H]-labeled neurotransmitter (50 μL, 20 nM final concentration) was added and the assay was initiated. After 10 min the incubation was terminated by filtration onto GF/C filters presoaked with 0.05% polyethylenimine using a Tomtech call harvester and radioactivity remaining on the filters was determined as described above. Specific uptake was defined as the difference in uptake in the presence and absence of 5 μM mazindol (hDAT and hNET) or 5 μM imipramine (hSERT).

4.1.15. PDE4 assay

PDE4 enzyme assays were carried out as described below. Briefly, 2'-Fluo-AHC-cAMP (100 nM), test compound (10 pM to 100 μM) and PDE4D2 (0.2 ng/incubation) were mixed and incubated in a black 96-well plate at room temperature for 1 h followed by addition of binding buffer per the manufacturers specification. The fluorescence polarization at excitation of 485 nm and emission of 520 nm with a microplate reader was done. Specific hydrolysis was defined as the difference in hydrolysis in the presence and absence of test compound. Two-three determinations were done at each of 10 test compound concentrations.

4.1.16. Data analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used to determine the kinetic data. IC_{50} values were converted to K_i values using the Cheng–Prusoff equation.

Acknowledgments

The analytical help of Luke Guo and John Buza is gratefully acknowledged. The financial support of the HBRI is gratefully acknowledged.

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