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Inhibition of serotonin and norepinephrine reuptake and inhibition of phosphodiesterase by multi-target inhibitors as potential agents for depression

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ABSTRACT

Compounds possessing more than one functional activity incorporated into the same molecule may have advantages in treating complex disease states. Balanced serotonin/norepinephrine reuptake inhibitors (SNRIs) (i.e., (*R*)- and (*S*)-norduloxetine) were chemically linked to a PDE4 inhibitor via a five carbon bridge. The new dual SNRI/PDE4 inhibitors (i.e., (***R***)-**15** and (***S***)-**15**) showed moderately potent serotonin reuptake inhibition (IC_{50} values of 442 and 404 nM, respectively) but low reuptake inhibition of norepinephrine (IC_{50} values of 2097 and 2190 nM, respectively) in vitro. The dual SNRI/PDE4 inhibitors (i.e., (***R***)-**15** and (***S***)-**15**) also inhibited PDE4D2 (i.e., K_i values of 23 and 45 nM, respectively). Due to their synergistic functional activity, SNRI/PDE4 inhibitors may be effective in treating diseases such as depression.

1. Introduction

Duloxetine is used in the treatment of major depression and is a potent inhibitor of serotonin and norepinephrine reuptake but only weakly inhibits dopamine reuptake.[1,2] The human serotonin transporter (hSERT) and norepinephrine transporter (hNET) are membrane proteins responsible for the re-uptake of 5-hydroxytryptamine (5-HT) and NE, respectively. Transport of 5-HT/NE is sensitive to nanomolar concentrations of dual SERT/NET reuptake inhibitors including serotonin norepinephrine reuptake inhibitors (SNRIs).[3] A hypothesis of duloxetine antidepressant action is that SNRI reuptake inhibition is due to elevation of excitatory neurotransmitters 5-HT and NE at post-synaptic sites of the brain. Balanced dual inhibition of monoamine reuptake processes possibly may offer advantages over other antidepressants by treating a wider range of symptoms.[4] Duloxetine is an SNRI that enhances 5-HT and NE neurotransmission in vitro and in vivo by decreasing 5-HT clearance via reuptake inhibition.[2] When animals are chronically treated repeatedly with duloxetine, cortical SERT, and NET density was down-regulated.[5] Repeated treatment of animals with duloxetine caused region-selective up-regulation of BDNF mRNA and protein in the brain.[6,7] Up-regulation of these genes may help explain the onset of antidepressant activity observed with SNRIs.

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.[8] PDE4 hydrolyzes cyclic AMP formed by stimulation of beta adrenergic receptor-linked adenylyl cyclase in brain cortical slices.[9] Rolipram or other selective inhibitors of PDE4 have antidepressant activity in both preclinical[10] and clinical tests[11] and produces memory-enhancing effects in animal models.[12,13] Repeated treatment of animals with SNRIs may up-regulate PDE4.[14] 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.[8] Thus, one adaptation of PDE4 that occurs in response to repeated treatment with SNRIs may be increased 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 balanced dual SNRI/PDE4 inhibitor may offer advantages beyond simple additive effects of administration of individual agents including providing greater symptomatic efficacy and

Abbreviations: SNRIs, serotonin norepinephrine reuptake inhibitors; 5-HT, 5-hydroxytryptamine; NE, norepinephrine; hDAT, human dopamine; hNET, human norepinephrine; hSERT, human serotonin transporters; PDEs, phosphodiesterases.

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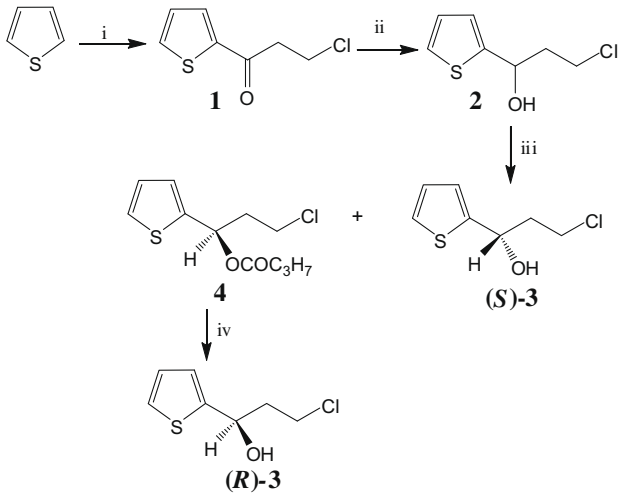
better utility. Dual SNRI/PDE4 inhibitors could affect local control in a stimulus-selective manner because of the compartmentalization of the PDE enzymes and monoamine transporters. Because central nervous system (CNS) disorders are recognized as poly-etiological in nature, drugs that modulate multiple targets may be more effective in contributing to the multi-factorial processes in disease treatment.

Dual SNRI/PDE4 inhibitors may offer an advantage of blocking the effect of up-regulation of PDE4 because 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 SNRI treatment. A strategy for synthesis and testing of multi-function compounds that selectively inhibit PDE4 and 5-HT/NE reuptake was undertaken. Compounds possessing SNRI and PDE4 inhibition potency were chemically synthesized and combined by a five carbon linker. Dual inhibitor diastereomers (**15**) were found to be moderate reuptake inhibitors of human SERT and potent inhibitors of PDE4D2. The successful synthesis of dual SNRI/PDE4 inhibitors based on duloxetine 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 SNRI/PDE4 inhibitors consisted of coupling norduloxetine through a five carbon linker with a known PDE4 inhibitor (i.e., a phthalazinone) to afford the target compound. The enantioselective synthesis of norduloxetine followed the general procedure for duloxetine.[15] The norduloxetine portion of the dual inhibitor was obtained by combining the requisite alkyl chloride (***R*)-3** or (***S*)-3** (Scheme 1) with the appropriate Mitsunobu reagent (i.e., made by combining triphenylphosphine, DIAD, and 1-naphthol[16]) followed by introduction of the nitrogen atom with potassium phthalimide, NaI and heat (Scheme 2). (*R*)- and (*S*)-norduloxetine (***R*)-10b** and (***S*)-10b**, respectively, were synthesized from the phthalamide **9** by reduction with hydrazine. The enantioselectivity of the norduloxetine synthesis of (***R*)-10b** and (***S*)-10b** was confirmed by converting individual isomer precursors (i.e., (***S*)-6** and (***R*)-6**) to their corresponding urea derivatives using (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate[17] (Scheme 3). The diastereomeric purity of the resulting urea was then judged by ^{1}H NMR. The methyl groups of the two diastereomers possess different chemical shifts ($\Delta \sim 0.2$ ppm). Based on the purity of the chiral isocyanate, the ^{1}H NMR spectra of compounds **7**(***S*,*R***) and **7**(***R*,*R***) showed that they were >90% diastereomerically pure.



Scheme 1. Synthetic scheme for asymmetric synthesis of norduloxetine. Reagents and conditions: (i) 3-chloropropionyl chloride, $SnCl_4$, benzene, 0 °C; (ii) $NaBH_4$, EtOH; (iii) Novozyme, vinylbutanoate, *n*-hexane; (iv) LiOH, H_2O/THF.

Based on the NMR studies described above, the enantiopurity of (***R*)-10b** and (***R*)-15** and (***S*)-10b** and (***S*)-15** was calculated to be >90%. The PDE4 inhibitor portion of the dual inhibitor (i.e., compound **13**) (Scheme 4) was synthesized by a previously described route.[18,19] The final target compounds (***R*)-15** and (***S*)-15** were prepared in a two step sequence involving: (1) attachment of a five carbon linker by treating the phthalazinone **13** with NaH and 1,5-dibromopentane to afford **14** and (2) linking **14** to (***R*)-10** and (***S*)-10** with the aid of CsOH (to diminish formation of the tertiary amine) to afford (***R*)-15** and (***S*)-15**, respectively (Scheme 2).

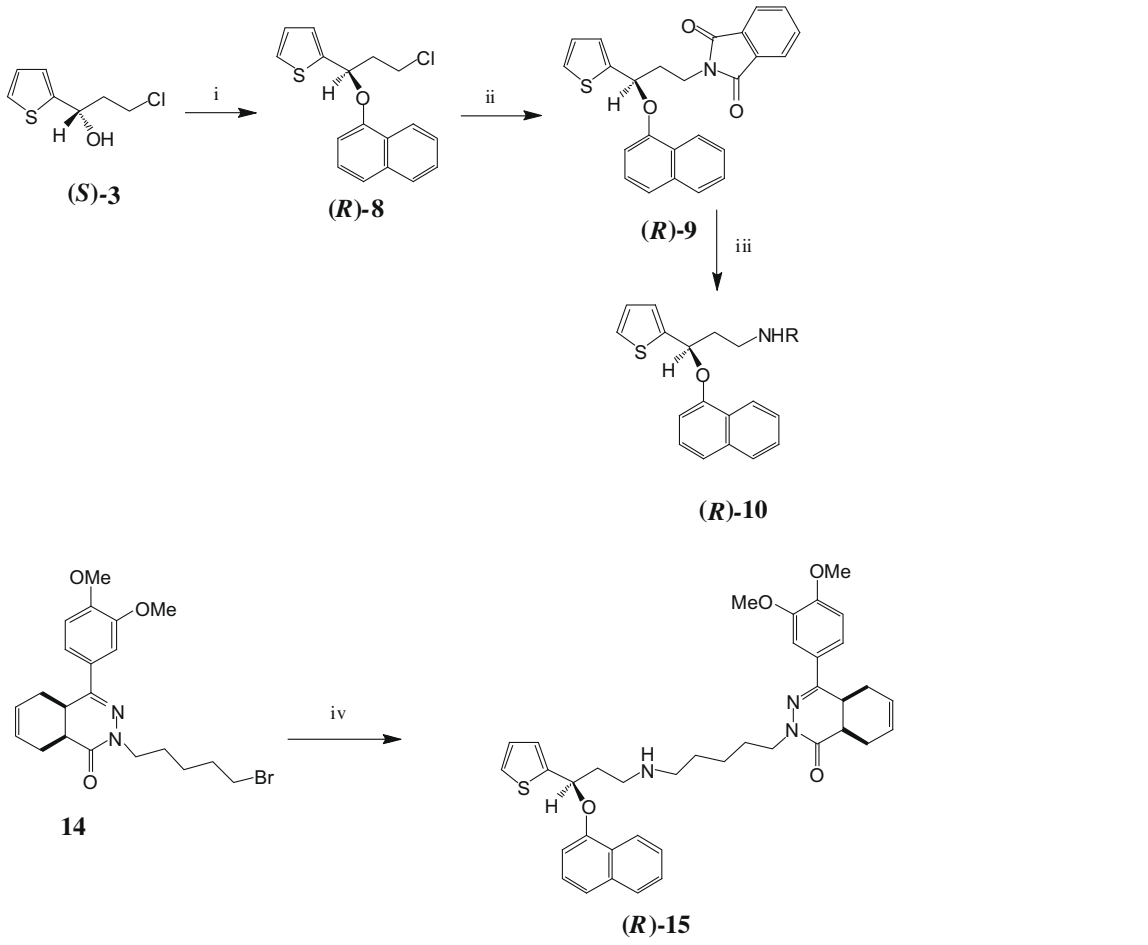
2.2. Pharmacology

Binding affinity (i.e., K_i value) of each compound examined was calculated from the IC_{50} value that was measured 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.[20–22] In vitro functional potency was measured by determining the reuptake 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.

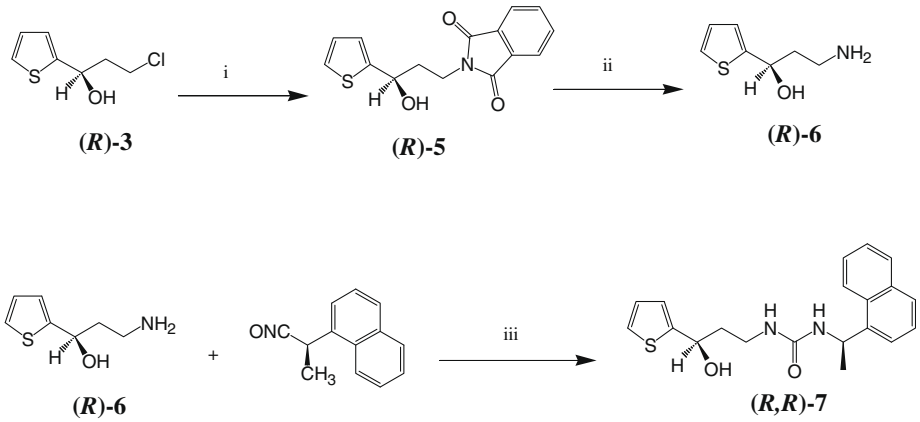
2.3. Serotonin/norepinephrine reuptake inhibitors

Compared with (−)-cocaine, (*R*)-duloxetine (i.e., compound **10a**) was a relatively potent inhibitor of radiolabeled RTI-55 binding to the hSERT and hNET (i.e., K_i values of 2.5 and 61 nM, respectively) (Table 1). (*R*)-Norduloxetine was a relatively potent inhibitor of the hSERT but not the hNET (i.e., K_i values of 8.3 and 953 nM, respectively). In contrast to the (*R*)-isomer, (*S*)-norduloxetine possessed 2–2.5-fold less potency for the hSERT and hNET, respectively (i.e., K_i values of 18 and 2402 nM, respectively) (Table 1). The dual SNRI/PDE4 inhibitors were also tested for affinity to the transporters. Similar to that observed for norduloxetine, the dual SNRI/PDE4 inhibitors also showed similar stereoselectivity. The (*R*)-dual SNRI/PDE4 reuptake inhibitor (***R*)-15** more potently inhibited radiolabeled RTI-55 binding to the hSERT and hNET (i.e., K_i values of 127 and 3236 nM, respectively) (Table 1) compared with the (*S*)-dual SNRI/PDE4 reuptake inhibitor (***S*)-15** (i.e., K_i values of 726 and >10 μM, respectively). The selectivity for hSERT and hNET binding inhibition by (*R*)-duloxetine and (*R* and *S*)-norduloxetine was significant compared to relatively little or no inhibition of binding to the hDAT. The selectivity of binding inhibition for both (*R* and *S*)-dual SNRI/PDE4 reuptake inhibitors (i.e., (***R*)-15** and (***S*)-15**) was hSERT ≫ hNET. Comparatively little to no binding of **15** was observed for the hDAT.

(*R*)-Duloxetine (i.e., compound **10a**) was a potent functional reuptake inhibitor for the hSERT and hNET (i.e., IC_{50} values of 2.6 and 13.2 nM, respectively) (Table 1). Compared to duloxetine, (*R*)- and (*S*)-norduloxetine largely retained hSERT reuptake inhibition potency (i.e., IC_{50} values of 6.8 and 25.6 nM, respectively) but lost >14-fold reuptake inhibition potency for hNET (i.e., IC_{50} values of 188 and 445 nM, respectively). In agreement with inhibition of binding, norduloxetine reuptake inhibition of the hSERT and hNET showed a 2–4-fold stereoselectivity (i.e., $R > S$) (Table 1). Dual SNRI/PDE4 reuptake inhibitors (i.e., compounds (***R*)-15** and (***S*)-15**) were moderately potent reuptake inhibitors of the hSERT (i.e., IC_{50} values of 442 and 404 nM, respectively) but showed only modest reuptake inhibition potency of the hNET (i.e., IC_{50} values of 2097 and 2190 nM, respectively) (Table 1). Compounds (***R*)-15** and (***S*)-15** were considerably less potent than duloxetine or norduloxe-

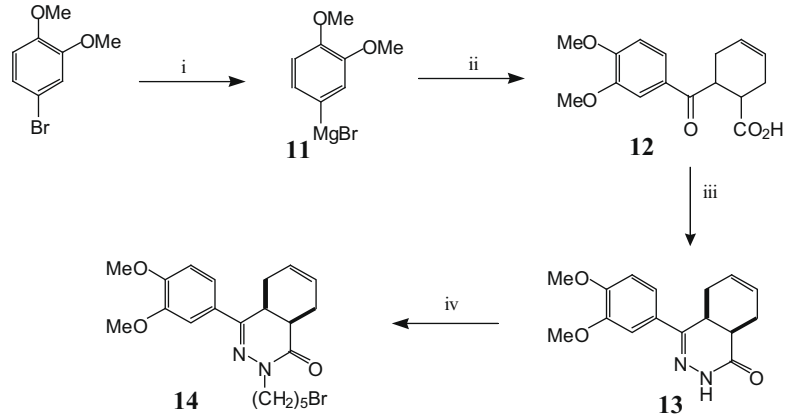


Scheme 2. Synthetic scheme for synthesis of a dual SNRI/PDE4 inhibitor using the (*R*)-isomer as an example. Reagents and conditions: (i) PPh_3, DIAD, 1-naphthol, THF, rt; (ii) NaI, potassium phthalimide, DMF, 85 °C; (iii) $N_2H_4{\cdot}H_2O$, MeOH, rt; (iv) compound (***R***)-**10**, CsOH H_2O/DMF, rt. As described in the synthesis, compound **10b** (R = H) is norduloxetine. Shown for illustration and unrelated to the synthesis, if R = CH_3 compound **10a** is duloxetine. Duloxetine was obtained by extractive isolation as described in Section 4.



Scheme 3. Synthetic scheme for synthesis of urea **7** using the (*R*)-isomer as an example. Reagents and conditions: (i) NaI, potassium phthalimide, DMF, 85 °C; (ii) $N_2H_4{\cdot}H_2O$, MeOH, rt; (iii), toluene, rt.

tine in reuptake inhibition of the hSERT and the hNET. In contrast to duloxetine, for the dual inhibitors (***R***)-**15** and (***S***)-**15**, the selectivity for either binding or reuptake inhibition for the hSERT was markedly greater than for hNET. In contrast to (*R*)- and (*S*)-norduloxetine, the stereoselectivity for binding and reuptake inhibition for both hSERT and hNET was largely abrogated (i.e., *R* = *S*) for the dual inhibitors (***R***)-**15** and (***S***)-**15**. Preliminary studies showed that a five carbon linker was optimal because a dual SNRI/PDE4 inhibitor possessing a three carbon linker between norduloxetine and the phthalazine had very little affinity or reuptake inhibition for the hDAT, hSERT, or the hNET (data not shown). The phthalazine with a pentyl bromide moiety attached (i.e., **14**) showed no detectable binding or re-uptake inhibition to the hSERT, hNET or hDAT. The PDE4 inhibitor **13** did not have any detectable potency at bind-



Scheme 4. Synthetic scheme for synthesis of phthalazine **14**. Reagents and conditions: (i) Mg turlings, THF, reflux; (ii) *cis*-1,2,3,6-tetrahydrophthalic anhydride, THF; (iii) H_2NNH_2, EtOH, reflux; (iv) (1,5-dibromopentane, NaH, DMF.

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

Compound	Description	Binding (K_i, nM)			Reuptake (IC_{50}, nM)		
		hDAT	hSERT	hNET	hDAT	hSERT	hNET
Cocaine		431 ± 14	525 ± 67	2377 ± 148	227 ± 62	338 ± 62	232±62
10a	Duloxetine	550 ± 13	2.5 ± 1.0	61.3 ± 2.1	809 ± 579	2.6 ± 1.5	13.2 ± 5.3
(***R***)-**10b**	(*R*)-Norduloxetine	3549 ± 201	8.3 ± 0.5	953 ± 41	>10,000	6.8 ± 5.5	188 ± 70
(***S***)-**10b**	(*S*)-Norduloxetine	>10,000	18 ± 0.5	2402 ± 387	>10,000	25.6 ± 7.7	445 ± 219
(***R***)-**15**	SNRI/PDE4 inhibitor	857 ± 35	127 ± 11	3236±192	1793 ± 4711	442 ± 76	2097 ± 537
(***S***)-**15**	SNRI/PDE4 inhibitor	4598 ± 509	726 ± 72	>100,000	6630 ± 951	404 ± 148	2190 ± 339
13	PDE4 inhibitor	>100,000	>100,000	>100,000	>100,000	>100,000	>100,000
14	PDE4 inhibitor-$(CH2)_5$-Br	>100,000	>100,000	39,119 ± 4915	>100,000	>100,000	>100,000

[a] Values are the mean ± SEM of at least three independent experiments conducted with duplicate (binding) or triplicate (reuptake) determinations, except when two independent experiments exceeded 100 μM.

ing or functional reuptake inhibition of the hDAT, hSERT, or hNET (Table 1).

2.4. PDE4 Inhibition

The PDE4 inhibitory potency of phthalazinone **13** (i.e., K_i value of 213 nM) was confirmed (Table 2). Previously it was reported that *N*-substitution in the phthalazinone series (i.e., *cis*-(±)-4a,5,8,8a-tetra- and *cis*-(±)-4a,5,6,7,8,8a-hexahydro-2*H*-phthalazin-1-ones) increased PDE4 inhibition potency.[18] We hypothesized that *N*-substituted derivatives distal to the fused ring system of **13** would increase PDE4 inhibitory potency and this is what was observed. As shown in Table 2, even though it was known that *cis*-(+) **13** was more potent than its (−)-counterpart at PDE4 inhibition, the racemic bromopentane derivative **14** was significantly more potent than **13** (i.e., K_i value 2.3 nM) and thus, the racemic bromopentane derivative **14** was judged to be a good starting point for elaboration of dual SNRI/PDE4 inhibitors.

Table 2
Inhibition of cAMP hydrolysis by PDE4D2 in the presence of SNRIs or dual SNRI-PDE4D2 inhibitors[a]

Compound	Description	PDE4D2 inhibition IC_{50} (nM)
10a	Duloxetine	>100,000
(***R***)-**10b**	(*R*)-Norduloxetine	>100,000
(***S***)-**10b**	(*S*)-Norduloxetine	>100,000
(***S***)-**15**	SNRI/PDE4 inhibitor	45 ± 10[a]
(***R***)-**15**	SNRI/PDE4 inhibitor	23 ± 10
13	PDE inhibitor	213 ± 1
14	PDE inhibitior-C_5-Br	2.0 ± 1

[a] Data is the average of three determinations ± SEM except when two independent experiments exceeded 100 μM.

2.5. Dual SNRI/PDE4 inhibitors

The dual SNRI/PDE4 inhibitors (i.e., compounds (***R***)-**15** and (***S***)-**15**) were moderately potent inhibitors of binding radiolabeled RTI-55 at the hSERT. For compounds (***S***)-**15** and (***R***)-**15**, inhibition of neurotransmitter reuptake was relatively potent and selective at the hSERT (i.e., IC_{50} values of 404 and 442 nM, respectively) (Table 1) because (***S***)-**15** and (***R***)-**15** did not potently interact with the hDAT or hNET (Table 1).

Compounds (***R***)-**15**, and (***S***)-**15** were examined as inhibitors of cAMP hydrolysis by recombinant human PDE4D2, in vitro. The K_i value for inhibition of human PDE4D2 for (***R***)-**15** and (***S***)-**15** were 23 nM and 45 nM, respectively (Table 2). The decrease in potency for (***S***)-**15** and (***R***)-**15** compared with **14** suggested that the *N*-substitution by norduloxetine makes an impact on inhibition of human PDE4D2. However, PDE4D2 inhibition was stereoselective (i.e., (***R***)-**15** > (***S***)-**15**) and this further suggests that additional interaction occur at sites distal from the *cis*-fused ring system of the phthalazinone to afford PDE4D2 inhibition.

3. Conclusions

In summary, the stereoselective synthesis of dual SNRI/PDE4 inhibitors based on norduloxetine 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 norduloxetine via a five

carbon linker to the phthalazinone PDE4 inhibitor decreased PDE4D2 inhibitory potency compared with the compound without the norduloxetine moiety. Additional interactions with PDE4D2 are likely present because the dual SNRI/PDE4 inhibitor with the (*R*)-stereochemistry in the norduloxetine moiety was more potent than the dual inhibitor with duloxetine possessing the (*S*)-stereochemistry. Attachment of the phthalazinone moiety to norduloxetine via a five carbon linker decreases hSERT binding and re-uptake inhibition potency but the hSERT can accommodate considerable lipophilicity in the region of the aliphatic nitrogen atom and (***R***)-**15** affords moderate functional activity. The results show that dual SNRI/PDE4 inhibitors (***S***)-**15** and (***R***)-**15** retain considerable potency for binding and reuptake inhibition of the hSERT but possessed little affinity or reuptake inhibition to the hNET or hDAT. Loss of affinity and reuptake inhibition of the hNET by **15** suggests this transporter is considerably more sensitive to steric bulk than the hSERT in agreement with previous studies.[17,20] However, the stereoselectivity for hSERT binding (i.e., $R > S$) observed for norduloxetine was considerably attenuated for the dual SNRI/PDE4 inhibitor **15**. Reuptake inhibition of the hSERT by **15** was not stereoselective suggesting that hSERT binding and reuptake inhibition are distinct. Regardless, it is likely that compounds such as (***R***)-**15** possess sufficiently suitable pharmacological and pharmaceutical properties to test in vivo as a new class of antidepressant or attention deficit disorder medications.

4. Experimental

4.1. Chemistry

4.1.1. General

Chemicals used in this study were of the highest purity available. Commercially available reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) or VWR (San Diego, CA) and were used as received. (*R*)-Duloxetine was purchased from a local pharmacy. 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. 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.[21] 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 Inc. (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 California (Irvine, CA) using ESI. Polarimetry was done with a Jasco P-1010 polarimeter. Solutions of compounds in methanol were examined through a 10 cm. path length cell at concentrations recorded in g/mL. Measurements were taken at 589 nm at room temperature.

4.1.1.1. 1-(2-Thienyl)-3-chloropropan-1-one,[15] 1. A mixture of thiophene (8.4 g, 0.1 mol) and 3-chloropropionyl chloride (9.7 mL, 0.1 mol) in benzene was cooled to 0 °C (ice bath) under Argon. Fuming $SnCl_4$ (11.7 mL, 0.1 mol) was added dropwise and the resulting mixture was stirred at room temperature for 2 h. The reaction was stopped by addition of concentrated HCl (6 mL) and ice water (45 mL). The organic layer was separated and the aqueous layer was extracted with benzene (2 × 20 mL). The combined organic extract was washed with H_2O, dried over $CaCl_2$, filtered, and concentrated to dryness. The crude product was purified by flash chromatography (pentane/ether, 3/1, v/v) to give 4.9 g, 28% yield of the product **1** as a yellow oil. $R_f = 0.36$; ^{1}H NMR ($CDCl_3$) δ 7.71 (m, $J = 12$ Hz, 1H), 7.65 (d, $J = 10.5$ Hz, 1H), 7.63 (m, $J = 10.5$ Hz, 1H), 3.86 (t, $J = 6.6$ Hz, 2H) 3.35 (t, $J = 6.6$ Hz, 2H).

4.1.1.2. 3-Chloro-1-(2-thienyl)-1-propanol, 2. The ketone **1** (4.4 g, 25 mmol) was dissolved in ethanol, cooled to 0 °C in an ice bath and $NaBH_4$ (0.9 g, 25 mmol) was added. The reaction mixture was stirred for 2 h. The reaction was stopped by slow addition of aqueous NH_4Cl (50 mL) and stirred for another 30 min at room temperature. The solution was extracted with dichloromethane (3 × 50 mL), dried over $MgSO_4$, filtered, and concentrated under vacuo. The residue was purified by flash chromatography (hexane/ether, 4/1, v/v) to provide 3.6 g, 82% of the alcohol, **2**, as a light yellow oil. $R_f = 0.38$; ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 1H), 6.99 (m, 2H), 5.21 (dd, $J = 4.8$, 8.1 Hz, 1H, CH), 3.73 (m, 1H), 3.6 (m, 1H), 2.24 (m, 2H).

4.1.1.3. (S)-3-Chloro-1-(2-thienyl)-1-propanol,[15] (*S*)-3. The racemic alcohol **2** (2 g, 11.3 mmol) and vinyl butyrate (7.2 mL, 56.5 mmol) were dissolved in *n*-hexane (25 mL). To this solution, Novozyme (265 mg) was added and the mixture was stirred at 30 °C for 22 h until 50% conversion was reached as determined by HPLC. The enzyme was removed by filtering and the reaction mixture was concentrated under vacuo. The alcohol ($R_f = 0.37$) and ester ($R_f = 0.74$) were separated by flash chromatography (pentane/ether, 4/1, v/v) to give 0.73 g, 37% yield of (***S***)-**3** and 1.32 g, 47% yield of the ester **4**. NMR data was in accordance with that of the racemate **2**. ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 1H), 6.99 (m, 2H), 5.21 (dd, $J = 4.8$, 8.1 Hz, 1H, CH), 3.73 (m, 1H), 3.6 (m, 1H), 2.24 (m, 2H).

4.1.1.4. (R)-3-Chloro-1-(2-thienyl)-1-propanol, (*R*)-3. The ester **4** (500 mg, 2.02 mmol) and LiOH (255 mg, 6.07 mmol) were dissolved in water/THF (2/1, v/v) and the solution was stirred at room temperature for 18 h. The mixture was acidified with 1 N HCl (15 mL) and then extracted with ether (3 × 75 mL). The combined ether extracts were dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give 0.22 g, 62% yield of the product (***R***)-**3** as a colorless oil. $R_f = 0.38$; ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 1H), 6.99 (m, 2H), 5.21 (dd, $J = 4.8$, 8.1 Hz, 1H, CH), 3.73 (m, 1H), 3.60 (m, 1H), 2.24 (m, 2H).

4.1.1.5. 2-(3-Hydroxy-3-thiophen-2-yl-propyl)-isoindole-1,3-dione, (*S*)-5. A mixture of 3-chloro-1-thiophen-propanol (***S***)-**3** (610 mg, 3.46 mmol), potassium phthalimide (1.9 g, 10.4 mmol), NaI (156 mg, 1.04 mmol) was dissolved in dry DMF (12 ml) under an atmosphere of argon and was stirred at 90 °C for 48 h. After the mixture was cooled to room temperature, water (12 ml) and satd sodium bicarbonate (12 ml) were added and the mixture was extracted with ethyl acetate (3 × 70 ml). The combined organic extract was washed with water and brine successively. The organic phase was dried over $MgSO_4$, filtered and the solvent was evaporated to dryness. The crude material was subjected to flash chromatography (hexane/ethyl acetate, 3/1, v/v) to afford 510 mg, 51% of the phthalimide product, (***S***)-**5** as a white powder. $R_f = 0.23$; mp = 128 °C. $[\alpha]_D^{25}$ +3.28 (*c* 5.0, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.87 (m, 2H), 7.74 (m, 2H), 7.19 (m, 1H), 6.96 (m, 1H), 6.9 (m,

1H), 4.95 (m, 1H), 3.93 (m, J = 4.5 Hz, 2H), 2.2 (m, 2H); HRMS calcd for $C_{15}H_{13}NNaO_3S$, 310.0514, found 310.0512.

4.1.1.6. 2-(3-Hydroxy-3-thiophen-2-yl-propyl)-isoindole-1,3-dione, (*R*)- 5. Compound (***R***)-**5** was synthesized according to the procedure described for compound (***S***)-**5**. Analytical data were in accord with compound (***R***)-**5**. R_f = 0.23; mp = 128 °C; $[\alpha]_D^{25}$ −3.55 (*c* 4.5, MeOH), ^{1}H NMR ($CDCl_3$) δ 7.87 (m, 2H), 7.74 (m, 2H), 7.19 (m, 1H), 6.96 (m, 1H), 6.9 (m, 1H), 4.95 (m, 1H), 3.93 (m, J = 4.5 Hz, 2H), 2.2 (m, 2H); HRMS calcd for $C_{15}H_{13}NNaO_3S$, 310.0514, found 310.0509.

4.1.1.7. 3-Amino-1-thiophen-2-yl-propan-1-ol, (*S*)-6. The phthalamide (***S***)-***5*** (170 mg, 0.59 mmol) was dissolved in methanol (5 mL) and hydrazine monohydrate (0.14 mL, 2.96 mmol) was added. The mixture was stirred at room temperature overnight. The precipitate was filtered off, washed with methanol and the solvent was removed to give a white solid. The white crude product was purified by a small plug of C_{18} silica gel (100% methanol) to afford 59 mg, 64% yield of compound (***S***)-**6** as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.18 (m, 1H), 6.96–6.85 (m, 2H), 6.05 (br s, 1H), 5.16 (m, 1H), 3.3 (m, 2H), 2.1 (m, 2H).

4.1.1.8. 3-Amino-1-thiophen-2-yl-propan-1-ol, (*R*)-6. The phthalamide (***R***)-**5** (250 mg, 0.87 mmol) was dissolved in methanol (20 mL) and hydrazine monohydrate (0.21 mL, 4.35 mmol) was added and the mixture was stirred at room temperature overnight. The precipitate was filtered off, the solid was washed with methanol and the solvent was removed to give a white solid. The white crude product was purified by a small plug of C_{18} silica gel (100% methanol) to afford 122 mg, 89% yield of compound (***R***)-**6** as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.18 (m, 1H), 6.96–6.85 (m, 2H), 6.05 (br s, 1H), 5.16 (m, 1H), 3.3 (m, 2H), 2.1 (m, 2H).

4.1.1.9. 1-((*S*)-3-Hydroxy-3-(thiophen-2-yl)propyl)-3-((*R*)-1-(naphthalen-1-yl)ethyl)urea, (*S*,*R*)-7. 3-Amino-1-thiophen-2-yl-propan-1-ol, (***S***)-**6** (10 mg, 0.06 mmol) was dissolved in toluene (0.2 mL). (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (11 mg, 0.06 mmol) was slowly added and the mixture was stirred at room temperature overnight. Solvent was removed and the resulting material was purified by PTLC (MeOH/DCM, 1/20, v/v) to give 20 mg of the product. R_f = 0.34, ESI/MS: m/z = 377 (MNa^+). ^{1}H NMR δ 8.31 (m, 1H), 8.15 (m, 1H), 7.85 (m, 2H), 7.56 (m, 3H), 7.18 (m, 1H), 6.9 (m, 2H), 5.96 (m, 1H), 4.75 (m, 2H), 4.5 (m, 1H), 3.66 (m, 1H), 3.08 (m, 1H), 1.78 (d, J = 6.6 Hz, 2.3H), 1.65 (d, J = 6.6 Hz, 0.7H).

4.1.1.10. 1-((*R*)-3-Hydroxy-3-(thiophen-2-yl)propyl)-3-((*R*)-1-(naphthalen-1-yl)ethyl)urea, (*R*,*R*)-7. 3-Amino-1-thiophen-2-yl-propan-1-ol, (***R***)-**6** (10 mg, 0.06 mmol) was dissolved in toluene (0.2 mL). (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (11 mg, 0.06 mmol) was slowly added and the mixture was stirred at room temperature overnight. Solvent was removed and the resulting material was purified by PTLC (MeOH/DCM, 1/20, v/v) to give 21 mg of the product. R_f = 0.34, ESI/MS: m/z = 377 (MNa^+). ^{1}H NMR δ 8.14 (t, J = 6.9 Hz, 1H), 7.88 (d, J = 7.5 Hz, 1H), 7.78 (d, J = 7.5 Hz, 1H), 7.46 (m, 4H), 7.2 (m, 1H), 6.88 (m, 2H), 5.63 (m, 1H), 4.75 (m, 2H), 4.5 (m, 1H), 3.66 (m, 1H), 3.08 (m, 1H), 1.85 (m, 1H), 1.65 (m, 3H).

4.1.1.11. 2-[3-Chloro-1-(naphthalen-1-yloxy)-propyl]-thiophene, (*R*)-8. Diisopropyl azodicarboxylate (0.33 ml, 1.7 mmol) was added to a solution of triphenylphosphine (0.45 g, 3.4 mmol) in freshly dried THF (1.4 ml) under Argon gas. The solution formed a yellow precipitate after 10 min stirring at room temperature. 1-Naphthol (0.51 g, 3.6 mmol) in THF (1.4 ml) was added to the mixture. The reaction turned into a clear solution and started forming a precipitate after 15 min stirring at room temperature. After stirring for another 2 h, 3-chloro-1-thiophene-2-yl-propan-1-ol (0.25 g, 1.4 mmol) in THF (1.5 ml) was added. The reaction mixture was stirred at room temperature for 36 h. The solvent was evaporated and diethyl ether was added to the residue. After a few minutes of stirring, the solution formed a white precipitate. The precipitate was filtered through filter paper. The ether was evaporated and the crude product was purified by flash chromatography (hexane/ether, 30/1, v/v) giving 60 mg, 14% yield of the product as a thick oil. R_f = 0.43; ^{1}H NMR ($CDCl_3$) δ 8.3 (m, 1H), 7.75 (m, 1H), 7.55–7.28 (m, 5H), 7.17 (m, 1H), 6.93 (m, 1H), 6.86 (m, 1H), 5.92 (m, 1H), 3.84 (m, 1H), 3.63 (m, 1H), 2.78 (m, 1H), 2.44 (m, 1H).

4.1.1.12. 2-[3-Chloro-1-(naphthalen-1-yloxy)-propyl]-thiophene, (*S*)-8. Compound (***S***)-**8** was prepared according to the procedure described for compound (***R***)-**8** giving 51 mg, 12% yield of (***S***)-**5** as a thick oil. ^{1}H NMR ($CDCl_3$) δ 8.3 (m, 1H), 7.75 (m, 1H), 7.55–7.28 (m, 5H), 7.17 (m, 1H), 6.93 (m, 1H), 6.86 (m, 1H), 5.92 (m, 1H), 3.84 (m, 1H), 3.63 (m, 1H), 2.78 (m, 1H), 2.44 (m, 1H).

4.1.1.13. 2-[3-(Naphthalen-1-yloxy)-3-thiophen-2-yl-propyl]-isoindole-1,3-dione, (*R*)-9. 2-[3-Chloro-1-(naphthalene-1-yloxy)-propyl]-thiophene (17 mg, 0.06 mmol) was dissolved in dry DMF (0.5 ml). To this solution, sodium iodide (1 mg, 0.006 mmol) and potassium phthalimide (31 mg, 0.17 mmol) were added and the reaction mixture was stirred at 85 °C for 48 h. The progress of the reaction was monitored by TLC (3:1, hexane/ethyl acetate). The solvent was evaporated and the crude product was purified by flash chromatography (hexane/ethyl acetate, 3/1, v/v) to give 15 mg, 65% yield of the desired product as a white powder. R_f = 0.32; ^{1}H NMR ($CDCl_3$) δ 8.28 (m, 1H), 7.87 (m, 1H), 7.77–7.71 (m, 3H), 7.63 (m, 2H), 7.42–7.35 (m, 4H), 7.18 (m, 1H), 7.12 (m, 1H), 6.81 (m, 1H), 5.78 (m, 1H), 4.08 (m, 1H), 3.95 (m, 1H), 2.73 (m, 1H), 2.43 (m, 1H); HRMS calcd for $C_{25}H_{20}NO_3S$ 436.0984, found 436.0985.

4.1.1.14. 2-[3-(Naphthalen-1-yloxy)-3-thiophen-2-yl-propyl]-isoindole-1,3-dione, (*S*)-9. Compound (***S***)-**9** was prepared according to the procedure described for compound (***R***)-**9** and afforded 40 mg, 75% yield of (***S***)-**9** as a white powder. (hexane/ethyl acetate, 3/1, v/v). R_f = 0.32; ^{1}H NMR ($CDCl_3$) δ 8.28 (m, 1H), 7.87 (m, 1H), 7.77–7.71 (m, 3H), 7.63 (m, 2H), 7.42–7.35 (m, 4H), 7.18 (m, 1H), 7.12 (m, 1H), 6.81 (m, 1H), 5.78 (m, 1H), 4.08 (m, 1H), 3.95 (m, 1H), 2.73 (m, 1H), 2.43 (m, 1H). HRMS calcd for $C_{25}H_{20}NO_3S$ 436.0984, found 436.0984.

4.1.1.15. 3-(Naphthalen-1-yloxy)-3-thiophen-2-yl-propylamine, (*R*)-10b. To a solution of 2-[3-(naphthalen-1-yloxy)-3-thiophen-2-yl-propyl]-isoindole-1,3-dione, (***R***)-**9** (52 mg, 0.13 mmol) in anhydrous methanol (2.5 ml) was added hydrazine monohydrate (0.06 ml, 1.3 mmol). The reaction mixture was stirred at room temperature overnight. The white precipitate was filtered through filter paper and the filtrate was concentrated. The crude product was subjected to flash chromatography (CH_2Cl_2/MeOH/0.1%NH_4OH, 8/1, v/v) to give 29 mg, 79% yield of compound (***R***)-**10b** as a thick light yellow oil. ESI/MS: m/z = 284 (MH^+); ^{1}H NMR ($CDCl_3$) δ 8.28 (m, 1H), 7.87 (m, 2H), 7.5 (m, 2H), 7.38 (m, 1H), 7.23 (m, 1H), 7.19 (m, 2H), 6.9 (m, 1H), 5.92 (m, 1H), 3.2 (m, 2H), 2.75 (m, 1H), 2. 54 (m, 1H).

4.1.1.16. 3-(Naphthalen-1-yloxy)-3-thiophen-2-yl-propylamine, (*S*)-10b. Compound (***S***)-**10b** was prepared according to the procedure described for compound (***R***)-**10b** providing 25 mg, 68% yield of (***S***)-**10b** as a thick light yellow oil. ESI/MS: m/z = 284 (MH^+); ^{1}H NMR ($CDCl_3$) δ 8.28 (m, 1H), 7.87 (m, 2H), 7.5 (m, 2H), 7.38 (m,

1H), 7.23 (m, 1H), 7.19 (m, 2H), 6.9 (m, 1H), 5.92 (m, 1H), 3.2 (m, 2H), 2.75 (m, 1H), 2.54 (m, 1H).

4.1.1.17. 3,4-Dimethoxyphenylmagnesium bromide (11). Magnesium turlings (3.73 g, 155.5 mmol) was suspended in dry THF (200 mL) under a flow of Argon gas. To this solution, 4-bromoveratrole (15 mL, 103.7 mmol) dissolved in THF (30 mL) was added slowly via a syringe. The resulting mixture was refluxed for 5 h under Argon. The Grignard reagent was cooled to room temperature and used immediately in the synthesis of **12**.

4.1.1.18. 6-(3,4-Dimethoxybenzoyl)-cyclohex-3-enecarboxylic acid (12). A solution of the Grignard reagent **11** (25 g, 103.7 mmol) was added dropwise to an ice-cooled solution of *cis*-1,2,3,6-tetrahydrophthalic anhydride (15.8 g, 103.7 mmol) in THF (100 mL) over a period of 1 h. After the addition was complete, the resulting mixture was stirred at 0 °C for another 30 min. The reaction mixture was then allowed to warm to room temperature and stirred overnight. The reaction was stopped by addition of satd NH_4Cl. The pH was adjusted to 2 with concentrated HCl and then extracted with diethyl ether. The organic layer was washed with water and subsequently extracted with 1 N NaOH. The combined aqueous extracts were 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 give a thick oil. The crude product was dissolved in dichloromethane and filtered through silica gel to remove the dicarboxylic acid formed during work up. The resulting product was recrystallized from diethyl ether to afford 5.01 g, 17% yield of pure product as a white powder. ESI $m/z = 289$ (MH^-); ^1H NMR ($CDCl_3$, 500 MHz): δ 7.6–7.53 (m, 2H), 6.92 (d, $J = 8.4$ Hz, 1H), 5.84–5.82 (m, 1H), 5.71–5.68 (m, 1H), 4.03–4.0 (m, 1H), 3.98 (s, 3H), 3.95 (s, 1H), 3.10–3.07 (m, 1H), 2.9–2.84 (m, 1H), 2.54–2.44 (m, 3H).

4.1.1.19. 4-(3,4-Dimethoxy-phenyl)-4a,5,8,8a-tetrahydro-2H-phthalazin-1-one (13). A mixture of compound **12** (3.7 g, 12.7 mmol) and hydrazine monohydrate (1.9 g, 38.1 mmol) in ethanol (50 mL) was refluxed overnight. The mixture was then cooled to room temperature. Upon cooling, the product crystallized out to give 2.97 g, 82% yield as a white precipitate. ESI/MS $m/z = 287$ (MH^+); ^1H NMR ($CDCl_3$, 500 MHz): δ 8.53 (br s, 1H), 7.46 (d, $J = 2.0$ Hz, 1H), 7.23 (dd, $J = 2.0$, 8.4 Hz, 1H), 6.87 (d, $J = 8.4$ Hz, 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).

4.1.1.20. 2-(5-Bromo-pentyl)-4-(3,4-dimethoxy-phenyl)-4a,5,8,8a-tetrahydro-2*H*-phthalazin-1-one, 14. A mixture of sodium hydride (83 mg, 2.1 mmol, 60% dispersion in mineral oil) and phthalazinone **13** (500 mg, 1.8 mmol) in DMF (20 mL) was stirred at room temperature under Argon (g) for 30 min. To this solution, 1,5-Dibromopentane (1.2 g, 5.25 mmol) was added and the reaction mixture was stirred at room temperature overnight. The reaction was stopped by addition of water (5 mL) and then extracted with diethyl ether (3×50 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an oil. The crude product was purified by flash chromatography (ether/hexane, 2/1, v/v) to afford 496 mg, 65% yield of the product as an oil. ESI/MS $m/z = 435$, 437 (MH^+); ^1H NMR ($CDCl_3$, 500 MHz): δ 7.47 (d, $J = 2.0$ Hz, 1H), 7.25 (dd, $J = 2.0$, 8.5 Hz, 1H), 6.87 (d, $J = 8.5$ Hz, 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).

4.1.1.21. (*R*)-4-(3,4-Dimethoxy-phenyl)-2-{5-[3-(naphthalene-1-yloxy)-3-thiophen-2-yl-propylamino]-pentyl}-4a,5,8,8a-tetrahydro-2*H*-phthalazin-1-one, (*R*)-15. To a solution of cesium hydroxide monohydrate (14 mg, 0.08 mmol) in dry DMF (0.2 mL) was added a solution of 3-(naphthalen-1-yloxy)-3-thiophen-2-yl-propylamine, (*R*)-**10b** (23 mg, 0.08 mmol) in DMF (0.2 mL) under an atmosphere of Argon. After stirring for 30 min at room temperature, 2-(5-bromo-pentyl)-4-(3,4-dimethoxy-phenyl)-4a,5,8,8a-tetrahydro-2*H*-phthalazin-1-one, **14** (53 mg, 0.12 mmol) in DMF (0.3 mL) was added. The reaction mixture was stirred at room temperature for 36 h under an atmosphere of Argon. The solvent was evaporated and the residue was purified by PTLC (CH_2Cl_2/MeOH, 8/1, v/v) to afford 10.8 mg, in 21% yield, of the desired product (*R*)-**15** as a thick oil. ESI/MS $m/z = 638$ (MH^+), ^1H NMR ($CDCl_3$) δ 8.22 (m, 1H), 7.69 (m, 1H), 7.41–7.37 (m, 3H), 7.31 (d, $J = 5.1$ Hz, 1H), 7.2–7.15 (m, 2H), 7.1 (m, 1H), 7.0 (m, 1H), 6.84–6.76 (m, 3H), 5.76 (m, 1H), 5.7–5.21 (m, 2H), 3.85 (m, 6H), 3.64 (m, 2H), 3.24 (m, 2H), 2.99–1.3 (m, 16H); HRMS calcd for $C_{38}H_{44}N_3O_4S$ 638.3052, found 638.3033.

4.1.1.22. (*S*)-4-(3,4-Dimethoxy-phenyl)-2-{5-[3-(naphthalene-1-yloxy)-3-thiophen-2-yl-propylamino]-pentyl}-4a,5,8,8a-tetrahydro-2*H*-phthalazin-1-one, (*S*)-15. (*S*)-**15** was synthesized according to the procedure described for compound (*R*)-**15**. To a solution of cesium hydroxide (7 mg, 0.04 mmol) in dry DMF (0.2 mL) was added a solution of 3-(naphthalen-1-yloxy)-3-thiophen-2-yl-propylamine, (*S*)-**10b** (12 mg, 0.04 mmol) in DMF (0.2 mL) under an atmosphere of Argon. After stirring for 30 min at room temperature, 2-(5-bromo-pentyl)-4-(3,4-dimethoxy-phenyl)-4a,5,8,8a-tetrahydro-2*H*-phthalazin-1-one, **14** (27 mg, 0.06 mmol) in DMF (0.2 mL) was added. The mixture was stirred at room temperature for 36 h under an atmosphere of Argon. The solvent was evaporated and the residue was purified by PTLC (CH_2Cl_2/MeOH, 8/1, v/v) to afford 5.1 mg, 19% yield, of the desired product (*S*)-**15** as a thick oil. ESI/MS $m/z = 638$ (MH^+), ^1H NMR ($CDCl_3$) δ 8.22 (m, 1H), 7.69 (m, 1H), 7.41–7.37 (m, 3H), 7.31 (d, $J = 5.1$ Hz, 1H), 7.20–7.15 (m, 2H), 7.1 (m, 1H), 7.0 (m, 1H), 6.84–6.76 (m, 3H), 5.76 (m, 1H), 5.7–5.21 (m, 2H), 3.85 (m, 6H), 3.64 (m, 2H), 3.24 (m, 2H), 2.99–1.3 (m, 16H); HRMS calcd for $C_{38}H_{44}N_3O_4S$ 638.3052, found 638.3059.

4.2. Biology

HEK-hDAT, -hSERT, or -hNET cells were grown until confluent as described previously.[21] Cells were scraped from plates and centrifuged for 20 min at 30,000*g* 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 through a Whatman GF/C filter 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 β-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).

4.3. Inhibition of substrate uptake

HEK-hDAT, -hSERT, or -hNET cells were grown as described above.[22] 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).

4.4. 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 recorded. Specific hydrolysis was defined as the difference in hydrolysis in the presence and absence of test compound. Two to three determinations were done at each of 10 test compound concentrations.

4.5. 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.

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