Characterization of Thien-2-yl 1*S*,2*R*-Milnacipran Analogues as Potent Norepinephrine/Serotonin Transporter Inhibitors for the Treatment of Neuropathic Pain

Brian Dyck,[†] Junko Tamiya,[†] Florence Jovic,[†] Rebecca R. Pick,[‡] Margaret J. Bradbury,[‡] Julie O'Brien,[§] Jenny Wen,[§] Michael Johns,[§] Ajay Madan,[§] Beth A. Fleck,[†] Alan C. Foster,[‡] Binfeng Li,[§] Mingzhu Zhang,[†] Joe A. Tran,[†] Troy Vickers,[†] Jonathan Grey,[†] John Saunders,[†] and Chen Chen*[,†]

Departments of Medicinal Chemistry, Pharmacology, Neuroscience, and Preclinical Development, Neurocrine Biosciences, Inc., 12790 El Camino Real, San Diego, California 92130

Received July 28, 2008

Thien-2-yl 1*S*,2*R*-milnacipran analogues were synthesized and characterized as norepinephrine/serotonin transporter inhibitors. These compounds possessed higher potencies than 1*S*,2*R*-milnacipran (2*R*-**1**) while maintaining low molecular weight and moderate lipophilicity, which are the important features for the pharmacological and pharmacokinetic characteristics of milnacipran (**1**). Thus, compound **5c** exhibited IC_{50} values of 2.3 and 32 nM, respectively, at NET and SERT, which were more than 10-fold better than those of **1** (NET IC_{50} = 77 nM, SERT IC_{50} = 420 nM). Moreover, **5c** achieved the same efficacy as **1**, but with much lower doses, in a rodent spinal nerve ligation pain model. In addition, **5c** displayed desirable pharmacokinetic properties in several species, including high oral availability and significant brain penetration.

Introduction

The cell membrane monoamine transporters—norepinephrine (NET[a]), serotonin (SERT), and dopamine transporter (DAT)—are important targets for many CNS drugs.[1] Since the synaptic actions of monoamine neurotransmitters such as norepinephrine (NE) and serotonin (SER) are terminated by reuptake into the nerve terminals from which they are released or by uptake into adjacent cells, inhibition of this action prolongs the function of these monoamines. Both selective serotonin reuptake inhibitors (sSRI) and selective norepinephrine reuptake inhibitors (sNRI) are effective against major depression and a range of other psychiatric illnesses. On the other hand, the dopamine transporter (DAT) is a key target for amphetamine and methylphenidate, which are used in the treatment of attention deficit hyperactivity disorder (ADHD).[2]

Many monoamine transporter inhibitors with various selectivity profiles have been used clinically for CNS-related diseases. For example, atomoxetine (**6**, Figure 1) was introduced into the market as a sNRI for ADHD.[3] Recently, duloxetine (**7**), a dual NET/SERT inhibitor (NSRI), has been approved by the FDA for both depression and diabetic neuropathy.[4] While the serotonin transporter inhibition is likely to improve depression,[5] the norepinephrine reuptake blockade probably improves chronic pain.[6] Available as an antidepressant, milnacipran (**1**, Figure 1)[7] inhibits norepinephrine and serotonin reuptake in a 3:1 ratio.[8] Compound **1** is currently in phase III clinical trials for fibromyalgia, and recent results showed its effectiveness in the treatment.[9,10]

The moderately lipophilic **1** (log $D \approx 0$) differs significantly from many CNS drugs such as **6**.[11] Compound **1**, with a low molecular weight of 247, exhibits almost ideal pharmacokinetics in humans, including low intersubject variability and high oral bioavailability (~85%).[12] It has very low plasma protein binding (~13%) and is mainly excreted in the urine as the parent and glucuronide (>80%). Only a small fraction (<10%) of **1** is metabolized via N-deethylation by the CYP3A4 enzyme.[13] This lack of potential for drug−drug interaction via cytochrome P450 enzymes is a quite attractive feature because many CNS drugs are highly lipophilic and rely heavily on liver enzymes for drug elimination.

As a mixture of 1*S*,2*R*- and 1*R*,2*S*-isomers with a NET/SERT ratio of about 3:1, **1** is known to be a NET/SERT dual inhibitor.[14] However, its potency at both NET and SERT is only moderate[15] and mainly resides in the 1*S*,2*R*-isomer (2*R*-**1**).[16,17] The structure−activity relationship (SAR) of **1** and its analogues based on in vivo efficacy was reported by Bonnaud and coworkers in 1987.[18] We have recently described the SAR of a series of *N*-alkylamides and *N*,*N*-dialkylamides of **1** (NET IC_{50} = 77 nM) and found that by replacing one of its ethyl groups with an allyl moiety resulted in an over 5-fold increase in NET potency (**2**, NET IC_{50} = 14 nM).[19] Very recently, Roggen et al. reported the SAR studies of a series of analogues as single stereoisomers with various aromatic groups.[16] Their results show that the IC_{50} values for the synaptosomal uptake inhibition of 1*S*,2*R*-milnacipran (2*R*-**1**, *r*NET IC_{50} = 7 nM) is much lower than the 1*R*,2*S*-isomer (2*S*-**1**, *r*NET = 200 nM), and 2*R*-**1** is also about 3.5-fold more potent than 2*S*-**1** at *r*SERT.

Results from the SAR study by Roggen et al. on the substituted phenyl analogues of 2*R*-**1** show no improvement in potency, although substantial increases in both *r*NET and *r*SERT activities are observed when the phenyl ring of 2*S*-**1** is replaced by a more lipophilic 2-naphthyl group.[16] We have found that the NET activity of the single isomeric 3,4-ethylenedioxyphenyl **3a** (1*S*,2*R*, IC_{50} = 6.3 nM) is 6-fold higher than 2*R*-**1** (IC_{50} = 40 nM, *r*NET IC_{50} = 16 nM).[17] The allyl analogue **3b** (1*S*,2*R*) has another 2-fold increase in both NET (IC_{50} = 2.9 nM) and SERT (IC_{50} = 65 nM). In comparison, the single isomeric thien-3-yl **4** (1*S*,2*R*) has a slight increase in SERT activity (IC_{50} =

* To whom correspondence should be addressed. Phone: 858-617-7600. Fax: 858-617-7925. E-mail: cchen@neurocrine.com.
[†] Department of Medicinal Chemistry.
[‡] Department of Neuroscience.
[§] Department of Preclinical Development.
[†] Department of Pharmacology.

[a] Abbreviation: NE, norepinephrine; SER, serotonin; DA, dopamine; NET, norepinephrine transporter; SERT, serotonin transporter; DAT, dopamine transporter; *r*NET, *r*SERT, and *r*DAT, rat synaptosomal transporters; sSRI, selective serotonin reuptake inhibitors; sNRI, selective norepinephrine reuptake inhibitors; NSRI, dual norepinephrine and serotonin reuptake inhibitor; CNS, central nerve system; log *P*, partition coefficient in log unit; log *D*, distribution coefficient in log unit; DCM, dichloromethane.

10.1021/jm8009537 CCC: $40.75
Published on Web 10/28/2008

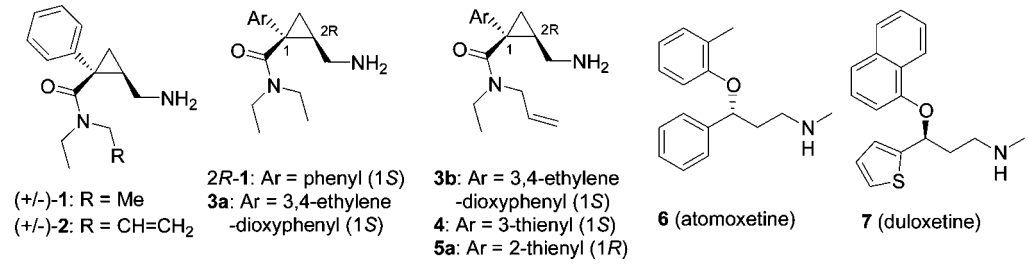
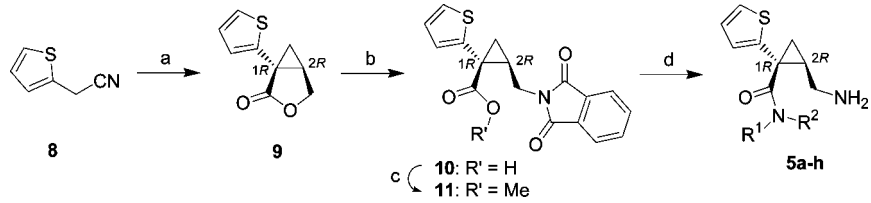


Figure 1. Chemical structures of milnacipran (**1**) and its analogues atomoxetine (**6**) and duloxetine (**7**).

Scheme 1[a]



[a] (a) (i) NaHMDS, THF, 0 °C; (ii) (*R*)-(−)-epichlorohydrin, 0 °C to room temp, 16 h; (iii) KOH, EtOH, reflux, 8 h; (iv) 12 N HCl, 0 °C to room temp, 2.5 h; (b) (i) potassium phthalamide, DMF, 120 °C, 16 h; (c) $(COCl)_2$, MeOH, room temp, 2 h; (d) (i) $SOCl_2$; (ii) R^1R^2NH, CH_2Cl_2, 0 °C to room temp, 16 h; (iii) $MeNHNH_2$, EtOH, room temp, 16 h.

150 nM) compared to 2*R*-**1** (SERT IC_{50} = 320 nM).[20] In our efforts to search potent NET-selective and NET/SERT dual inhibitors, we studied a series of thien-2-yl analogues of 2*R*-**1**. Here, we report the pharmacological and pharmacokinetic characterization of these compounds as NET and SERT inhibitors for the treatment of neuropathic pain.

Chemistry

The targeted compounds **5a**−**h** as 1*R*,2*R*-isomers were synthesized from 2-thienylacetonitrile **8** using the procedure shown in Scheme 1.[21] The stereoselective cyclization to form the lactone **9** was modified to improve its ee value.[22] Thus, treatment of **8** with NaHMDS in THF at 0 °C followed by *R*-(−)-epichlorohydrin gave **9** (94% ee) after KOH hydrolysis. Ring opening of **9** with potassium phthalamide afforded the corresponding acid **10**. A small sample of the methyl ester **11** was obtained for X-ray crystal structure determination. Coupling reactions of **10** with several secondary amines gave the final products **5a**−**h** after deprotection with methylhydrazine.

The absolute stereochemistry (1*R*,2*R*) of these compounds, which matches that of 1*S*,2*R*-milnacipran 2*R*-**1** but with an opposite nomenclature due to the high priority of sulfur in the 2-thiophene, was confirmed by the X-ray structure of **11** (Figure 2).

Pharmacology

The targeted compounds **5a**−**h** were tested in functional transporter assays evaluating their inhibition of cloned human NET, SERT, and DAT using a procedure similar to that described by Owens et al.[23] These results are summarized in Table 1.

The NET activity of the thien-2-yl compound **5a** was slightly lower than that of the thien-3-yl **4** (IC_{50} = 5.6 nM). Incorporating a fluorine at the allyl group of **5a** improved its potency at NET (**5b**, NET IC_{50} = 4.4 nM). Replacing the ethyl group of **5a** with an allyl moiety (**5c**) increased the NET and SERT activity by 5- to 6-fold. In comparison, the cyclopropyl analogue **5d** only improved SERT potency from **5a**. Similarly, the 2-thiazole **5e** showed improved SERT potency, resulting in a 1:1 ratio of NET/SERT inhibitory activity. The propargyl compounds **5f** and **5g** displayed similar profiles to the allyl

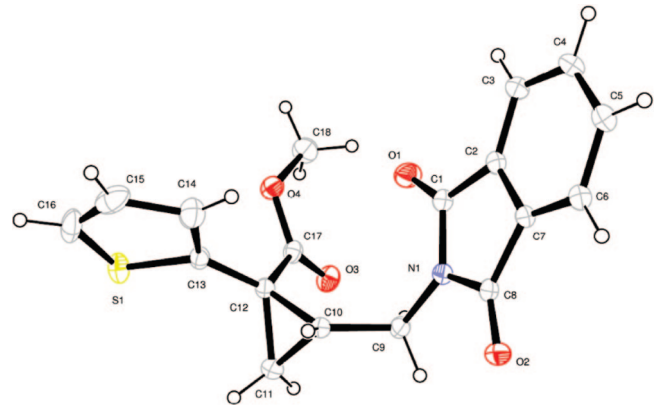


Figure 2. X-ray crystal structure of **11**.

analogues **5a** and **5c**, respectively. Finally, the isoindole **5h** exhibited potency and selectivity similar to the thiazole **5e** as a NET/SERT dual inhibitor, except **5h** showed a weak activity at DAT. As a reference, compound **6** had IC_{50} values of 5.1 and 190 nM, respectively, at NET and SERT, while **7** had a 1:1 ratio at these transporters (Table 1).

At rat synaptosomes, **5c** showed about a 3-fold increase in both NET and SERT compared to 2*R*-**1**, while **5b** was less active (Table 2).

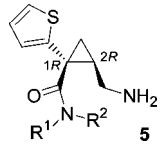
The selectivity of compounds **5b** and **5c** was tested at a concentration of 10 μM in a panel of radioligand binding assays for approximately 70 receptors, ion channels, enzymes and transporters; no significant activity was seen. This profile is quite similar to the high selectivity of milnacipran.[15]

Metabolism and Pharmacokinetics

Compounds **5a**−**h** were profiled in an in vitro liver microsomal assay to assess their metabolic stability in comparison with **1** and **6**, and the results are summarized in Table 3.

Compound **1** was quite stable metabolically in human liver microsomes (CL_{sys} = 3.1 (mL/(min·kg)), supporting the low metabolic clearance of **1** observed in humans (nonrenal clearance = 273 mL/min).[24,25] The thien-2-yl analogue **5a** was less stable than 2*R*-**1**, as was the diallyl analogue **5c**, indicating that the C−C double bond might be a major site for oxidative

Table 1. Potencies of Compounds **5a**−**h** at the Human Monoamine Transporters (IC_{50}, nM)[a]



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compd	R^1NR^2	IC_{50}, nM NET	SERT	DAT	calcd log P[b]
1[c]		77	420	6100	1.2
2*R*-**1**		40	320	3200	1.2
5a	$EtNCH_2CH{=}CH_2$	15	140	>10000	1.2
5b	$EtNCH_2CF{=}CH_2$	4.4	97	>10000	2.0
5c	$N(CH_2CH{=}CH_2)_2$	2.3	32	>10000	1.7
5d	$cPrNCH_2CH{=}CH_2$	17	45	>10000	1.1
5e	2-thiazole$CH_2NCH_2CH{=}CH_2$	13	16	>10000	1.9
5f	$EtNCH_2C{\equiv}CH$	19	81	>10000	0.8
5g	$N(CH_2C{\equiv}CH)_2$	6.0	26	>10000	0.7
5h	1-isoindoline	21	13	5539	1.7
6		5.1	190	3100	3.3
7		8.9	6.6	660	3.7

[a] Data are the average of two or more independent measurements. [b] Calculated using ACD software. [c] The following values at the cloned human transporters are reported by Neil Vaishnavi et al.: NET IC_{50} = 68 nM, SERT IC_{50} = 151 nM, DAT IC_{50} > 100 000 nM; see ref 8.

Table 2. Inhibition of Compounds **1**, 2*R*-**1**, **4**, **5b**,**c**, **5f**, and **5h** at Rat Synaptosomes (IC_{50}, nM)[a]

compd	*r*NET	*r*SERT	*r*DAT
1[b]	30	47	37000
2*R*-**1**[c]	16	28	20000
4	6.4	27	>10000
5b	21	160	>10000
5c	4.6	11	19000
5f	160	160	>10000
5h	15	25	2000

[a] Data are the average of two independent measurements. [b] The following values are reported by Mochizuki et al.: *r*NET IC_{50} = 29.6 nM, *r*SERT IC_{50} = 28 nM, *r*DAT IC_{50} > 10 000 nM; see ref 15. [c] The following values are reported by Roggen et al.: *r*NET IC_{50} = 7 nM, *r*SERT IC_{50} = 120 nM, *r*DAT IC_{50} = 10 000 nM; see ref 16.

Table 3. In Vitro Metabolic Profiles of 2-Thienyl Analogues **5a**−**h** in Comparison with **1** and **6**

compd	measured value[a] log P	log D	pK_a	Cl_{sys}[b] (mL/(min·kg)) HLM	RLM	Caco-2[c] P_{app} (nm/s)	efflux ratio	hERG[d] IC_{50} (μM)
1	1.7	0.2	9.5	3.1	24	89	4.5	ND[e]
2*R*-**1**	1.6	−0.2	9.6	5.0	24	51	7.4<	>10
5a		ND[e]		11	62	137	4.3	ND[e]
5b	1.8	0.2	9.4	2.6	54	167	3.2	>10
5c	2.0	0.3	9.4	9.9	61	167	3.8	>10
5d		ND[e]		5.0	63	178	3.2	>10
5e	1.6	−0.4	9.4	7.0	47	64	14	>10
5f	1.6	0.1	9.4	2.6	44	206	4.2	ND[e]
5g	1.5	0.2	9.3	2.6	43	250	2.9	>10
5h	2.2	0.5	9.5	8.8	64	84	9.0	ND[e]
6	3.5	0.8	10.1	14.3	ND[e]	437	1.0	2.1

[a] Determined using pION's Gemini Profiler. [b] Determined in vitro using liver microsomes; see Experimental Section for details. HLM: human liver microsomes. RLM: rat liver microsomes. [c] Digoxin was used as a control in the Caco-2 assay, and it showed on average P_{app} of 10 nm/s and efflux ratio of 20−30. [d] Determined using a patch-clamp assay; see Experimental Section for details. [e] ND: not determined.

metabolism. The metabolic stability of **5b** was, however, significantly increased from **5a** because of the addition of a fluorine to the allyl group. Incubation of **5c** with liver microsomes resulted in two detectable metabolites with molecular

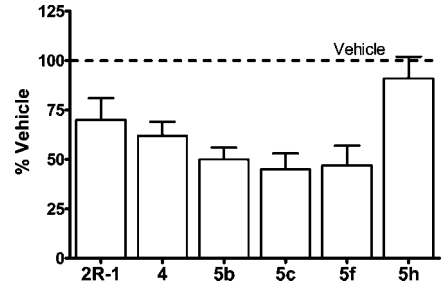

Figure 3. Compounds 2*R*-**1**, **4**, **5b**,**5c**, **5f**, and **5h** in rat formalin test (phase IIA) after 60 mg/kg oral administration.

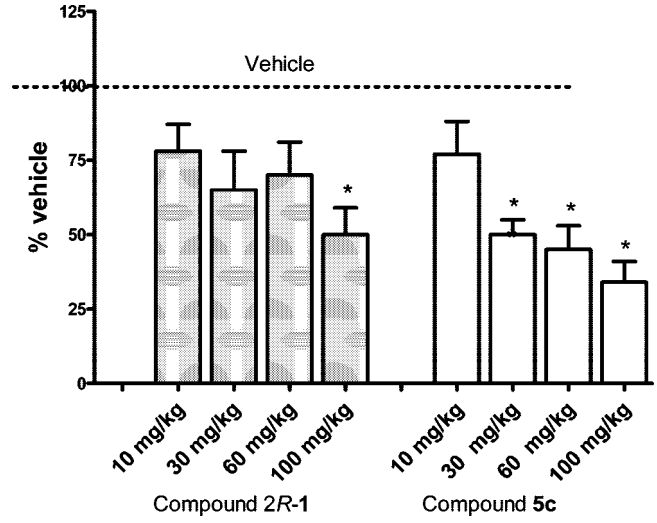

Figure 4. Dose−response of compound **5c** in rat formalin test (phase IIA) in comparison with 2*R*-**1**.

mass of +16 and +18, respectively, of the parent. Mass fragmentation studies indicated that the metabolism is at the amide side chain, suggesting that a reduction of the olefin bond followed by an oxidation could be a major metabolic pathway. Replacing the ethyl group of **5a** with cyclopropyl or thiazolemethyl resulted in analogues (**5d** and **5e**) with increased stability. Replacing the allyl group of **5a** and **5c** with a propargyl moiety significantly improved metabolic stability of these compounds (**5f** and **5g**). Finally, the lipophilic isoindoline (**5h**) showed only moderate stability in this assay; however, it was still better than the more lipophilic **6** (CL_{sys} = 14.3 mL/(min·kg)). Compounds **5** possessed similar lipophilicity as **1** based on the measured log P or log D values (Table 3).

In a Caco-2 assay measuring cell permeability of these compounds, **5** displayed similar or slightly better profiles compared to 2*R*-**1**. Thus, 2*R*-**1** (log D = −0.2) had a P_{app} of 51 nm/s from apical to basolateral direction and an efflux ratio (basolateral to apical versus apical to basolateral direction) of 7.4. In comparison, the slightly more lipophilic **5b** (log D = 0.2) and **5c** (log D = 0.3) showed a P_{aap} of 167 nm/s and an efflux ratio of 3.2 and 3.8, respectively.

None of the tested compounds showed significant inhibition (IC_{50} > 10 μM) at hERG potassium channels, indicating a low potential for possible QT prolongation liability.[26] In contrast, **6** displayed an IC_{50} of 2.1 μM in this assay (Table 3). Compounds **5b** and **5c** had no significant inhibitory activity at the major cytochrome P450 enzymes (1A2, 2C9, 2C19, and 3A4) except CYP2D6 ($IC_{50} \approx 6$ μM), implying a low risk of potential drug−drug interactions via major liver enzymes.

Compounds **5b**,**c**, **5f**, and **5h** were studied for their plasma and brain exposure after oral administration in comparison with

Table 4. Pharmacokinetic Parameters of Compounds 2*R*-**1**, **5b**,**c**, **5f**, and **5h** in Sprague−Dawley Rats ($N = 3$) after Oral Administration (30 mg/kg)

compd	C_{max} (ng/mL)	T_{max} (h)	AUC (ng/(mL·h))	apparent $t_{1/2}$ (h)	CL/F (mL/(min·kg))	C_{brain} (ng/g) at 1, 4 h	b/p ratio at 1, 4 h
2*R*-**1**[a]	232	0.3	566	1.3	294	103, 5	0.42, 0.86
5b	974	0.4	1498	0.9	334	70, 31	0.73, 1.1
5c	522	0.5	1126	1.2	444	127, 26	0.6, 1.4
5f	649	1.7	3208	1.9	156	230, 112	0.3, 0.3
5h	58	1.1	272	2.3	1838	28, 24	1.0, 1.8

[a] Dosed at 10 mg/kg.

Table 5. Pharmacokinetic Parameters of 2*R*-**1** and **5b**,**c** in Sprague−Dawley Rats ($N = 3$) after iv Adminisration (5 mg/kg)

compd	AUC (ng/(mL·h))	$t_{1/2}$(h)	MRT (h)	CL_p(mL/(min·kg))	CL_r(ml/(min·kg))	Vd_{ss}(L/kg)	Vd (L/kg)	*F* (%)
2*R*-**1**[a]	897	0.8	0.52	58	nd	2.0	4.3	16.0
5b	532	0.6	0.7	140	59	5.5	7.5	47.0
5c	721	0.6	0.7	102	11	4.4	5.3	12.0

[a] Dosed at 2.5 mg/kg.

Table 6. Pharmacokinetic Properties of **5b** and **5c** in Mice, Monkeys, and Dogs[a]

compd	species	CL_p (mL/(min·kg))	CL_r (mL/(min·kg)	$t_{1/2}$ (h)	V_{ss} (L/kg)	C_{max} (ng/mL)	T_{max} (h)	AUC (ng/mL·h)	*F*
5b	Monkey	34	6	4.3	4.4	2440	2.3	9050	70
	Dog	10.8	2.2	3.5	2.4	5570	1.2	47500	109
5c	Mouse	135	ND[b]	0.79	5.4	1731	0.3	3020	92
	Monkey	22.9	3.9	2.1	3.8	905	4.5	6316	34
	Dog	19.2	2	2.4	3.6	2546	1.8	17059	78

[a] Data obtained following the administration of a single intravenous bolus (5 mg/kg) or an oral gavage (30 mg/kg) dose of compounds **5b** or **5c** as a hydrochloride salt. [b] ND: not determined.

2*R*-**1** in Sprague−Dawley rats (Table 4). On the basis of the in vitro rat liver microsomal studies (Table 3), these compounds might be highly accessible to metabolic extraction when passing the liver in rats. Compound 2*R*-**1** had a C_{max} of 232 ng/mL and an area under the curve (AUC) of 566 ng/(mL·h) in plasma when dosed at 10 mg/kg to rats. Its brain concentrations measured at 1 and 4 h postdosing were 42% and 86% of those in plasma, indicating its fairly high brain penetration. Its apparent elimination half-life of 1.3 h was moderate in this species. The thien-2-yl compound **5b** or **5c** displayed a very similar profile to 2*R*-**1**, despite that they were much more metabolically labile based on the in vitro rat liver microsomal studies. The propargyl analogue **5f** had the highest AUC values, while the most lipophilic compound in this group, **5h**, had a very low C_{max} and AUC. As a reference, the following parameters were reported by Mattiuz et al. after an oral dose of 50 mg/kg **6** in Fischer 344 rats: $C_{max} = 170$ ng/mL, $T_{max} = 2.0$ h, AUC = 910 ng/(mL·h), apparent $t_{1/2} = 2.8$ h,[27] indicating a high first pass effect in this species.

The pharmacokinetic parameters of **5b** and **5c** were determined after intravenous administration to Sprague−Dawley rats to compare with those of 2*R*-**1**, and the results are summarized in Table 5. 2*R*-**1** had a high plasma clearance and a volume of distribution (V_d) of 4.3 L/kg, resulting in a short half-life ($t_{1/2} = 0.8$ h). Its oral bioavailability (F = 16%) was low. In comparison, compound **5b** had a very high plasma clearance ($CL_p = 140$ mL/(min·kg)). Despite its high V_d of 7.5 L/kg, **5b** had a short half-life ($t_{1/2} = 0.6$ h) in this species. Part of the high plasma clearance of **5b** was attributed by its high renal clearance ($CL_r = 59$ mL/(min·kg)), since over 30% of **5b** was excreted to the urea as unchanged parent. In contrast, compound **5c** had a lower renal clearance ($CL_r = 11$ mL/(min·kg)). As a reference, **6** has a moderate plasma clearance ($CL_p = 36.4$ mL/(min·kg)) but a very low oral bioavailability (1.5%).[27] It is worth nothing that **6** is not detectable in the urine.[27]

The plasma protein binding of **1** in rats is unknown but expected to be low based on the human data (13%) and low lipophilicity (log $D = 0.2$). The volume of distribution at steady state of 2*R*-**1** ($V_{ss} = 2.0$ L/kg) was not significantly lower than that of **6** ($V_{ss} = 4.4$ L/kg),[27] In contrast, the lipophilic **6** is reported to have a plasma protein binding of 89% in rats.[27]

Compounds **5b** and **5c** were also profiled in other species for their PK properties (Table 6). Compound **5b** had high oral bioavailability in both monkeys and beagle dogs, which were better than **5c**, although **5c** had high oral bioavailability in mice. Oral bioavailability of **5c** was comparable to **6** (73%) in dogs.[27]

In Vivo Efficacy in Pain Models

Previous studies have shown that both NE and SER may contribute to antinociception: NE in supraspinal descending pathway and SER in spinal pathway.[28−30] Furthermore, multiple studies have also suggested that the action of NE and SER in these pathways may produce synergistic effects.[31] To examine the contribution of SER and NE to antinociception, the present series of compounds were used to examine the relative contributions of NE and SER to antinociception in a model of persistent pain.

Compounds **4**, **5b**, **5c**, **5f**, and **5h** were studied in a rat formalin test to compare with 2*R*-**1**. Oral administration of these compounds at 60 mg/kg reduced the number of hindpaw flinches as much as 56% compared to vehicles (Figure 3). Compounds **5b**, **5c**, and **5f** showed significant better efficacy than 2*R*-**1**, while **5h** did not display an effect.

Compound **5c** was further studied with dose response in comparison with 2*R*-**1**, and the results are shown in Figure 4. 2*R*-**1** demonstrated statistical significance in reduction of the number of hindpaw flinches only at 100 mg/kg. In comparison, at a dose of 30 mg/kg, **5c** showed the same effect as the 100 mg/kg of 2*R*-**1**.

Acutely administrated **5c** (3, 10, and 30 mg/kg, po) dose-dependently reversed mechanical hyperalgesia induced by pinprick in spinal nerve ligation (SNL) rats (Figure 5). The highest dose (30 mg/kg) of **5c** produced about a 60% reversal. In

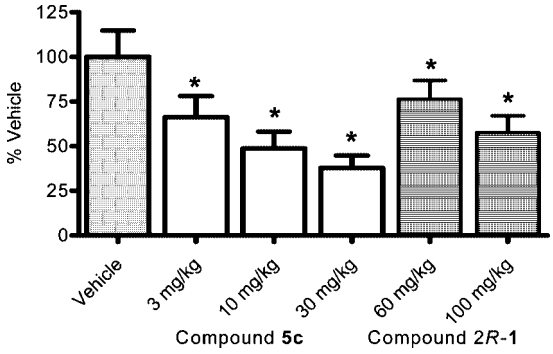

Figure 5. Dose-dependent reversal of pin-prick-induced mechanical hyperalgesia by compounds **5c** and 2*R*-**1** in rat SNL model.

comparison, 2*R*-**1** also produced a dose-dependent reversal but with much higher doses (60 and 100 mg/kg, Figure 5).

Conclusion

A series of thien-2-yl 1*S*,2*R*-milnacipran analogues were synthesized and studied as norepinephrine/serotonin transporter inhibitors. These compounds showed higher potencies than milnacipran at NET and SERT. Compounds **5b** and **5c** showed high oral bioavailability in several species. In addition, compound **5c** demonstrated efficacy in persistent and neuropathic pain models. Therefore, compounds such as **5c** will have the potential for the clinical treatment of neuropathic pain.

Experimental Section

Chemistry. General Methods. NMR spectra were recorded on a Varian 300 MHz spectrometer with TMS as an internal standard. ^{13}C NMR spectra were recorded at 75 MHz. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Purity measurements were performed on an HP Agilent 1100 HPLC−MS (detection at 220 and 254 nm). Chiral purity analyses were performed on a Chiralpak AD-H ADHOCE-KD016 column, with a mobile phase of 10% isopropanol/90% hexane + 0.1% diethylamine at 1 mL/min flow rate.

Typical Procedure: Synthesis of *N*,*N*-Diallyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarbamide Hydrochloride (5c). (*Z*)-1*R*-(2-Thienyl)-3-oxa-bicyclo[3,1,0]hexan-2-one (9). To a stirred solution of NaHMDS (1 M in THF, 9.581 L) was added dropwise 2-thienylacetonitrile (**8**, 536 g, 4.351 mol) in THF (2.1 L) at 0 °C over 2 h under nitrogen atmosphere. After additional 30 min of stirring, *R*-(−)-epichlorohydrin (97% ee, 400.93 g, 4.351 mol) in THF (2.4 L) was added dropwise (3 h) while maintaining a reaction temperature below 8 °C. The mixture was stirred for 2 h at 0 °C and then allowed to warm to room temperature overnight. Aqueous KOH (1M, 13.4 mL) and EtOH (26.8 mL) were added to the reaction mixture, and solvent volume was reduced under vacuum to approximately 3 L. Aqueous KOH (1 M, 415 mL) and EtOH (1.072 L) were added, and the mixture was refluxed under nitrogen atmosphere for 8 h and then stirred at room temperature for 13 h. The reaction mixture was placed in an ice bath, and hydrochloric acid (12 M) was added dropwise (2 h) under a stream of nitrogen until the pH was 1. The mixture was stirred for 2 h at 0 °C and then allowed to warm to room temperature. The material was extracted (ethyl acetate 2 L × 1, DCM 1.5 L × 3), and the combined organic fractions were concentrated under reduce pressure. The resulting tar residue was dissolved in ethyl acetate (2 L) and washed twice with aqueous sodium bicarbonate (1.5 L). The organic layer was dried over Na_2SO_4, filtered, and concentrated to give a thick brown oil (578 g), which was purified by chromatography using hexanes and ethyl acetate to afford the title compound (410 g, 52%) as a brown oil. HPLC: 94% ee. ^{1}H NMR ($CDCl_3$): 1.48 (t, J = 5.8 Hz, 1H), 1.71 (dd, J = 5.8, 7.5 Hz, 1H), 2.61 (m, 1H), 4.28 (d, J = 9.3 Ha, 1H), 4.48 (dd, J = 4.5, 9.3 Hz, 1H), 6.98 (dd, J = 3.9, 5.1 Hz), 7.07 (dd, J = 0.9, 3.6 Hz, 1H), 7.23 (dd, J = 1.2, 5.1 Hz, 1H).

(1*R*,2*R*)-2-(1,3-Dioxo-1,3-dihydroisoindol-2-ylmethyl)-1-(2-thienyl)cyclopropanecarboxylic Acid (10). A mixture of (*Z*)-1*R*-(2-thienyl)-3-oxabicyclo[3,1,0]hexan-2-one (**9**, 118 g, 0.655 mmol) and potassium phthalimide (122 g, 0.655 mmol) in DMF (500 mL) was heated at 145 °C in a sealed vessel overnight. The mixture was cooled to room temperature, poured over 5 L of cold water, which was washed with ethyl acetate (2 L × 3), acidified at 0 °C with hydrochloric acid (1 M), and extracted with ethyl acetate (2 L, then 1 L). The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the crude product, which was purified by chromatography using hexanes and ethyl acetate to afford the title compound (66 g, 31%) as a light-brown oil. HPLC: 96.5% ee. ^{1}H NMR ($CDCl_3$): 1.61 (dd, J = 4.8, 9.3 Hz, 1H), 2.00 (dd, J = 4.8, 7.5 Hz, 1H), 2.16 (m, 1H), 4.11 (d, J = 6.6 Hz, 2H), 6.88 (dd, J = 3.6, 5.1 Hz, 1H), 6.94 (dd, J = 1.5, 3.6 Hz, 1H), 7.17 (dd, J = 1.5, 5.4 Hz, 1H), 7.72 (dd, J = 3.3, 5.7 Hz, 2H), 7.86 (dd, J = 2.7, 5.4 Hz, 2H). MS: 328 (MH^+).

Methyl (1*R*,2*R*)-2-(1,3-Dioxo-1,3-dihydroisoindol-2-ylmethyl)-1-(2-thienyl)cyclopropanecarboxylate (11). (1*R*,2*R*)-2-(1,3-Dioxo-1,3-dihydroisoindol-2-ylmethyl)-1-(2-thienyl)cyclopropanecarboxylic acid (**10**, 7.90 g, 24 mmol) and DMF (0.25 mL) were combined in DCM (100 mL) and cooled in an ice bath. Oxalyl chloride in DCM (2.0 M, 30 mL, 60 mmol) was added over 5 min, the ice bath was removed, and the resulting mixture was stirred at room temperature for 3 h. The mixture was concentrated in vacuo to afford the crude acid chloride (8.41 g, 100%), which was used without further purification.

A portion of the above material (2.21 g, 6.4 mmol) was quenched slowly with methanol (10 mL). After 10 min, ethyl acetate (100 mL) was added and the mixture was left standing for several days. After the slow evaporation of approximately half of the reaction volume, large brown rhomboid crystals formed which were isolated by filtration. One of the crystals was retained for X-ray analysis, and the remainder were crushed to afford the titled compound (1.82 g, 83%) as a tan solid: ^{1}H NMR ($CDCl_3$): 1.53 (dd, J = 9.0, 4.8 Hz, 1 H), 1.94 (dd, J = 7.5, 4.8 Hz, 1 H), 2.14−2.04 (m, 1 H), 3.78 (s, 3 H), 4.09−3.96 (m, 2 H), 6.88−6.84 (m, 2 H), 7.16−7.13 (m, 1 H), 7.76−7.69 (m, 2 H), 7.90−7.83 (m, 2 H). MS: 342.0 (MH^+).

***N*,*N*-Diallyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5c).** Thionyl chloride (52.16 g, 0.438 mol) in DCM (90 mL) was added dropwise, over 15 min, to a stirred solution of (1*R*,2*R*)-2-(1,3-dioxo-1,3-dihydroisoindol-2-ylmethyl)-1-(2-thienyl)cyclopropanecarboxylic acid (10, 15 g, 0.046 mol) in DCM (150 mL) under a nitrogen atmosphere at room temperature. After 3 h, the mixture was concentrated in vacuo. The resulting residual was dissolved in DCM (200 mL) and cooled to 0 °C under a nitrogen atmosphere. To this stirred solution was added diallylamine (17.6 mL, 0.24 mol) in DCM (50 mL) dropwise over 30 min, then triethylamine (15 mL, 0.21 mol) in DCM (50 mL) dropwise over 30 min. The mixture was stirred at 0 °C for 1 h and then at room temperature for 2 h, washed with concentrated aqueous ammonium chloride (3 × 100 mL) and brine (2 × 50 mL), dried over sodium sulfate, filtered, and concentrated in vacuo to give a red oil (15.58 g) that partially solidified after 12 h. This material was triturated in 5:1 EtOH/EtOAc to give an off-white solid (6.44 g). The solid was chromatographed on silica gel (20% ethyl acetate in hexanes) to afford *N*,*N*-diallyl (1*R*,2*R*)-2-(1,3-dioxo-1,3-dihydroisoindol-2-ylmethyl)-1-(2-thienyl)-cyclopropanecarboxamide (6.16 g, 32%) as a white solid.

The above compound (4.98 g, 12.0 mmol) was dissolved in a mixture of toluene (100 mL) and ethanol (15 mL) and purged with nitrogen (3 min). To the stirred mixture under a stream of nitrogen was added methylhydrazine (10 mL) slowly. The clear yellow mixture was purged with nitrogen (1 min), and the reaction vessel was sealed and stirred for 17 h. The white precipitate (2.24 g) was removed by vacuum filtration and rinsed with toluene/ethanol (10:1). The filtrate was concentrated in vacuo to give a clear amber oil

(5.75 g) which was purified to afford *N*,*N*-diallyl (1*R*,2*R*)-2-(aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide (2.34 g) as a yellowish oil, which was dissolved in DCM (90 mL) and treated with HCl (2 M in diethylether) until the solution was slightly acific based on dampened pH paper. The solution was concentrated in vacuo to afford the title compound (1.98 g, 51%) as a white solid. Mp = 148.6 °C (crystallized from isopropanol−ethyl acetate−diethyl ether). HPLC purity: 100% (220 nm) and 99.3% (254 nm). Chiral HPLC: 98.6% ee. $[\alpha]_D^{20}$ +13.76° (*c* 1.06 g/100 mL, EtOH). ^{1}H NMR (D_2O): 1.45 (t, *J* = 5.7 Hz, 1H), 1.62 (m, 1H), 1.71 (dd, *J* = 5.7, 6.0 Hz, 1H), 2.88 (dd, *J* = 6.0, 12.0 Hz, 1H), 3.01 (dd, *J* = 6.0, 12.0 Hz, 1H), 3.64 (dd, *J* = 6.0, 15.0 Hz, 1H), 3.91 (m, 2H), 4.16 (dd, *J* = 3.0, 15.0 Hz, 1H), 4.90 (d, *J* = 18.0 Hz, 1H), 5.00 (dd, *J* = 1.0, 9.0 Hz, 1H), 5.39 (m, 1H), 5.62 (m, 1H), 6.86 (m, 2H), 7.19 (m, 1H). ^{13}C NMR: 19.92, 25.67, 31.33, 41.66, 47.72, 50.93, 117.11, 118.47, 125.28, 125.43, 127.60, 131.51, 131.97, 142.86, 171.33. IR (KBr): 3423, 3191, 3074, 2834, 1616, 1469. MS: 277 (MH^+). Anal. for ($C_{15}H_{20}N_2OS\cdot HCl$) C, H, N, S.

***N*-Allyl-*N*-ethyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5a).** White powder. HPLC purity: 100% (220 nm) and 99.2% (254 nm). ^{1}H NMR ($CDCl_3$, mixture of *E*/*Z* amides, ∼58:42): 0.98 and 1.09 (t, *J* = 7.2 Hz, 3H), 1.25 and 1.30 (t, *J* = 6.3 Hz, 1H), 1.70 and 1.81 (dd, *J* = 5.7, 9.0 Hz, 1H), 1.95 (m, 1H), 2.38 (m, 1H), 3.10 and 3.47 (dq, *J* = 6.9 Hz, 1H), 3.57 (1H), 3.77 (m, 1H), 3.84 and 3.90 (dd, *J* = 5.4, 16.5 Hz, 1H), 4.04 and 4.20 (dd, *J* = 4.8, 16.2 Hz, 1H), 5.10 (d, *J* = 18.5 Hz, 1H), 5.18 (m, 1H), 5.53 and 5.72 (m, 1H), 6.92 (m, 2H), 7.13 (m, 1H), 8.82 (brs, 3H). MS: 265 (MH^+). Anal. for ($C_{14}H_{20}N_2OS\cdot HCl$) C, H, N, S.

***N*-Ethyl-*N*-(2-fluoroallyl) (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5b).** White powder, mp = 140 °C (crystallized from isopropanol−ethyl acetate−diethyl ether). HPLC purity: 99.8% (220 and 254 nm). Chiral HPLC: 99.6% ee. $[\alpha]_D^{20}$ +24.37° (*c* 1.04 g/100 mL, EtOH). ^{1}H NMR (D_2O, mixture of *E*/*Z* amides, ∼60:40): 0.76 and 0.94 (t, *J* = 7.2 Hz, 3H), 1.45 (m, 1H), 1.68 (m, 2H), 2.90 (dd, *J* = 6.0, 12.0 Hz, 0.4H), 2.93 (m, 1.2H), 3.00 (dd, *J* = 6.0, 12.0 Hz, 0.4H), 3.17 and 3.38 (dq, *J* = 6.0 Hz, 1H), 3.33 and 3.57 (dq, *J* = 6.0 Hz, 1H), 3.87 and 4.00 (dd, *J* = 12, 18 Hz, 1H), 4.07 and 4.28 (dd, *J* = 12, 18 Hz, 1H), 4.27 and 4.44 (dd, *J* = 3.0, 9.0 Hz, 1H), 4.62 and 4.63 (dd, *J* = 3.0, 18.0 Hz, 1H), 6.85 (m, 2H), 7.17 (m, 1H). ^{13}C NMR: 11.15 and 12.01, 20.17 and 26.09, 25.68 and 26.09, 31.41, 40.98 and 43.52, 41.50 and 41.55, 44.58 and 48.14 (d, *J* = 33.0 Hz), 92.89 and 94.38 (d, *J* = 17.3 Hz), 124.94, 125.29 and 125.39, 127.63, 142.76, 159.58 and 159.88 (d, *J* = 255.8 Hz), 171.32 and 171.47. MS: 283 (MH^+). Anal. for ($C_{14}H_{19}FN_2OS\cdot HCl$) C, H, N, S, Cl.

***N*-Allyl-*N*-cyclopropyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5d).** White powder. HPLC purity: 98% (220 and 254 nm). ^{1}H NMR ($CDCl_3$, mixture of *E*/*Z* amides, ∼38:62): 0.55−0.86 (m, 4H), 1.32 and 1.42 (t, *J* = 6.9 Hz, 1H), 1.68−2.00 (m, 2H), 2.24−2.58 (m, 1H), 2.65 (m, 1H), 3.72 (m, 1H), 3.82−4.16 (m, 2H), 5.10 (m, 2H), 5.58 and 5.78 (m, 1H), 6.84 (m, 2H), 7.11 (m, 1H), 8.66 (brs, 3H). MS: 277 (MH^+). Anal. for ($C_{15}H_{20}N_2OS\cdot HCl\cdot 0.3H_2O$) C, H, N, S.

***N*-Allyl-*N*-(2-thiazolyl)methyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5e).** White powder. HPLC purity: 100% (220 and 254 nm). Chiral HPLC 99.1% ee. ^{1}H NMR (DMSO-d_6, mixture of *E*/*Z* amides, ∼76:34): 1.52−1.76 (m, 3H), 2.74 (m, 1H), 2.96 (m, 1H), 3.65−4.32 (m, 2H), 4.52 (d, *J* = 15.6 Hz, 0.76H), 4.87 (d, *J* = 15.6 Hz, 1H), 4.95−5.18 (m, 2.24H), 5.43 and 5.65 (m, 1H), 6.92−7.15 (m, 2H), 7.40 and 7.42 (dd, *J* = 1.5, 4.8 Hz, 1H), 7.67 (d, *J* = 3.6 Hz, 1H), 7.71 and 7.73 (d, *J* = 3.6 Hz, 1H), 8.04 (brs, 3H). MS: 334 (MH^+). Anal. for ($C_{16}H_{19}N_3OS_2\cdot 1.8HCl\cdot 0.2H_2O$) C, H, N, S, Cl.

***N*-Ethyl-*N*-propargyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5f).** White powder. HPLC purity: 98.7% (220 and 254 nm). ^{1}H NMR (DMSO-d_6, mixture of *E*/*Z* amides, ∼56:44): 0.86 and 1.07 (t, *J* = 7.2 Hz, 3H), 1.50−1.75 (m, 3H), 2.64 (m, 1H), 2.95 (m, 1H), 3.16 and 3.30 (t, *J* = 2.4 Hz, 1H), 3.20−3.68 (m, 2H), 3.98 and 4.00 (dd, *J* = 2.4, 17.7 Hz, 1H), 4.23 and 4.28 (dd, *J* = 2.4, 17.7 Hz, 1H), 6.94 (m, 2H), 7.39 (m, 1H), 8.12 (brs, 3H). ^{13}C NMR ($CDCl_3$): 12.13 and 12.68, 21.38 and 21.48, 32.39 and 33.14, 33.64, 37.58, 40.55 and 42.13, 43.47 and 43.91, 71.56 and 72.82, 123.46, 123.79, 124.38, 127.26 and 127.35, 141.5, 171.0. MS: 263 (MH^+). Anal. for ($C_{14}H_{18}N_2OS\cdot HCl\cdot 0.3H_2O$) C, H (calcd 6.49%, found 7.06%), N, S, Cl.

***N*,*N*-Dipropargyl (1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarboxamide Hydrochloride (5g).** White powder. HPLC purity: 99% (220 and 254 nm). Chiral HPLC 98% ee. ^{1}H NMR (DMSO-d_6): 1.54−1.78 (m, 3H), 2.57 (m, 1H), 3.05 (m, 1H), 3.25 (t, *J* = 2.1 Hz, 1H), 3.36 (t, *J* = 2.1 Hz, 1H), 4.07 and 4.19 (dd, *J* = 2.1, 17.1 Hz, 1H), 4.40 and 4.36 (dd, *J* = 1.8, 18.0 Hz, 1H), 6.96 (m, 2H), 7.41 (m, 1H), 8.04 (brs, 3H). MS: 273 (MH^+). Anal. for ($C_{15}H_{16}N_2OS\cdot 1.2HCl\cdot 0.1H_2O$) C, H, N (calcd 8.91%, found 9.50%), S, Cl.

2-[(1*R*,2*R*)-2-(Aminomethyl)-1-(2-thienyl)cyclopropanecarbonyl]isoindoline Hydrochloride (5h). White powder. HPLC purity: 100% (220 and 254 nm). Chiral HPLC 96% ee. ^{1}H NMR ($CDCl_3$): 1.41 (t, *J* = 5.7 Hz, 1H), 1.81 (dd, *J* = 5.7, 9.0 Hz, 1H), 2.00 (m, 1H), 2.66 (m, 1H), 3.69 (m, 1H), 4.70 (d, *J* = 14.7 Hz, 1H), 4.77 (s, 2H), 4.92 (d, *J* = 14.4 Hz, 1H), 6.87 (dd, *J* = 3.6, 5.8 Hz, 1H), 6.97 (dd, *J* = 1.2, 3.3 Hz, 1H), 7.10 (dd, *J* = 0.9, 5.4 Hz, 1H), 7.15−7.30 (m, 4H), 8.78 (brs, 3H). ^{13}C NMR: 20.31, 26.82, 32.14, 32.87, 52.92, 53.34, 122.77, 123.24, 124.80, 125.74, 127.60, 127.91, 128.10, 135.66, 136.00, 142.01, 169.11. MS: 299 (MH^+). Anal. for ($C_{17}H_{18}N_2OS\cdot 1.2HCl$) C, H, N, S.

General Monoamine Transporter Inhibition Assay Methods. The norepinephrine, dopamine, and serotonin transporters were individually expressed in stably transfected HEK293 cell lines and grown in Dulbecco's modified Eagles medium (DMEM) (Cellgro, 15-013-CV) with the following supplements: 1% HEPES (Cellgro, MT 25-060-Cl), 1% L-glutamine (Cellgro, MT 25-005-Cl), 1% sodium pyruvate (Cellgro, MT 25−000Cl), 1% Pen/Strep (Cellgro, MT 30-001-Cl), 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 250 μg/mL G418 (Cellgro, 61-234-RG).

The day before the assay, solid white 96-well TC-treated sterile plates (Costar, 3917) that had been coated with 0.01% poly-D-lysine (Sigma, P6407) and 0.01% collagen (BD Biosciences, 354236) were seeded with cells at a density of 20 000 cells/well. The cells were allowed to attach overnight in a 37 °C incubator (7.5% CO_2). On the day of the assay, media were removed, and cells were washed with phosphate buffered saline. The cells were then incubated at room temperature for 20 min with varying concentrations of competing ligand in a total volume of 150 μL of transport buffer (20 mM HEPES, 122 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.4 mM $MgSO_4$, 1 mM ascorbic acid, 0.1 mM pargyline, 0.1 mM tropolone). Radioligand was then added to the cells for a total volume of 200 μL, and cells were incubated at room temperature for an additional 20 min. (Levo[ring-2,5,6-^{3}H]norepinephrine (52 Ci/mmol, PerkinElmer, NET-678) was used for NET, 3,4-[ring-2,5,6-^{3}H]dihydroxyphenylethylamine (50 Ci/mmol, PerkinElmer, NET-673) was used for DAT, and [α,β-^{3}H(N)]-5-hydroxytryptamine (30 Ci/mmol, PerkinElmer, NET-498) was used for SERT.) After incubation, the transport buffer was quickly aspirated from the plates, and the cells were washed twice with 4 °C wash buffer (20 mM HEPES, 280 mM D-mannitol, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM ascorbic acid, 0.1 mM pargyline, 0.1 mM tropolone). Cells were treated with 50 μL of 5% sodium dodecyl sulfate solution (Sigma, L4522) and 200 μL of Microscint scintillation fluid. Plates were shaken vigorously overnight before monitoring radioligand in a TopCount-NXT (Packard) microplate scintillation counter. Data were analyzed by nonlinear, least-squares curve fitting algorithms using ActivityBase (IDBS, Guildford, Surrey, U.K.).

hERG Patch-Clamp Assay. The hERG potassium current was recorded from a hERG/HEK cell line using established patch-clamp methods. The effects of test compounds on the hERG current were determined at the end of a 5 min application. Test compounds were tested at six concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM). Cisapride (30 nM) was used as a positive control.

Metabolic Stability in Rat and Human Liver Microsomes. Pooled male human and rat liver microsomes (0.5 mg/mL for human and 0.1 mg/mL for rat; $n > 10$; mixed gender) were incubated at 37 °C with the test compound in the presence of an NADPH-generating system containing 50 mM, pH 7.4, potassium phosphate buffer, 3 mM magnesium chloride, 1 mM EDTA, 1 mM NADP, 5 mM G-6-P, and 1 unit/mL G-6-PD. Incubations were conducted with 1 μM of each compound (0.01% DMSO) with a total volume of 250 μL, in duplicate at each time point (0, 5, 10, 20, 40, and 60 min). Reactions were stopped by the addition of 0.3 mL of acetonitrile (ACN) containing a proprietary internal standard. Precipitated proteins were removed by centrifugation for 15 min at 3000 rpm, and the supernatant fluid (~0.1 mL) was analyzed by LCMS for the percentage of parent compound remaining. The in vitro initial rates of metabolism were scaled using constants, such as microsomal protein/g of liver, g of liver/kg of body weight, and liver blood flow, to predict systemic clearance and maximum predicted percent bioavailability. These calculations from nonlinear regression assume that liver metabolism alone is the determinant of bioavailability.

Pharmacokinetic Characterization. The pharmacokinetic profiles of selected compounds were determined in animals ($N = 3$ time points at a typical dose of 30 mg/kg for po or 5 mg/kg for iv). Compounds were dosed as a hydrochloride salt in water. Composite sampling was used to collect samples. Terminal blood samples were taken from treated animals at 9 time points ranging from predose to 24 h (0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h) postdose. Brain samples were collected at 1 and 4 h after po dosing in rats for all compounds.

For plasma samples, the compound was extracted via a protein precipitation assay by adding 130 mL of acetonitrile (CAN) and 30 mL of internal standard in 50 μL of mouse plasma (EDTA). Standards and QCs were prepared by drying 50 mL of spiked aqueous solutions and reconstituting with 50 mL of blank plasma. For brain samples, the whole brain was homogenized in 2.0 mL of ACN/H_2O/formic acid (v/v, 60/40/0.1) containing 50 mL of internal standard, and the supernatant was collected and injected into an LC−MS/MS system for analysis. Brain standards and QCs were prepared by adding 1.0 mL of spiked aqueous solutions (prepared in 60/40 ACN/H_2O), 1.0 mL of ACN/H_2O/formic acid (v/v, 60/40/0.1) and 50 mL of internal standard into a whole blank brain. Standards and QCs for both plasma and brain were processed and analyzed at the same time and in exactly the same way as the analytical samples. The compound was measured using a specific and sensitive HPLC/MS assay that offered linear ranges of 1−1000 ng/mL for plasma sample analysis and 1−500 ng/mL for brain sample analysis. The lower limit of quantitation (LLOQ) was 1 ng/mL for the study. Quantification for both plasma and brain samples was performed by fitting peak area ratios to a weighted ($1/x$) linear calibration curve.

Descriptive pharmacokinetics were derived and evaluated on the basis of the mean plasma concentrations ($N = 3$ time points). A noncompartmental model in ActivityBase with linear trapezoidal rule was used to perform all pharmacokinetic analyses pertaining to this manuscript.

Bioanalysis. The LC−MS/MS system equipped with Agilent 1100 HPLC system was coupled to a CTC PAL autoinjector (Leap Technologies, Carrboro, NC) and a Sciex API 4000 triple quadruple mass spectrometer. Samples were eluted at 0.45 mL/min using a mobile phase consisting of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile) with a fast gradient (0−0.1 min, 5% B; 0.1−1.0 min, 5−95% B; hold to 2.5 min; 2.5−2.7 min, 95−5% B; hold to 3.5 min) and a column temperature of 20 °C. The HPLC column used for this assay was Agilent Zorbax SB-C18 column, 2.1 mm × 50 mm, with a particle size of 5 μm. Electrospray ionization source was used for detection at a positive MRM (multiple reaction monitoring) mode. Quantification was performed by fitting peak area ratios (peak of parent analyte vs peak of the internal standard) to a weighted ($1/x$) linear calibration curve.

Formalin Model. All animal studies were conducted using protocols approved by the Neurocrine IACUC committee. The formalin test was conducted using the automated nociception analyzer (Department of Anesthesiology, University of California, San Diego) and performed as described by Yaksh et al.[32] One hour prior to testing, male rats (CD, Charles River Laboratories, 250−300 g) were dosed orally with either vehicle (5% Cremophor EL, Sigma, St. Louis, MO in MilliQ water) or test compound at indicated doses and fitted with a metal band on their left hind paw. At testing, rats were injected subcutaneously on dorsal surface of the left hind paw with 50 μL of 5% formalin in saline and placed on the nociception analyzer. The number of flinches per minute was tabulated for 1 h. Here, we report flinches from 10−40 min after formalin injection, the phase referred to as phase IIA, the portion of the response that corresponds to sensitization of central processing of nociceptive inputs. Compound effects on the number of flinches in phase IIA were analyzed by a one-way analysis of variance (ANOVA) (NCSS 2001, Kaysville, UT). Significant effects were analyzed by Dunnett's post hoc comparison to vehicle treatment.

Mechanical Hyperalgesia in Spinal Nerve Ligated Rats. Spinal nerve ligation (SNL) neuropathy was induced in male rats (Sprague−Dawley, Harlan, 150−175 g). Briefly, the left L5 and L6 spinal nerves distal to the dorsal root ganglion were tightly ligated with 6−0 silk suture.[33] SNL rats were tested for mechanical hyperalgesia using the pin-prick method.[34] The length of time the paw was held off the grid floor was measured with the computer program Xnote Stopwatch, version 1.4. Zero seconds was assigned when there was no paw withdrawal. The baseline score was determined from the average of five trials. Rats that met criteria (withdrawal durations greater than 1.5 s)[34] were assigned to treatment groups by counterbalancing their baseline times. Animals were dosed orally with either vehicle (5% Cremophor in MilliQ water) or test compound at indicated doses. One hour later withdrawal response to the pin prick was assessed by the average of three trials. Drug effects are analyzed by a two-way ANOVA with treatment and time as variables. Significant effects are analyzed by Dunnett's post hoc comparison (NCSS 2001, Kaysville, UT).

Acknowledgment. The authors are indebted to Dr. Robert Petroski for performing the *h*ERG assay and Paddi Ekhlassi for the measurement of physicochemical properties.

Supporting Information Available: Elemental analysis data of final compounds and HPLC chromatograms of **5g** and **5h**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM8009537