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First-in-Class, Dual-Action, 3,5-Disubstituted Indole Derivatives Having Human Nitric Oxide Synthase (nNOS) and Norepinephrine Reuptake Inhibitory (NERI) Activity for the Treatment of Neuropathic Pain

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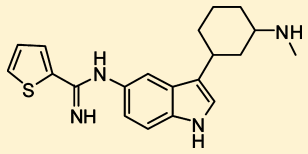
Supporting Information

ABSTRACT: A family of different 3,5-disubstituted indole derivatives having 6-membered rings were designed, synthesized, and demonstrated inhibition of human nitric oxide synthase (NOS) with norepinephrine reuptake inhibitory activity (NERI). The structure−activity relationship (SAR) within the cyclohexane ring showed the *cis*-isomers to be more potent for neuronal NOS and selective over endothelial NOS compared to their *trans*-counterparts. Compounds, such as *cis*-(+)-**37**, exhibited dual nNOS and NET inhibition (IC_{50} of 0.56 and 1.0 μM, respectively) and excellent selectivity (88-fold and 12-fold) over eNOS and iNOS, respectively. The lead compound (*cis*-(+)-**37**) showed lack of any direct vasoconstriction or inhibition of ACh-mediated vasorelaxation in isolated human coronary arteries. Additionally, *cis*-(+)-**37** was effective at reversing both allodynia and thermal hyperalgesia in a standard Chung (spinal nerve ligation) rat neuropathic pain model. Overall, the data suggest that *cis*-(+)-**37** is a promising dual action development candidate having therapeutic potential for the treatment of neuropathic pain.



INTRODUCTION

Nitric oxide (NO) is a biological signaling molecule, and since its discovery it has been under investigation as a potential therapeutic for the treatment of neurodegenerative disorders such as inflammation and pain. Nitric oxide synthase (NOS) inhibitors have repeatedly been shown to be active in multiple models of neuropathic pain.[1] NO is synthesized by catalysis from three isoforms of nitric oxide synthase (NOS), a constitutive form in endothelial cells (eNOS), a constitutive form in neuronal cells (nNOS), and an inducible form found in macrophage cells (iNOS). These enzymes are homodimeric proteins that catalyze a five-electron oxidation of L-arginine, yielding NO and L-citrulline. The role of NO produced by each of the NOS isoforms is quite unique, and its functions in normal and pathological processes are well established.[2] For example, NO produced from overstimulation of individual NOS isoforms, especially nNOS and iNOS, can lead to several disorders such as septic shock, arthritis,[3] pain,[4] and various neurodegenerative disorders.[5] On the other hand, stimulation of eNOS produces NO which has mainly a physiological role in maintaining normal blood pressure and flow, while it is inhibition (for example *N*-methyl-L-arginine (L-NMMA)) can lead to enhanced white cell, platelet activation, and hypertension.[6−9]

Norepinephrine (NE) is a monoamine neurotransmitter found in the CNS and plays an important role in human physiology and pathology.[10] It is involved in mood and sleep regulation, expression of behavior, and the general degree of alertness. It is believed that by inhibiting norepinephrine transporter (NET), pain is attenuated by blocking reuptake of NE, leading to increased postsynaptic NE levels and sustained activation of the descending pain inhibitory pathway. In addition, animal studies and clinical observations suggest that drugs with NERI activity are more effective for the treatment of pain.[11−13]

Neuropathic pain is a complex phenomenon characterized by burning pain coupled with hyperalgesia and allodynia that involves several mechanisms in both the peripheral and central nervous system. At present, first-line treatment options for this pathology are represented by the so-called "analgesic adjuvants" such as antidepressants, anticonvulsivants, and local anesthetics (gabapentin, lidocaine, tramadol, nortriptyline, doxepine).[14−17]

In recent years, an increased understanding of the mechanism of action has led to the use of antidepressant medication for the treatment of neuropathic pain.[18−21] Further, there has been a growing interest in the design of drugs that act specifically on multiple targets ("targeted polypharmacology") with the aim of improving drug efficacy and safety. These types of compounds, with defined multitarget profiles, have been classified as designed multiple ligands (DMLs) to distinguish them from nonselective drugs that often possess activities irrelevant to disease management, with the potential to elicit undesirable side effects. Success with the DML approach has

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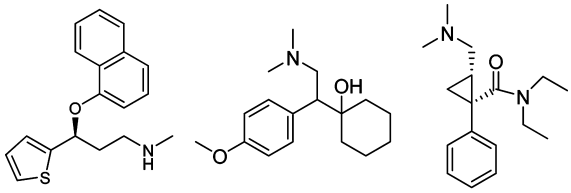
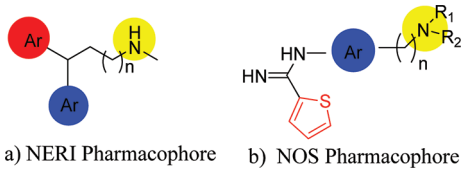
been achieved in the development of dual inhibitors of serotonin and norepinephrine reuptake for the treatment of depression or pain[22] such as Duloxetine (**1**)[23,24] Venlafaxine (**2**),[25] and Milnacipran (**3**).[26] In general, these new dual action antidepressants show superior efficacy[22] via the action of both ascending and descending noradrenergic and serotonergic pathways.



(*S*)-Duloxetine (**1**) Venlafaxine (**2**) Milnacipran (**3**)

Considering that both norepinephrine and nitric oxide are involved in the progression of pain, the plan was to design and synthesize dual action selective neuronal nitric oxide synthase (nNOS) inhibitors with NERI activity. Both nNOS and NET, an enzyme and membrane transporter, respectively, are completely distinct biological targets. Given that a sufficient overlap of pharmacophores must exist between the two targets of interest in order for a drug to interact sufficiently, it may be more challenging to find suitable dual action compounds. The goal of this project was to establish the SAR based on our NOS pharmacophore model and to optimize the potency and selectivity for nNOS versus eNOS as well as the relative balance of activity for nNOS versus NET (Figure 1).



a) NERI Pharmacophore b) NOS Pharmacophore

Figure 1. (a) NET pharmacophore model: basic amine linked (flexible or constrained) to two aromatic systems. Generally *N*-methyl compounds are more active; (b) nNOS pharmacophore scaffold is usually an aromatic ring. Main interaction is usually achieved through a 2° or 3° amine, and the anchoring point is made with an amidine group.

On the basis of our previous findings,[27−30] bulkier groups showed better potency and selectivity for the nNOS inhibitors and therefore a number of different 3,5-disubstituted indole derivatives having 1,4-disubstituted or 1,3-disubstituted cyclohexane rings were designed, synthesized, and tested for their potencies at these two targets. The synthesis would also provide an insight into the SAR within the series. Specifically, it would enable an examination of the effect of stereochemistry within the cyclohexane ring (*cis* versus *trans*) and the relative potencies of secondary versus tertiary amines.

RESULTS AND DISCUSSION

Chemistry. The syntheses started with commercially available 5-nitroindole (**4**) and are outlined in Schemes 1 and 2. Compounds **6** and (±)-**29** are the key intermediates in the preparation of our final targets consisting of 3,5-disubstituted indoles with 1,4-disubstituted cyclohexanes or 1,3-disubstituted cyclohexanes, respectively. Compound **6** was prepared from the condensation of **4** and **5** under basic conditions followed by the acid hydrolysis of the ketal (Scheme 1). The resulting ketone was subjected to reductive amination with the appropriate amine to give di- and trisubstituted amines as racemic mixtures in 60−70% yield. The separation of the enatiomers was not attempted at this time. After protection of the secondary amines ((±)-**8** and (±)-**10**), compounds (±)-**7**, (±)-**9**, and (±)-**11** underwent two separate transformations. In the first pathway, only the nitro group was reduced using hydrazine in the presence of Raney nickel as catalyst to obtain anilines (±)-**12**−(±)-**14**, which were then coupled with methyl thiophene-2-carbimidothioate **15**. After deprotection of (±)-**17** and (±)-**19** under acid conditions, the first set of target compounds, (±)-**16**, (±)-**18**, and (±)-**20**, was obtained. Only compound (±)-**16** was separated into its enantiomers in high optical purity using preparative chiral HPLC. The absolute configurations were not assigned.

To obtain the saturated analogues, compounds (±)-**7**, (±)-**9**, and (±)-**11** were subjected to hydrogenation in the presence of palladium on carbon at 1 atm, where the nitro groups were reduced as well. Mixtures of inseparable diastereomers were obtained. The anilines were further coupled with **15** as previously mentioned, and after removal of the Boc-protecting group a new set of compounds **24**, **26**, and **28** was obtained. At this time the final compounds were separated into their corresponding *cis* and *trans* isomers using HPLC. Although NOE data were obtained for all isomers, the 2D data proved difficult for stereochemical assignment owing to the fact that the cyclohexane ring would undergo interconversion between the boat and chair conformations on the NMR time scale. This interconversion would tend to average the chemical shift values of the axial and equatorial protons and decrease the resolution of the couplings. Therefore, in all three cases 1D spectra were used to assign the *cis* and *trans* stereochemistry (see Supporting Information). The argument is based on the fact that there is good chemical shift dispersion for the protons of the *trans* isomers as well as good resolution of the coupling constants and similar observations have been previously reported.[31,32] The *cis* isomers did not have the same chemical shift differences, and the coupling constants were not as well resolved. Using the above argument, the proton chemical shift range for the *cis*-configurations in compounds **24**, **26**, and **28** is 0.3−0.5 and 0.8−0.93 ppm for the *trans*-configurations.

Compound (±)-**29**, the second key intermediate, was obtained from the Michael addition of **4** to cyclohex-2-enone with $Bi(NO_3)_3$ as the catalyst (Scheme 2). Reductive amination of the ketone with the appropriate amine yielded compounds (±)-**30** (*cis*/*trans* 1:3) and (±)-**32** (*cis*/*trans* 1:3) as separable isomers. The enantiomers were not separated at this time. The *cis*/*trans* stereochemistry was assigned by using COSY and NOESY spectroscopic techniques and proved much easier in the stereochemical assignment compared to the 1,4-disubstituted analogues (see Supporting Information). Each of the isomers underwent further transformation separately, first protection, followed by reduction of the nitro group with hydrazine hydrate and Raney nickel and coupling with **15** as previously. After removal of the Boc- group, the final set of target compounds *cis*/*trans*-(±)-**37** and *cis*/*trans*-(±)-**39** was obtained as racemates. Because *cis*-(±)-37 showed a better in vitro profile, it was further separated into its enatiomers with high optical purity using chiral supercritical fluid chromatography, however, the absolute configuration was not established at this time.

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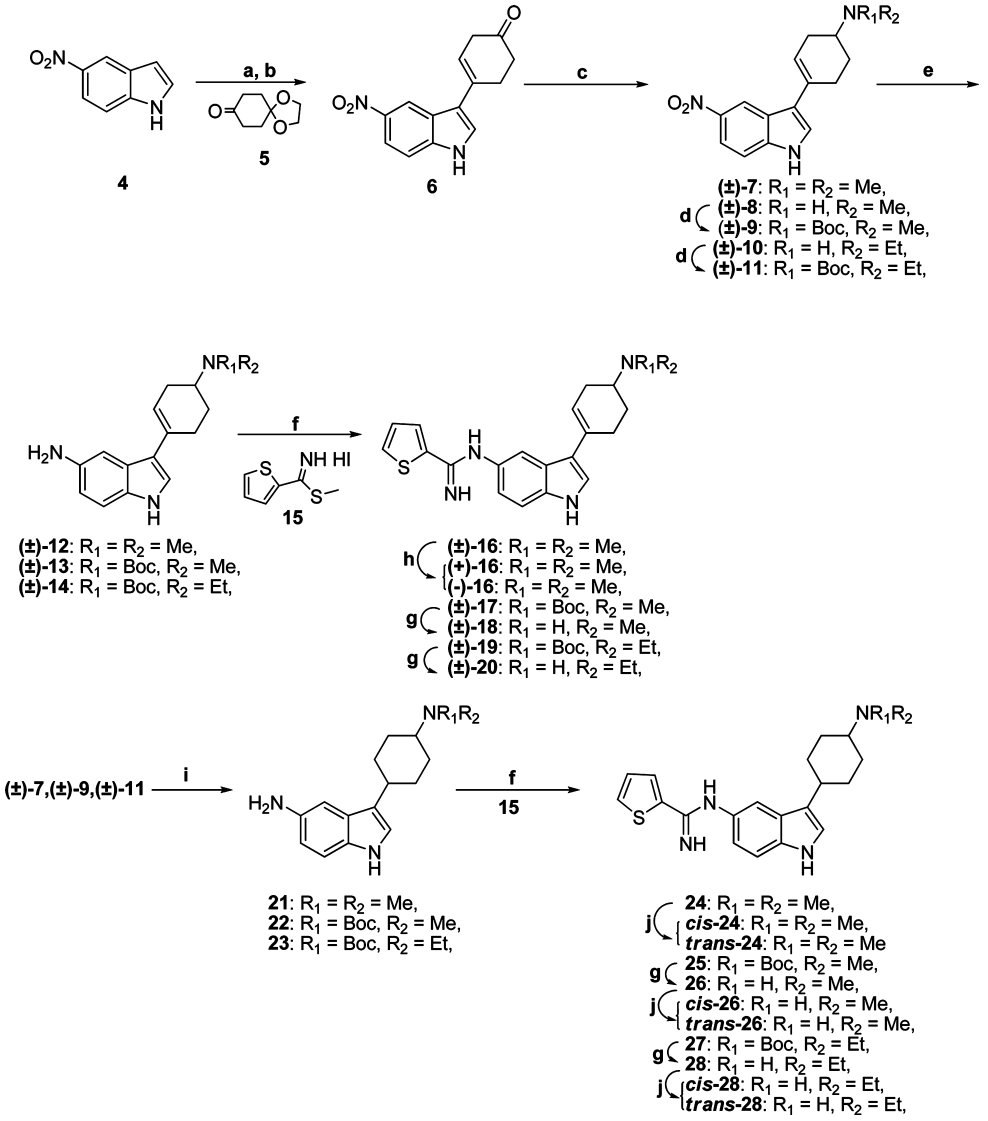
Scheme 1. 3,5-Disubstituted Indoles with 1,4-Disubstituted Cyclohexane Rings[a]



[a]Reagents and Conditions: (a) **5**, KOH, MeOH, reflux; (b) 10% HCl, acetone, rt; (c) amine, AcOH, $NaBH(OAC)_3$, 1,2-DCE; (d) $(Boc)_2O$, Et_3N, 1,4-dioxane; (e) hydrazine hydrate, Raney nickel, MeOH, reflux; (f) **15**, EtOH, rt; (g) 1N HCl, reflux or TFA/CH_2Cl_2 rt; (h) chiral HPLC separation; (i) EtOH, Pd/C, H_2, 1 atm; (j) preparative HPLC.

All final compounds were converted into their hydrochloride salts prior to in vitro and in vivo testing.

Structure–Activity Relationship (SAR) Studies. The synthesized compounds were screened for their in vitro human NOS and human NET inhibitory activities by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline using a radiometric method for NOS inhibition and the inhibition of [^{3}H] nisoxetine binding to CHO cells in the presence of the test compound for NET inhibition (Table 1). Initial look into the SAR of 1,4-disubstituted cyclohexane analogues for both the unsaturated and their saturated counterparts of all compounds showed very good potency for nNOS and NET and excellent selectivity for nNOS over eNOS and iNOS.

It is interesting to note that compared to the racemate (±)-**16**, the separated enantiomers (+)-**16** and (−)-**16** became more potent for nNOS (i.e., from IC_{50} = 3.0 to $IC_{50} \sim 0.8$ μM) while the potencies for NET were reduced by half (i.e., $IC_{50} \sim 1$ from IC_{50} = 0.55 μM).

An apparent difference in both potency and selectivity was observed for the *cis*/*trans* pairs of compounds **24**–**28**. The *cis* analogues are 5–15 times more potent for the nNOS isoform as well as for NET compared to their *trans* counterparts. For example, *cis*-**26** exhibited an IC_{50} value of 0.39 μM for nNOS, while the corresponding trans isomer was 5-fold less potent. Similar difference in activity was observed for NET, with IC_{50} value of 0.4 μM for *cis*-**26** and IC_{50} value of 2.0 μM for *trans*-**26**. The *cis* analogues also showed better selectivity for nNOS over eNOS. This is consistent with our previous observations where the *cis* isomers were found to be more potent and selective for the nNOS over eNOS isoform,[30] and the conformational difference between these stereoisomers appears to be just as important on NET activity. This provides additional support for the suggested pharmacophore model, where a bulky or cyclic side chain with a basic amine is necessary to obtain the submicromolar potency for nNOS[33] and selectivity over eNOS isoform and to achieve potency for NET. Also, translocation of the basic amine out of the cyclic ring can make the functional

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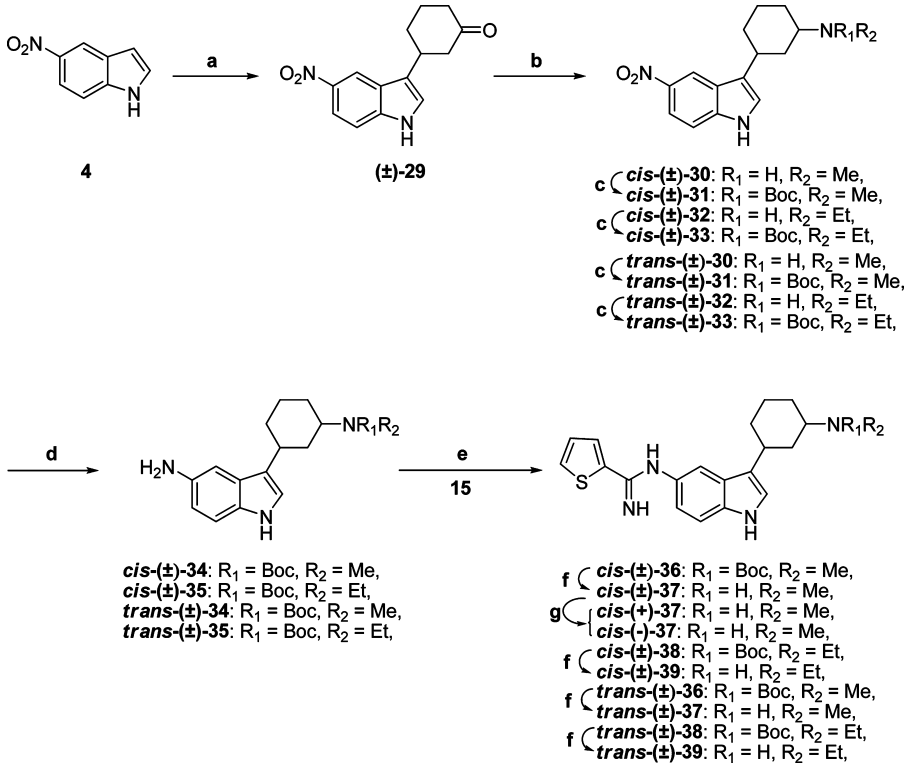
Scheme 2. 3,5-Disubstituted Indoles with 1,3-Disubstituted Cyclohexane Rings



Reagents and Conditions: (a) cyclohex-2-enone, $Bi(NO_3)_3$, MeCN, rt; (b) amine, AcOH, $NaBH(OAC)_3$, 1,2-DCE; (c) $(Boc)_2O$, Et_3N, 1,4-dioxane; (d) hydrazine hydrate, Raney nickel, MeOH, reflux; (e) **15**, EtOH, rt; (f) TFA, CH_2Cl_2, rt; (g) chiral HPLC

group less rigid and orient its position to the active site of the NOS enzyme.

In the 1,3-disubstituted cyclohexane series, the *cis* and *trans* isomers did not show drastic differences in potency for nNOS and NET, however the selectivity over eNOS was better for the *cis* isomers. In contrast, the NET potency of the *trans* isomers increased over their *cis* analogues (ex. *cis*-(±)-**37** IC_{50} = 2.4 μM vs *trans*-(±)-**37** IC_{50} = 0.88 μM). With separated enantiomers *cis*-(+)-**37** and *cis*-(−)-**37** and comparison to their corresponding racemate, *cis*-(+)-**37** demonstrated an increase in potency over its antipode for both nNOS and NET (2- and 5-fold, respectively) and better selectivity over eNOS.

It is interesting to note that the size of the alkyl group on the basic amine did not play a major role in the potency and selectivity of these compounds. For example, *cis*-**26** and *cis*-(±)-**37** (R_2 = Me), *cis*-**28** and *cis*-(±)-**39** (R_2 = Et) all had comparable potencies for nNOS (IC_{50} = 0.38, 0.49, 0.54, and 0.73 μM, respectively) and comparable selectivity over eNOS (IC_{50} = 25.4, 77.6, 19.0, and 19.2 μM, respectively). Similarly, comparing secondary versus tertiary amines the overall selectivity for nNOS and potency over eNOS also remained unchanged as in *cis*-**24** (R_1 = R_2 = Me) and *cis*-**26** (R_2 = Me, R_1 = H) (IC_{50} = 0.34 and 0.39 μM for nNOS and 62.8 and 35.2 μM for eNOS, respectively).

Several compounds *cis*-**26**, *cis*-**28**, *cis*-(+)-**37**, and *cis*-(±)-**39** exhibited the expected in vitro profile and were subject to further investigation. Compounds *cis*-**26** and *cis*-(+)-**37** demonstrated low hERG inhibition ($IC_{50} \geq 30$ μM), which suggests a low potential for QTc prolongation.[34] However *cis*-**28** and *cis*-(±)-**39** were more selective at hERG with IC_{50} of 23 and 16.3 μM (Table 2).

The selected compounds also showed a low potential for drug−drug interaction with low cytochrome P450 (CYP450) inhibition against multiple isozymes (Table 2). This rules out the inhibitory activity with CYP450 enzymes, where compounds that bind to the heme iron (for example, imidazole-containing compounds)[35] have shown to be potent inhibitors of CYP450.

Because *cis*-(+)-**37** exhibited excellent potency for nNOS and NET and excellent selectivity over eNOS, the compound was considered for further evaluation in a variety of in vitro and in vivo assays. In addition to the low hERG inhibition and low potential for drug−drug interaction, *cis*-(+)-**37** also possess drug-like molecular properties such as polar surface area (PSA) less than 70[36] and cLogP less than 4, which are expected to be beneficial to the PK and the in vivo efficacy of this drug candidate.[37,38]

Mediated Vasoconstriction Effect on Isolated Human Resistance Arteries. To investigate potentially undesirable cardiovascular effects associated with the inhibition of eNOS, *cis*-(+)-**37** was assessed for the contractile response (inhibition of acetylcholine-mediated vasorelaxation) on isolated human resistance arteries. Acetylcholine (Ach), an endothelium and nitric oxide dependent vasodilator, slows the heart rate when functioning as an inhibitory neurotransmitter by inhibiting contraction of cardiac muscle fibers.[39,40] The test was performed in the absence and presence of L-arginine (substrate for eNOS) with positive (L-NMMA) and negative (vehicle) controls (Figure 3). The arteries were preconstricted with U46619, a thromboxane A2 (TxA2) mimetic agent, and then exposed to acetylcholine. The response to ACh provides information on the activity of eNOS in an active human biological tissue and thereby any inhibitory effect of *cis*-(+)-**37**

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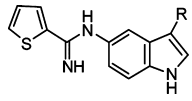
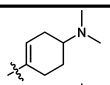
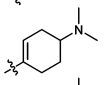
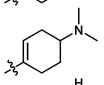
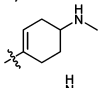
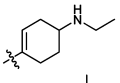
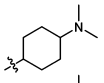
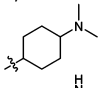
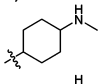
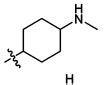
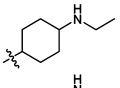
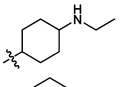
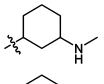
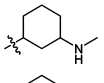
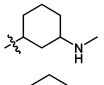
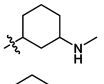
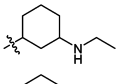
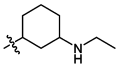
Table 1. Human NOS and NET Inhibitory Activity by 3,5-Disubstituted Indole Derivatives



Compound	R	Human NOS, IC_{50} (μM)[a]			Selectivity		Human NET binding, IC_{50} (μM)[b]
		nNOS	eNOS	iNOS	eNOS/nNOS	iNOS/nNOS	
(±)-16		3.0 (2.03-4.45)	51.4 (38.60-68.46)	24 (19.16-31.67)	17	8.0	0.55
(-)-16		0.86 (0.44-1.68)	47.1 (27.84-79.58)	NT[d]	54	NC[e]	1.1
(+)-16		0.84 (0.32-2.18)	96 (80.9-113.9)	NT[d]	114	NC[e]	0.96
(±)-18		1.73 (1.24-2.41)	32 (19.83-51.71)	NT[d]	19	NC[e]	1.3
(±)-20		1.42 (0.95-2.12)	40.3 (27.16-59.97)	NT[d]	28	NC[e]	0.28
cis-(24)		0.35 (0.22-0.55)	17.5 (7.74-39.69)	26.5 (11.46-62.30)	51	77	0.16
trans-(24)		4.52 (2.12-9.62)	284 (184.8-437.6)	149 (96.54-228.6)	63	33	2.2
cis-(26)		0.4 (2.07-7.20)	25.4 (41.23-118.0)	36.3 (18.33-72.06)	64	91	0.4
trans-(26)		1.8 (3.98-7.64)	6.24 (127.6-320.7)	142 (74.66-269.5)	3.4	77	2.0
cis-(28)		0.54 (0.23-0.58)	19.0 (10.49-26.09)	493 (335.7-731.5)	35	913	0.38
trans-(28)		7.52 (3.70-8.14)	216 (51.15-108.7)	155 (99.73-241.2)	29	21	2.8
cis-(±)-37		0.49 (0.33-0.75)	77.6 (26.35-228.6)	54.1 (34.57-84.59)	158	110	2.4
cis-(+)-37		0.56 (0.39-0.83)	49.3 (3.47-696.7)	6.76 (3.87-11.80)	88	12	1.0
cis-(-)-37		1.37 (0.71-2.66)	75 (45.96-122.0)	70 (27.52-177.5)	55	51	5.2
trans-(±)-37		0.30 (0.20-0.47)	7.76 (4.26-14.15)	14.5 (10.69-19.59)	26	48	0.88
cis-(±)-39		0.73 (0.38-1.41)	32 (15.78-64.06)	19.2 (35.32-77.67)	44	26	1.7
trans-(±)-39		0.2 (0.19-0.37)	11 (4.85-24.04)	54.2 (14.54-25.47)	42	208	0.76
L-NMMA[c]		0.95 (0.63-1.4)	0.65 (0.45-0.94)	1.8 (0.47-6.7)	0.7	2.0	-
Protriptyline		-	-	-	-	-	0.0017

Values reported in parentheses are 95% confidence intervals. [a]In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline. [b]Inhibition of [^{3}H] nisoxetine binding to CHO cells. Protriptyline was used as a reference compound (K_i = 2.0 ± 0.5 nM). Each NET value is within accepted limits of the historic average ±0.5 log units. [c]L-NMMA is a known nonselective NOS inhibitor; tested for comparison. [d]NT not tested. [e]Not calculable

on eNOS. Furthermore, if inhibition of relaxation is due to the inhibition of eNOS, the addition of L-arginine (substrate for eNOS) would recover the relaxation due to ACh. As seen in Figure 3a, no significant inhibition with *cis*-(+)-**37** on the ability of the blood vessels to respond to ACh was observed at 100 μM concentration. Also, the addition of L-arginine did not significantly affect the response to ACh in the presence of the compound, providing further evidence that *cis*-(+)-**37** does not

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Table 2. Inhibition of Human Cytochrome P450 Enzymes and hERG Inhibition Activity with Selected 3,5-Disubstituted Indole Derivatives

		CYP450 (IC_{50} μM)					
compd	hERG (IC_{50} μM)	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4	CYP3A4
cis-**26**	39	>100	>100	26.4	3.48	~57.3	>100
cis-**28**	23	>100	~21.5	23.4	0.927	23.1	>100
cis-(+)-**37**	33	n/a[a]	n/a[a]	>100	5.14	~100	n/a[a]
cis-(±)-**39**	16.3	NT[b]	NT	NT	NT	NT	NT

[a]n/a: no inhibition observed over the concentration range tested. [b]NT: not tested.

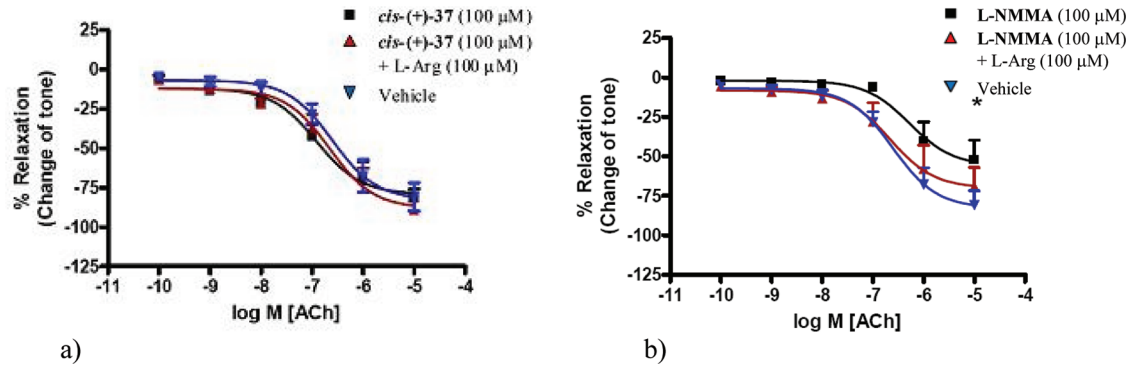

Figure 2. (a) Effect of *cis*-(+)-**37** on the responses to ACh in absence and presence of L-arginine. (b) Effect of L-NMMA on the responses to ACh in absence and presence of L-arginine.

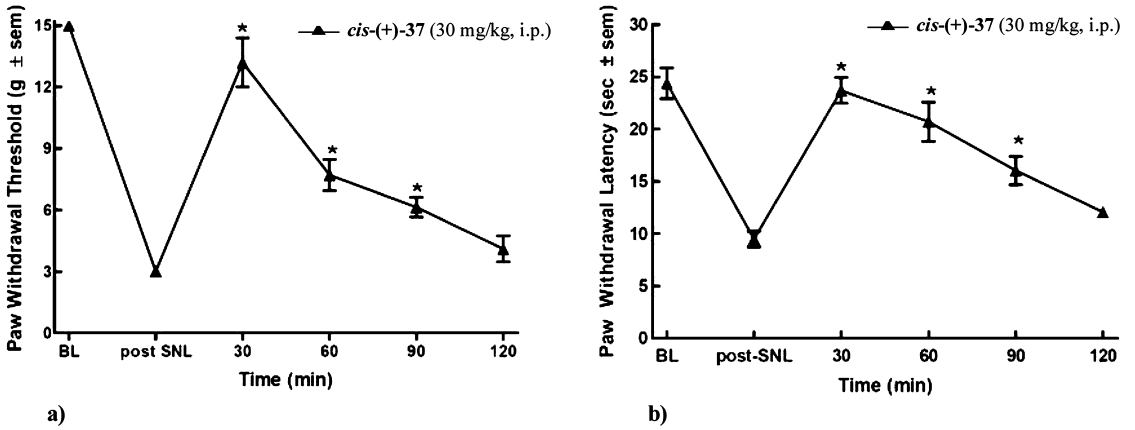

Figure 3. Neuropathic pain model. (a) Antiallodynic effects of *cis*-(+)-**37** in the L5/L6 SNL SD rats. (b) Antihyperalgesia effects of *cis*-(+)-**37** in the L5/L6 SNL SD rats.

have an effect on agonist-mediated activation of human eNOS. The in vitro data of *cis*-(+)-**37** is therefore consistent with the weak inhibitory potency in human eNOS with IC_{50} of 49.3 μM. On the other hand, L-NMMA, a nonselective NOS inhibitor, causes vasoconstriction at 100 μM concentration, and this effect is reversed with the addition of L-arginine (100 μM) (Figure 2b).

Chung or Spinal Nerve Ligation (SNL) Model Studies with *cis*-(+)-37. The activity of *cis*-(+)-**37** was evaluated in a rat spinal nerve ligation (SNL) model of neuropathic pain. Animal models of disease serve as a useful tool in assessing efficacy in a disease state as it relates to drug potency and PK properties of the molecule. The Chung model of neuropathic pain is an animal model that involves spinal nerve ligation and the development of mechanical or tactile allodynia and thermal hyperalgesia in the paws of the animal. This model is widely used for various investigative studies on pain mechanisms and for assessing the potential of a drug analgesic effectiveness. Allodynia is pain due to a stimulus that does not normally provoke pain and are distinct from analgesia, which is defined as reduction in the intensity of pain that occurs in response to a normally painful stimulus, and hyperalgesia is defined as an increased response to a stimulus that is normally painful. Allodynia and hyperalgesia can be assessed by measuring the paw withdrawal times from a thermal stimulus on the paws of the animals (hyperalgesia) or by application of a mechanical force using a calibrated filament applied to the paws (allodynia).

In this model,[41] surgery is performed to tightly ligate the L5 and L6 spinal nerves on one side of the rat. This produces mechanical allodynia and thermal hyperalgesia of the affected foot. Withdrawal threshold to application of mechanical force and withdrawal latency to application of radiant heat to the foot was tested. Intraperitoneal administration of *cis*-(+)-**37** at 30

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mg/kg resulted in both reversal of allodynia and thermal hyperalgesia. Maximum reversal effect of 90% for allodynia and 96% for hyperalgesia was observed at 30 min. (Figure 3). The results clearly show that *cis*-(+)-**37** is effective at reversing both hyperalgesia and allodynia, suggesting it may be efficacious in treating neuropathic pain. This is also a strong evidence for the dual action activity of *cis*-(+)-**37** in comparison to our previous published pure nNOS inhibitors, where only reversal of thermal hyperalgesia was observed.[27,30]

Encouraged by the later results, the metabolic profile of *cis*-(+)-**37** was investigated and its stability tested in liver hepatocytes of several species (human, primate, dog, and rat). No turnover was observed over 120 min, indicating a great metabolic stability of this compound.

High Throughput Profile of *cis*-(+)-37. The high throughput profile is useful tool for identifying off-target activities of compounds with potential drawbacks associated with the drug development process. This drawback can include the interaction of the lead candidate with a specific protein or a biological pathway and thus contribute to undesirable side effects once administered in an individual. This process is also a rapid and cost-effective way of prioritizing the most promising compound such as *cis*-(+)-**37** for further evaluation in various preclinical toxicology studies during the selection process. To identify off-target activities, *cis*-(+)-**37** was tested in several validated in vitro pharmacological assays at a single concentration of 10 μM (40 nonpeptidic receptors, 30 peptidic receptors, two nuclear receptors, five ion channels, and three transporters).[30] The compound was found to be selective for most of the targets tested (<50% inhibition at 68 out of 80 receptors) with weak inhibition at: opioid receptors μ (53%) and sigma receptor (σ, 61%). The compound showed strong inhibition at: human muscarinic M_1 (80%), M_2 (87%), M_3 (88%), M_4 (93%), and M_5 (87%). Overall *cis*-(+)-**37** possesses an excellent safety profile.

CONCLUSION

Novel 3,5-disubstituted indole derivatives with various 1,3- and 1,4-disubstituted cyclohexane side chains were synthesized and shown to be selective nNOS inhibitors with NERI activity. Further, with respect to potential cardiovascular effects, excellent selectivity for nNOS over eNOS was also achieved. In terms of potency, the *cis*-isomers were found to be more potent for nNOS and selective over eNOS compared to the *trans*-isomers. In the case of 1,4-disubstitution, *cis*-isomers were found to have better NERI activity than the *trans*-isomer, whereas the reverse was seen in the 1,3-disubstituted cyclohexane rings.

As seen in the broad chemical series, the lead compound, *cis*-(+)-**37**, showed lack of any direct vasoconstriction or inhibition of ACh-mediated vasorelaxation in isolated human coronary arteries, suggesting that a cardiovascular effect associated with the inhibition of human eNOS would be greatly diminished. *cis*-(+)-**37** does not have any off-target activities (80 receptors), suggesting that clinically effective concentrations would engender a minimal side effect profile. The compound was active in the neuropathic pain model. Intraperitoneal administration of *cis*-(+)-**37** was shown to reverse the thermal hyperalgesia and mechanical allodynia in the SNL model, indicating the potential of *cis*-(+)-**37** to treat neuropathic pain. Overall, the chemistry, safety pharmacology, and animal efficacy data support the continued advancement of *cis*-(+)-**37** as a novel pain therapeutic.

EXPERIMENTAL SECTION

General. All the reactions were performed under an atmosphere of argon and stirred magnetically unless otherwise noted. Commercial reagents and anhydrous solvents were used as received without further purification. Reactions were monitored by analytical TLC using precoated silica gel aluminum plates (0.2 mm, 60 Å) and were visualized with UV light or stained appropriately. Flash column chromatography was performed using Silicycle Siliaflash F60 (40−63 μm) silica gel. The ^{1}H NMR spectra were performed on a Bruker 300 and 600 MHz (for 2D data) spectrometers. Low and high resolution mass spectra were performed on an applied Biosystems/MDS Sciex QstarXL hybrid quadrupole/TOF instrument using electrospray ionization. The chemical purity of all final compounds was determined by Agilent 1100 series HPLC system using reverse phase column, and the purity was determined to be >95% for all final compounds, unless otherwise indicated (see Supporting Information for complete analytical methods and retention times). No attempts were made to optimize the yields.

*4-(5-Nitro-1H-indol-3-yl)cyclohex-3-enone (**6**).* (a) A solution of **4** (3.0 g, 18.50 mmol) in dry MeOH (50 mL) was treated with KOH (5.6 g, 100 mmol) at rt. After stirring for 10 min, 1,4-cyclohexanedione monoethylene acetal (7.22 g, 46.25 mmol) was added and the resulting solution was refluxed for 36 h. The reaction was brought to rt, and solvent was evaporated. Crude was diluted with water (50 mL), and precipitated solid was filtered off and washed with water (2 × 10 mL). The precipitate was dried under vacuum to obtain 5-nitro-3-(1,4-dioxaspiro[4.5]dec-7-en-8-yl)-1*H*-indole (4.7 g, 85%) as a solid. ^{1}H NMR ($CDCl_3$) δ 8.78 (d, 1H, J = 2.1 Hz), 8.36 (brs, 1H), 8.05 (dd, 1H, J = 2.1, 9.0 Hz), 7.32 (d, 1H, J = 8.7 Hz), 7.22 (d, 1H, J = 2.4 Hz), 6.12 (t, 1H, J = 3.9 Hz), 2.66−2.49 (m, 2H), 4.00−3.96 (m, 4H), 2.49 (brs, 2H), 1.91 (t, 2H, J = 6.6 Hz). ESI-MS (m/z, %) 301 (MH^+, 100).

(b) A solution of 5-nitro-3-(1,4-dioxaspiro[4.5]dec-7-en-8-yl)-1*H*-indole (4.7 g, 15.65 mmol) in acetone (50 mL) was treated with 10% aq HCl (50 mL) at rt and stirred overnight (14 h). Acetone was evaporated, and crude was basified using 10% aq NH_4OH solution (100 mL). The precipitate was filtered off and washed with 10% NH_4OH solution (2 × 10 mL) and water (2 × 10 mL). The product was dried under vacuum to obtain compound **6** (4.0 g, quantitative) as a solid. ^{1}H NMR ($CDCl_3$) δ, 6.24 (t, 1H, J = 3.6 Hz), 7.57 (d, 1H, J = 9.0 Hz), 7.76 (d, 1H, J = 2.1 Hz), 8.03 (dd, 1H, J = 2.1, 9.0 Hz), 8.71 (d, 1H, J = 2.1 Hz), 3.19−3.18 (m, 2H), 2.97−2.88 (m, 2H), 2.71 (t, J = 6.9 Hz, 2H). ESI-MS (m/z, %) 279 (M + Na, 36), 257 (MH^+, 100).

*N,N-Dimethyl-4-(5-nitro-1H-indol-3-yl)cyclohex-3-enamine ((±)-**7**).* A solution of compound **6** (1.0 g, 3.90 mmol) in dry 1,2-dichloroethane (10 mL) was treated with *N*,*N*-dimethyl amine hydrochloride (0.31 g, 3.902 mmol), AcOH (0.22 mL, 3.90 mmol), and $NaBH(OAc)_3$ (1.24 g, 5.85 mmol) at rt, and the resulting mixture was stirred overnight (14 h). The reaction was diluted with 1 N NaOH (30 mL), and product was extracted into ethyl acetate (2 × 50 mL). The combined ethyl acetate layer was washed with brine (20 mL) and dried (Na_2SO_4). Solvent was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-7 (0.73 g, 66%) as a brown solid. ^{1}H NMR (DMSO-d_6) δ 11.82 (s, 1H), 8.67 (d, 1H, J = 2.1 Hz), 8.00 (dd, 1H, J = 2.1, 9.0 Hz), 7.62 (s, 1H), 7.54 (d, 1H, J = 9.0 Hz), 6.15 (t, 1H, J = 1.5 Hz), 2.62−2.39 (m, 4H), 2.23−2.12 (m, 7H), 2.06−1.98 (m, 1H), 1.57−1.43 (m, 1H). ESI-MS (m/z, %) 286 (MH^+, 100).

*N-Methyl-4-(5-nitro-1H-indol-3-yl)cyclohex-3-enamine ((±)-**8**).* A solution of compound **6** (1.44 g, 5.62 mmol) in 1,2-dichloroethane (20 mL) was treated with AcOH (0.31 mL, 5.62 mmol), methylamine hydrochloride (0.37 g, 5.62 mmol), and $NaBH(OAC)_3$ (1.78 g, 8.43 mmol) at rt and stirred overnight (14 h). Same workup as in (±)-7. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-**8** (0.82 g, 54%) as a solid. ^{1}H NMR (DMSO-d_6) δ 11.85 (brs, 1H), 8.67 (s, 1H), 8.00 (d, 1H, J = 7.5 Hz), 7.63 (s, 1H), 7.54 (d, 1H, J = 9.0 Hz), 6.13 (brs, 1H), 2.70−2.60 (m, 1H), 2.57−2.40 (m, 3H), 2.35 (s, 3H), 2.01−1.97 (m, 2H), 1.53−1.44 (m, 1H). ESI-MS (m/z, %) 272 (MH^+, 100).

*tert-Butyl Methyl(4-(5-nitro-1H-indol-3-yl)cyclohex-3-enyl)-carbamate ((±)-**9**).* A solution of compound (±)-**8** (0.8 g, 2.95

mmol) in dry 1,4- dioxane (20 mL) was treated with Et_3N (0.82 mL, 5.90 mmol) followed by $(Boc)_2O$ (0.67 g, 3.10 mmol) at rt, and the resulting solution was stirred overnight (16 h). Solvent was evaporated, and crude was purified by column chromatography (EtOAc:hexanes 1:1) to obtain compound (±)-**9** (1.0 g, 91%) as a solid. ^{1}H NMR (DMSO-d_6) δ 11.87 (s, 1H), 8.68 (d, 1H, J = 2.1 Hz), 8.01 (dd, 1H, J = 2.4, 9.0 Hz), 7.66 (s, 1H), 7.55 (d, 1H, J = 9.0 Hz), 6.17 (brs, 1H), 4.16−4.10 (m, 1H), 2.74 (s, 3H), 2.70−2.60 (m, 2H), 2.45−2.29 (m, 2H), 1.87−1.81 (m, 2H), 1.42 (s, 9H). ESI-MS (m/z, %) 394 (M.Na$^+$, 100), 316 (44), 272 (82).

*N-Ethyl-4-(5-nitro-1H-indol-3-yl)cyclohex-3-enamine ((±)-**10**).* A solution of compound **6** (1.0 g, 3.902 mmol) in dry 1,2-dichloroethane (10 mL) was treated with ethyl amine hydrochloride (0.31 g, 3.902 mmol), AcOH (0.22 mL, 3.902 mmol), and $NaBH(OAc)_3$ (1.24 g, 5.853 mmol) at room temperature, and the resulting mixture was stirred for overnight (14 h). Same workup as in (±)-**7**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-**10** (1.08 g, 97%) as a dark-yellow solid. ^{1}H NMR (DMSO-d_6) δ 11.83 (brs, 1H), 8.67 (d, 1H, J = 2.4 Hz), 8.00 (dd, 1H, J = 2.4, 9.0 Hz), 7.62 (s, 1H), 7.54 (d, 1H, J = 9.0 Hz), 6.13 (s, 1H), 4.07 (brs, 1H), 3.16 (s, 2H), 2.80−2.40 (m, 3H), 2.00−1.94 (m, 2H), 1.52 −1.39 (m, 2H), 1.03 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 286 (MH$^+$, 100).

*tert-Butyl Ethyl(4-(5-nitro-1H-indol-3-yl)cyclohex-3-enyl)-carbamate ((±)-**11**).* A solution of compound (±)-**10** (1.05 g, 3.68 mmol) in dry 1,4-dioxane (20 mL) was treated with Et_3N (1.02 mL, 7.36 mmol) followed by $(Boc)_2O$ (0.84 g, 3.863 mmol) at rt, and the resulting solution was stirred for overnight (14 h). Solvent was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:1) to obtain compound (±)-**11** (1.1 g, 78%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 11.85 (s, 1H), 8.67 (d, 1H, J = 2.1 Hz), 8.01 (dd, 1H, J = 2.1, 8.7 Hz), 7.64 (s, 1H), 7.55 (d, 1H, J = 9.0 Hz), 6.16 (s, 1H), 4.05 (brs, 1H), 3.18−3.14 (m, 2H), 2.62−2.56 (m, 2H), 2.43 −2.27 (m, 2H), 1.96−1.83 (m, 2H), 1.42 (s, 9H), 1.09 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 408 (M + Na, 95), 386 (MH$^+$, 9), 330 (73), 286 (100).

*3-(4-(Dimethylamino)cyclohex-1-enyl)-1H-indol-5-amine ((±)-**12**).* A solution of compound (±)-**7** (0.21 g, 0.74 mmol) in dry MeOH (5 mL) was treated with Raney nickel (0.05 g) followed by hydrazine hydrate (0.22 mL, 7.36 mmol) at rt. The reaction was placed in a preheated oil bath and refluxed for 5 min. The reaction brought to room temperature, filtered through a Celite bed, and washed with methanol (2 × 10 mL). The solvent was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-**12** (0.185 g, quantitative) as a foam. ^{1}H NMR (DMSO-d_6) δ 10.55 (s, 1H), 7.13 (d, 1H, J = 2.4 Hz), 7.04 (d, 1H, J = 8.7 Hz), 6.99 (d, 1H, J = 0.9 Hz), 6.47 (dd, 1H, J = 1.8, 8.4 Hz), 5.99 (brs, 1H), 4.47 (s, 2H), 2.57−2.08 (m, 11H), 2.02−1.97 (m, 1H), 1.52−1.40 (m, 1H). ESI-MS (m/z, %) 256 (MH$^+$, 100), 211 (41).

*tert-Butyl 4-(5-Amino-1H-indol-3-yl)cyclohex-3-enyl(methyl)-carbamate ((±)-**13**).* A solution of compound (±)-**9** (0.5 g, 1.35 mmol) in dry MeOH (20 mL) was treated with hydrazine hydrate (0.41 mL, 13.46 mmol) followed by Raney nickel (0.1 g), and the resulting mixture was refluxed for 30 min. Same workup as in (±)-**12**. The crude was purified by column chromatography (EtOAc:hexanes 1:1) to obtain compound (±)-**13** (0.43 g, 94%) as a foam. ^{1}H NMR (DMSO-d_6) δ 10.60 (s, 1H), 7.16 (d, 1H, J = 2.7 Hz), 7.05 (d, 1H, J = 8.4 Hz), 6.99 (d, 1H, J = 1.5 Hz), 6.48 (dd, 1H, J = 1.8, 8.2 Hz), 6.00 (brs, 1H), 4.49 (s, 2H), 4.15−4.05 (m, 1H), 2.73 (s, 3H), 2.42−2.14 (m, 2H), 1.86−1.76 (m, 2H), 1.41−1.38 (m, 11H). ESI-MS (m/z, %) 364 (MNa$^+$, 7), 342 (MH$^+$, 11), 286 (100).

*tert-Butyl 4-(5-Amino-1H-indol-3-yl)cyclohex-3-enyl(ethyl)-carbamate ((±)-**14**).* A solution of compound (±)-**11** (0.5 g, 1.30 mmol) in dry MeOH (10 mL) was treated with Raney nickel (0.05 g) followed by hydrazine hydrate (0.4 mL, 12.971 mmol) at rt. The reaction was placed in a preheated oil bath and refluxed for 5 min. Same workup as in (±)-**12**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 5:95) to obtain compound (±)-**14** (0.46 g, quantitative) as a foam. ^{1}H NMR (DMSO-d_6) δ 10.58 (s, 1H), 7.15 (d, 1H, J = 2.7 Hz), 7.04 (d, 1H, J = 8.4 Hz), 6.99 (d, 1H, J = 1.5 Hz), 6.48 (dd, 1H, J = 2.1, 8.5 Hz), 6.00−5.98 (m, 1H), 4.48 (s, 2H), 4.06 (brs, 1H), 3.18−3.12 (m, 2H), 2.60−2.20 (m, 4H), 1.91- 1.80 (m, 2H), 1.41 (s, 9H), 1.08 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 356 (MH$^+$, 10), 300 (100).

*N-(3-(4-(Dimethylamino)cyclohex-1-enyl)-1H-indol-5-yl)-thiophene-2-carboximidamide ((±)-**16**).* A solution of compound (±)-**12** (0.18 g, 0.70 mmol) in dry EtOH (10 mL) was treated with compound **15** (0.4 g, 1.41 mmol) at rt and stirred for 24 h. Solvent was evaporated, and crude was diluted with satd $NaHCO_3$ solution (20 mL), and product was extracted into CH_2Cl_2 (2 × 25 mL). The combined CH_2Cl_2 layer was washed with brine (20 mL) and dried (Na_2SO_4). Solvent was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-**16** (0.24 g, 90%) as a solid. ^{1}H NMR (DMSO-d_6) δ 10.88 (s, 1H), 7.71 (d, 1H, J = 2.7 Hz), 7.58 (d, 1H, J = 4.5 Hz), 7.31−7.28 (m, 2H), 7.20 (s, 1H), 7.09 (t, 1H, J = 4.2 Hz), 6.65 (dd, 1H, J = 1.2, 8.4 Hz), 6.21 (brs, 2H), 6.03 (s, 1H), 2.60−2.31 (m, 3H), 2.22−2.08 (m, 8H), 2.02−1.97 (m, 1H), 1.53−1.42 (m, 1H). ESI-MS (m/z, %) 365 (MH$^+$, 39), 320 (38), 183 (76), 160 (100). ESI-HRMS calcd for $C_{21}H_{25}N_4S$ (MH$^+$), 365.1813; obsd 365.1794.

The enantiomeric mixture was separated by using preparative chiral HPLC column chromatography to obtain (−)-**16** and (+)-**16**.

*First Enantiomer (−)-**16**).* ESI-MS (m/z, %) 365 (MH$^+$, 35), 320 (43), 160 (82), 119 (100). ESI-HRMS calcd for $C_{21}H_{25}N_4S$ (MH$^+$), 365.1794; obsd, 365.1794. Chiral purity: 98.87%. Chemical purity: 95.3%.

*Second Enantimer (+)-**16**).* ESI-MS (m/z, %) 365 (MH$^+$, 34), 320 (46), 160 (89), 119 (100). ESI-HRMS calcd for $C_{21}H_{25}N_4S$ (MH$^+$), 365.1794; obsd, 365.1795. Chiral purity: 99.38%. Chemical purity: 95%.

*tert-Butyl Methyl(4-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohex-3-enyl)carbamate ((±)-**17**).* A solution of compound (±)-**13** (0.42 g, 1.22 mmol) in dry EtOH (20 mL) was treated with compound **15** (0.69 g, 2.43 mmol) at room temperature, and the resulting solution was stirred for 24 h. Same workup as in (±)-**16**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 5:95) to obtain compound (±)-**17** (0.37 g, 68%) as foam. ^{1}H NMR (DMSO-d_6) δ 10.94 (s, 1H), 7.72 (d, 1H, J = 3.3 Hz), 7.60 (d, 1H, J = 4.8 Hz), 7.32−7.25 (m, 2H), 7.22 (s, 1H), 7.10 (t, 1H, J = 4.2 Hz), 6.66 (d, 1H, J = 8.4 Hz), 6.28 (brs, 1H), 6.06 (s, 1H), 4.16−4.06 (m, 1H), 2.72 (s, 3H), 2.40−2.22 (m, 2H), 1.87−1.77 (m, 2H), 1.40 (s, 9H), 1.26−1.20 (m, 1H), 0.85 (t, 1H, J = 7.2 Hz). ESI-MS (m/z, %) 451 (MH$^+$, 100).

*N-(3-(4-(Methylamino)cyclohex-1-enyl)-1H-indol-5-yl)thiophene-2-carboximidamide ((±)-**18**).* A solution of compound (±)-**17** (0.35 g, 0.78 mmol) was treated with 20% TFA in CH_2Cl_2 (20 mL) at 0 °C, and stirring was continued for 1 h at same temperature. Solvent was evaporated, crude was diluted with 10% aq NH_3 (15 mL), and product was extracted into CH_2Cl_2 (3 × 20 mL). The combined CH_2Cl_2 layer was washed with brine (10 mL) and dried (Na_2SO_4). Solvent was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-**18** (0.2 g, 74%) as a solid. ^{1}H NMR (DMSO-d_6) δ 10.87 (s, 1H), 7.71 (d, 1H, J = 3.3 Hz), 7.59 (d, 1H, J = 4.2 Hz), 7.31−7.28 (m, 2H), 7.20 (s, 1H), 7.09 (dd, 1H, J = 4.2, 4.9 Hz), 6.65 (dd, 1H, J = 1.5, 8.2 Hz), 6.19 (brs, 2H), 6.01 (s, 1H), 2.61−2.57 (m, 1H), 2.46−2.40 (m, 1H), 2.33 (s, 3H), 1.96−1.88 (m, 3H), 1.47−1.39 (m, 2H). ESI-MS (m/z, %) 351 (MH$^+$, 66), 320 (54), 160 (63), 119 (100). ESI-HRMS calcd for $C_{20}H_{23}N_4S$ (MH$^+$), 351.1654; obsd, 351.1637. HPLC purity 84.6%.

*tert-Butyl Ethyl(4-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohex-3-enyl)carbamate ((±)-**19**).* A solution of compound (±)-**14** (0.44 g, 1.24 mmol) in dry EtOH (20 mL) was treated with compound **15** (0.7 g, 2.48 mmol) at rt and stirred for 24 h. Same workup as in (±)-**16**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 5:95) to obtain compound (±)-**19** (0.49 g, 85%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 10.92 (s, 1H), 7.72−7.70 (m, 1H), 7.59 (d, 1H, J = 5.1 Hz), 7.36−7.27 (m, 2H), 7.20 (s, 1H), 7.09 (t, 1H, J = 4.2 Hz), 6.66 (d, 1H, J = 7.8 Hz), 6.22 (s, 2H), 6.04 (brs, 1H), 4.02 (brs, 1H), 3.17−3.13 (m,

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2H), 2.58 −2.26 (m, 4H), 1.92−1.82 (m, 2H), 1.41 (s, 9H), 1.07 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 465 (MH^+, 100).

*N-(3-(4-(Ethylamino)cyclohex-1-enyl)-1H-indol-5-yl)thiophene-2-carboximidamide ((±)-**20**).* A solution of compound (±)-**19** (0.3 g, 0.645 mmol) was treated with 20% TFA in CH_2Cl_2 (20 mL) at 0 °C, and stirring was continued for 2 h at same temperature. Same workup as in (±)-**18**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound (±)-**20** (0.125 g, 53%) as a solid. ^{1}H NMR (DMSO-d_6) δ 10.87 (s, 1H), 7.70 (d, 1H, J = 2.7 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.31−7.26 (m, 2H), 7.19 (s, 1H), 7.09 (dd, 1H, J = 3.9, 4.9 Hz), 6.64 (d, 1H, J = 8.4 Hz), 6.19 (s, 2H), 6.01 (s, 1H), 2.74−2.40 (m, 5H), 1.96 −1.88 (m, 2H), 1.48−1.37 (m, 2H), 1.02 (t, 3H, J = 7.2 Hz). ESI-MS (m/z, %) 365 (MH^+, 22), 320 (44), 160 (66), 127 (41), 119 (100). ESI-HRMS calcd for $C_{21}H_{25}N_4S$ (MH^+), 365.1794; obsd, 365.1811. HPLC purity 94.2%.

*tert-Butyl 4-(5-Amino-1H-indol-3-yl)cyclohex-3-enyl(methyl)-carbamate (**22**).* A solution of compound (±)-**9** (0.5 g, 1.36 mmol) in 2 M NH_3 in MeOH (20 mL) was treated with Pd−C (0.05 g) and flushed with hydrogen gas. The reaction was stirred at rt for overnight (16 h) under hydrogen atm (balloon pressure). The solution was filtered using Celite bed and washed with CH_2Cl_2:MeOH (1:1, 3 × 20 mL). The solvent was evaporated, and crude was purified by column chromatography (EtOAc:hexanes 1:1) to obtain compound **22** (0.46 g, quantitative) as a solid in 1:2 ratio of diastereomers. ^{1}H NMR (DMSO-d_6) δ 10.28, 10.23 (2s, 1H), 6.87−6.85, 7.06−6.99 (2 m, 2H), 6.68−6.66 (m, 1H), 6.50−6.42 (m, 1H), 4.41 (brs, 2H), 3.85−3.82 (m, 1H), 2.72−2.60 (2s, 3H), 2.57−2.53 (m, 1H), 2.17−2.02 (m, 2H), 1.84−1.46 (m, 6H), 1.41, 1.38 (2s, 9H). ESI-MS (m/z, %) 366 (M + Na^+, 8), 344 (MH^+, 10), 288 (100).

*tert-Butyl 4-(5-Amino-1H-indol-3-yl)cyclohexyl(ethyl)carbamate (**23**).* A solution of compound (±)-**11** (0.55 g, 1.43 mmol) in 2 M NH_3 in MeOH (10 mL) was treated with Pd−C (0.05 g) and flushed with hydrogen gas. The reaction was stirred at rt for overnight (16 h) under hydrogen atm (balloon pressure). Same workup as in **22**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 2.5:97.5) to obtain compound **23** (0.43 g, 84%) as a solid in 2:3 ratio of diastereomers. ^{1}H NMR (DMSO-d_6) δ 10.27, 10.23 (2s, 1H), 6.88−6.86, 7.06−6.99 (2 m, 2H), 6.70−6.66 (m, 1H), 6.47−6.44 (m, 1H), 4.52 (brs, 2H), 3.80−3.68 (m, 1H), 3.17−3.11 (m, 2H), 3.04−2.98 (m, 1H), 2.18−2.01 (m, 2H), 1.78−1.63 (m, 4H), 1.51−1.37 (m, 11H), 1.07, 0.99 (2t, 3H, J = 7.2, 6.6 Hz). ESI-MS (m/z, %) 380 (M + Na, 6), 358 (MH^+, 5), 302 (100), 258 (54). ESI-HRMS calcd for $C_{21}H_{32}N_3O_2$ (MH^+), 358.2507; obsd, 358.2489.

*N-(3-(4-(Dimethylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (**24**).* A solution of compound (±)-7 (0.43 g, 1.51 mmol) in dry EtOH (5 mL) was treated with Pd−C (0.04 g) and purged with hydrogen gas at rt. The reaction was stirred at same temperature under hydrogen atm (balloon pressure) for overnight (14 h). The reaction was filtered using Celite bed and washed with dry EtOH (2 × 5 mL). The combined EtOH layer was treated with compound **15** (0.85 g, 3.01 mmol) at room temperature and stirred for 24 h. Solvent was evaporated, crude was diluted with satd $NaHCO_3$ solution (20 mL), and product was extracted into CH_2Cl_2 (2 × 25 mL). The combined CH_2Cl_2 layer was washed with brine (20 mL) and dried (Na_2SO_4). Solvent was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain compound **24** (0.4 g, 72%, over two steps) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 10.57 (s, 1H), 7.71 (d, 1H, J = 3.3 Hz), 7.60 (d, 1H, J = 5.4 Hz), 7.27 (d, 1H, J = 8.4 Hz), 7.10 (t, 1H, J = 4.2 Hz), 7.05−6.99 (m, 2H), 6.64 (dd, 1H, J = 1.5, 8.4 Hz), 6.48 (brs, 1H), 2.96−2.91 (m, 1H), 2.71−2.64 (m, 1H), 2.34 (s, 3H), 2.23 (s, 3H), 2.08−2.05 (m, 1H), 1.94−1.82 (m, 3H), 1.72−1.66 (m, 1H), 1.60−1.39 (m, 3H). ESI-MS (m/z, %) 367 (MH^+, 31), 322 (18), 184 (100). ESI-HRMS calcd for $C_{21}H_{27}N_4S$ (MH^+), 367.1965; obsd, 367.1950.

*Compound **24** Was Separated by Preparative SFC. cis-**24**.* ^{1}H NMR ($CDCl_3$) δ 8.29 (brs, 1H), 7.45−743 (m, 2H), 7.33 (d, 1H, J = 12.6 Hz), 7.26 (s, 1H), 7.10 (dd, 1H, J = 5.8, 7.3 Hz), 7.06 (d, 1H, J = 2.8 Hz), 6.88 (dd, 1H, J = 2.4, 12.6 Hz), 4.88 (brs, 1H), 3.11−3.06 (m, 1H), 2.31 (s, 6H), 2.19−2.16 (m, 1H), 2.07−1.98 (m, 2H), 1.89−1.81 (m, 4H), 1.71−1.64 (m, 2H). ESI-MS (m/z, %) 367 (MH^+, 100) 322 (70). ESI-HRMS (MH^+) calcd for $C_{21}H_{27}N_4S$, 367.1950; found, 367.1940. HPLC purity 99.3%.

*trans-**24**.* ^{1}H NMR ($CDCl_3$) δ 8.00 (brs, 1H), 7.46−7.43 (m, 2H), 7.34 (d, 1H, J = 12.7 Hz), 7.28−7.26 (m, 1H), 7.11 (dd, 1H, J = 5.8, 7.3 Hz), 6.96 (d, 1H, J = 3.3 Hz), 6.89 (dd, 1H, J = 2.3, 12.7 Hz), 4.88 (brs, 1H), 2.79−2.72 (m, 1H), 2.35 (s, 6H), 2.33−2.27 (m, 1H), 2.22−2.19 (m, 2H), 2.05−2.02 (m, 2H), 1.60−1.58 (m, 2H), 1.48−1.38 (m, 2H). ESI-MS (m/z, %) 367 (MH^+, 100) 322 (75). ESI-HRMS (MH^+) calcd for $C_{21}H_{27}N_4S$, 367.1950; found, 367.1951. HPLC purity 95.3%.

*tert-Butyl Methyl(4-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohexyl)carbamate (**25**).* A solution of compound **22** (0.44 g, 1.28 mmol) in dry EtOH (20 mL) was treated with compound **15** (0.73 g, 2.56 mmol) at rt and stirred for 24 h. Same workup as in (±)-**16**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 5:95) to obtain compound **25** (0.425 g, 73%) as a foam in 1:2 ratio of diastereomers. ^{1}H NMR (DMSO-d_6) δ 10.63, 10.59 (2s, 1H), 7.71 (d, 1H, J = 3.6 Hz), 7.59 (d, 1H, J = 5.1 Hz), 7.29−7.22 (m, 1H), 7.11−6.95 (m, 3H), 6.66−6.62 (m, 1H), 6.27 (brs, 1H), 3.90−3.80 (m, 1H), 2.70−2.62 (m, 4H), 2.18−2.06 (m, 2H), 1.82−1.64 (m, 4H), 1.56−1.38 (m, 11H). ESI-MS (m/z, %) 453 (MH^+, 100).

*N-(3-(4-(Methylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (**26**).* Compound **25** (0.2 g, 0.44 mmol) was treated with 1 N HCl solution at room temperature, and the resulting solution was refluxed for 2 h. The reaction was brought to room temperature, filtered, and washed with water (5 mL). The solvent was evaporated, and crude was recrystallized from ethanol/ether to obtain compound **26** (0.18 g, 94%) as HCl salt in 1:2 ratio of diastereomers.

Compound **26** was separated (as a free base) using normal phase HPLC semipreparative column chromatography.

*cis-**26** (Less Polar Isomer).* ^{1}H NMR (DMSO-d_6) δ 10.52 (s, 1H), 7.70 (d, 1H, J = 2.7 Hz), 7.58 (d, 1H, J = 5.4 Hz), 7.25 (d, 1H, J = 8.4 Hz), 7.09 (dd, 1H, J = 3.9, 5.1 Hz), 6.96−7.00 (m, 2H), 6.61 (d, 1H, J = 8.4 Hz), 6.18 (brs, 2H), 2.80−2.73 (m, 1H), 2.64−2.59 (m, 1H), 2.27 (s, 3H), 1.96−1.53 (m, 6H), 1.13−1.08 (m, 1H), 1.01−0.94 (m, 1H), 0.91−0.81 (m, 1H). ESI-MS (m/z, %) 353 (MH^+ for free base, 30) 322 (100), 119 (51). ESI-HRMS calcd for $C_{20}H_{25}N_4S$ (MH^+ for free base), 353.1794; obsd, 353.1777. HPLC purity 97%.

*trans-**26** (More Polar Isomer).* ^{1}H NMR (DMSO-d_6) δ 10.54 (s, 1H), 7.70 (d, 1H, J = 2.7 Hz), 7.58 (d, 1H, J = 5.4 Hz), 7.25 (d, 1H, J = 8.4 Hz), 7.09 (t, 1H, J = 4.5 Hz), 6.99−6.95 (m, 2H), 6.61 (dd, 1H, J = 1.2, 8.2 Hz), 6.18 (brs, 2H), 2.71−2.61 (m, 1H), 2.35−2.24 (m, 4H), 2.02−1.90 (m, 3H), 1.52−1.40 (m, 1H), 1.28−1.08 (m, 3H), 1.01−0.94 (m, 1H), 0.91−0.81 (m, 1H). ESI-MS (m/z, %) 353 (MH^+ for free base, 28) 322 (100), 119 (47). ESI-HRMS calcd for $C_{20}H_{25}N_4S$ (MH^+ for free base), 353.1794; obsd, 353.1799. HPLC purity 92%.

*tert-Butyl Ethyl(4-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohexyl)carbamate (**27**).* A solution of compound **23** (0.4 g, 1.12 mmol) in dry EtOH (20 mL) was treated with compound **15** (0.63 g, 2.24 mmol) at rt and stirred for 24 h. Same workup as in (±)-**16**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 5:95) to obtain compound **27** (0.4 g, 60%) as a yellow solid in 2:3 ratio of diastereomers. ^{1}H NMR (DMSO-d_6) δ 10.62, 10.59 (2s, 1H), 7.72−7.70 (m, 1H), 1.08−0.98 (m, 3H), 7.60 (d, 1H, J = 5.1 Hz), 7.30−7.22 (m, 2H), 7.11−7.09 (m, 1H), 7.01−6.96 (m, 1H), 6.67−6.62 (m, 1H), 6.31 (brs, 2H), 3.76−3.70 (m, 1H), 3.17 −3. 02 (m, 3H), 2.18−2.05 (m, 2H), 1.85−1.68 (m, 4H), 1.56 −1.38 (m, 11H). ESI-MS (m/z, %) 467 (MH^+, 100).

*N-(3-(4-(Ethylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (**28**).* Compound **27** (0.26 g, 0.557 mmol) was treated with 1 N aqueous HCl solution at rt, and the resulting solution was refluxed for 2 h. Same procedure as for compound **26**. The crude was recrystallized from ethanol/ether to obtain compound **28** (0.23 g, 94%) as a solid in 2:3 ratio of diastereomers. Compound **28** was separated (as a free base) using reverse phase semipreparative column on HPLC.

*cis-**28***. ^{1}H NMR (CD_3OD) δ 7.61 (dd, 1H, J = 1.2, 3.9 Hz), 7.54 (d, 1H, J = 5.4 Hz), 7.34 (d, 1H, J = 8.4 Hz), 7.16 (d, 1H, J = 1.5 Hz), 7.12 (d, 1H, J = 3.9 Hz), 7.10 (brs, 1H), 6.78 (dd, 1H, J = 1.8, 8.4 Hz), 2.79−2.74 (m, 1H), 3.06−3.00 (m, 1H), 1.98−1.81 (m, 4H), 2.65 (q, 2H), 1.75−1.71 (m, 4H), 1.13 (t, 3H, J = 7.5 Hz). ESI-MS (m/z, %) 367 (MH^+ for free base, 18), 322 (100), 184 (19), 119 (39). ESI-HRMS calcd for $C_{21}H_{27}N_4S$ (MH^+, free base), 367.1959; obsd, 367.1950. HPLC purity 93.5%.

*trans-**28***. ^{1}H NMR (CD_3OD) δ 7.62 (d, 1H, J = 3.6 Hz), 7.54 (d, 1H, J = 5.4 Hz), 7.34 (d, 1H, J = 8.4 Hz), 7.15 (d, 1H, J = 1.2 Hz), 7.12 (t, 1H, J = 4.2 Hz), 6.98 (s, 1H), 6.77 (dd, 1H, J = 1.5, 8.5 Hz), 2.81−2.64 (m, 3H), 2.59−2.52 (m, 1H), 2.10 (t, 4H, J = 13.8 Hz), 1.63−1.51 (m, 2H), 1.38−1.28 (m, 2H), 1.13 (t, 3H, J = 7.2 Hz). ESI-MS (m/z, %) 367 (MH^+ for free base, 18), 322 (100), 184 (19), 119 (39). ESI-HRMS calcd for $C_{21}H_{27}N_4S$ (MH^+, free base), 367.1959; obsd, 367.1950. HPLC purity 98.5%.

*3-(5-Nitro-1H-indol-3-yl)cyclohexanone ((±)-**29**)*. To a solution of **4** (4.00 g, 25.61 mmol) in dry MeCN (5.00 mL) was added cyclohex-2-enone (7.40 mL, 76.83 mmol) and $Bi(NO_3)_3$ (0.12 g, 0.26 mmol) and the mixture stirred overnight at rt The solvent then was evaporated, and the crude was purified by column chromatography (hexane:EtOAc 1:1) to obtain compound (±)-**29** (2.70 g, 41%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 8.59 (d, 1H, J = 2.1 Hz), 8.51 (s, 1H, NH), 8.12 (dd, 1H, J = 2.1, 9.0 Hz), 7.41 (d, 1H, J = 9.0 Hz), 7.15 (d, 1H, J = 2.1 Hz), 3.56 −3.47 (m, 1H), 2.85−2.77 (m, 1H), 2.65 (t, 1H, J = 9.9 Hz), 2.55−2.37 (m, 2H), 2.34−2.26 (m, 1H), 2.09−1.81 (m, 3H). EI-MS (m/z, %) 258 (M^+, 100).

*N-Methyl-3-(5-nitro-1H-indol-3-yl)cyclohexanamine ((±)-**30**)*. To a solution of (±)-**29** (1.20 g, 4.65 mmol) in 1,2-dichloroethane (50 mL) was added AcOH (0.28 mL, 4.65 mmol), $MeNH_2$·HCl (0.38 g, 4.65 mmol), and $NaBH(OAc)_3$ (1.50 g, 7.00 mmol) and the mixture left to stir overnight at rt. Same workup as in (±)-7. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain two diastereomers as yellow solids.

*trans-(±)-**30** (Less Polar Diastereomer): (0.58 g, 46%)*. ^{1}H NMR ($CDCl_3$) δ 8.63 (d, 1H, J = 2.1 Hz), 8.44 (s, 1H, NH), 8.09 (dd, 1H, J = 2.1, 9.0 Hz), 7.36 (d, 1H, J = 9.0 Hz), 7.12 (s, 1H), 3.37−3.26 (m, 1H), 2.97−2.87 (m, 1H), 2.41 (s, 3H), 2.08−2.04 (m, 2H), 1.88−1.69 (m, 3H), 1.65−1.49 (m, 3H). EI-MS (m/z, %) 273 (M^+, 10), 242 (100).

*cis-(±)-**30** (More Polar Diastereomer): (0.21 g, 16%)*. ^{1}H NMR ($CDCl_3$) δ 8.93 (s, 1H, NH), 8.54 (d, 1H, J = 2.4 Hz), 8.06 (dd, 1H, J = 2.1, 9.0 Hz), 7.35 (d, 1H, J = 9.0 Hz), 7.06 (s, 1H), 2.93−2.75 (m, 2H), 2.56 (s, 3H), 2.44−2.33 (m, 1H), 2.17−2.13 (m, 1H), 2.08−2.01 (m, 1H), 1.99−1.85 (m, 1H), 1.57−1.45 (m, 2H), 1.38−1.26 (m, 2H). EI-MS (m/z, %) 273 (M^+, 30), 230 (100).

The stereochemistry of both diastereomers was determined using COSY and NOESY spectroscopic techniques (see supporting info).

*tert-Butyl Methyl(3-(5-nitro-1H-indol-3-yl)cyclohexyl)carbamate (trans-(±)-**31**)*. To a solution of *trans*-(±)-**30** (0.55 g, 2.0 mmol) in 1,4-dioxane (10 mL) was added $(Boc)_2O$ (0.48 g, 2.21 mmol) and triethyl amine (0.56 mL, 4.10 mmol) and the resulting mixture left to stir overnight at rt. The solvent was evaporated and the crude purified on column chromatography (hexane:EtOAc 1:1) to give *trans*-(±)-**31** as a yellow solid (0.73 g, quantitative). ^{1}H NMR ($CDCl_3$) δ 8.57 (d, 1H, J = 2.1 Hz), 8.50 (s, 1H, NH), 8.08 (dd, 1H, J = 2.1, 9.0 Hz), 7.35 (d, 1H, J = 9.0 Hz), 7.26 (s, 1H), 4.52−4.35 (m, 1H), 3.63−3.57 (m, 1H), 2.78 (s, 3H), 2.18−2.09 (m, 2H), 1.98−1.86 (m, 1H), 1.81−1.64 (m, 3H), 1.57−1.49 (m, 2H), 1.43 (s, 9H). EI-MS (m/z, %) 299 (M^+, 100).

*tert-Butyl Methyl(3-(5-nitro-1H-indol-3-yl)cyclohexyl)carbamate (cis-(±)-**31**)*. Same procedure as for *trans*-(±)-**31**. (0.40 g, 73%). ^{1}H NMR ($CDCl_3$) δ8.61 (d, 1H, J = 2.1 Hz), 8.37 (s, 1H, NH), 8.10 (dd, 1H, J = 2.1, 9.0 Hz), 7.38 (d, 1H, J = 9.0 Hz), 7.11 (d, 1H, J = 1.8 Hz), 4.27−3.96 (m, 1H), 3.06−2.95 (m, 1H), 2.78 (s, 3H), 2.10−2.03 (m, 2H), 2.00−1.92 (m, 1H), 1.86−1.78 (m, 1H), 1.69−1.57 (m, 3H), 1.49 (s, 9H), 1.44−1.34 (m, 1H). EI-MS (m/z, %), 373 (M^+, 20), 242 (100).

*N-Ethyl-3-(5-nitro-1H-indol-3-yl)cyclohexanamine ((±)-**32**)*. To a solution of (±)-**29** (1.20 g, 4.65 mmol) in 1,2-dichloroethane (50 mL) was added AcOH (0.28 mL, 4.65 mmol), $EtNH_2$·HCl (0.38 g, 4.65 mmol), and $NaBH(OAc)_3$ (1.50 g, 7.00 mmol) and the mixture left to stir overnight at rt. Same workup as in (±)-7. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain two diastereomers as yellow solids.

*trans-(±)-**32** (Less Polar Diastereomer): (0.70 g, 52%)*. ^{1}H NMR ($CDCl_3$) δ 8.64 (d, 1H, J = 2.1 Hz), 8.34 (s, 1H, NH), 8.09 (dd, 1H, J = 2.1, 9.0 Hz), 7.37 (d, 1H, J = 9.0 Hz), 7.12 (d, 1H, J = 2.1 Hz), 3.42−3.24 (m, 1H), 3.06−3.01 (m, 1H), 2.70 (q, 2H, J = 7.2, 7.2 Hz), 2.07−2.01 (m, 2H), 1.82−1.74 (m, 2H), 1.70−1.55 (m, 4H), 1.17 (t, 3H, J = 8.4 Hz). EI-MS (m/z, %) 287 (M^+, 10), 242 (100).

*cis-(±)-**32** (More Polar Diastereomer): (0.21 g, 16%)*. ^{1}H NMR ($CDCl_3$) δ 8.61 (d, 1H, J = 2.1 Hz), 8.37 (s, 1H, NH), 8.10 (dd, 1H, J = 2.1, 9.0 Hz), 7.37 (d, 1H, J = 9.0 Hz), 7.10 (d, 1H, J = 1.8 Hz), 3.00−2.89 (m, 1H), 2.75 (q, 2H, J = 7.2 Hz), 2.32−2.28 (m, 1H), 2.11−2.04 (m, 2H), 1.97−1.84 (m, 1H), 1.63−1.47 (m, 2H), 1.44−1.29 (m, 3H), 1.14 (t, 3H, J = 7.1 Hz). EI-MS (m/z, %) 287 (M^+, 15), 244 (100).

The stereochemistry of both diastereomers was determined using COSY and NOESY spectroscopic techniques (see Supporting Information).

*tert-Butyl Ethyl(3-(5-nitro-1H-indol-3-yl)cyclohexyl)carbamate (trans-(±)-**33**)*. To a solution of *trans*-(±)-**32** (0.67 g, 2.36 mmol) in 1,4-dioxane (10 mL) was added $(Boc)_2O$ (0.57 g, 2.60 mmol) and triethyl amine (0.66 mL, 4.74 mmol) and the resulting mixture left to stir overnight at rt. The solvent was evaporated and the crude purified on column chromatography (EtOAc:hexane 1:1) to obtain *trans*-(±)-**33** as a yellow solid (0.72 g, 78%). ^{1}H NMR ($CDCl_3$) δ 8.57 (d, 1H, J = 2.1 Hz), 8.08 (dd, 1H, J = 2.1, 9.0 Hz), 7.63 (s, 1H, NH), 7.35 (d, 1H, J = 9.0 Hz), 7.26 (s, 1H), 3.61−3.57 (m, 1H), 3.28−3.07 (m, 2H), 2.17−2.07 (m, 2H), 1.96−1.86 (m, 1H), 1.79−1.62 (m, 3H), 1.49−1.45 (s, m, 9H, 3H), 1.14 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 410 (NaM^+, 50), 288 (100).

*tert-Butyl Ethyl(3-(5-nitro-1H-indol-3-yl)cyclohexyl)carbamate (cis-(±)-**33**)*. Same procedure was used as for *trans*-(±)-**33** (0.26 g, 97%). ^{1}H NMR (DMSO-d6) δ 8.55 (d, 1H, J = 2.1 Hz), 7.97 (dd, 1H, J = 2.1, 9.0 Hz), 7.50 (d, 1H, J = 9.0 Hz), 7.39 (s, 1H), 3.14 (q, 2H, J = 6.9 Hz), 3.04−2.96 (m, 1H), 1.95−1.86 (m, 2H), 1.75−1.64 (m, 2H), 1.57−1.51 (m, 2H), 1.42 (s, 9H), 1.49−1.23 (m, 2H), 1.04 (t, 3H, J = 6.9 Hz). EI-MS (m/z, %) 387 (M^+, 20), 270 (100).

*tert-Butyl 3-(5-Amino-1H-indol-3-yl)cyclohexyl(methyl)-carbamate (trans-(±)-**34**)*. To a solution of *trans*-(±)-**31** (0.70, g 1.87 mmol) in dry MeOH (15 mL) was added Raney nickel (0.1 g 50% as a slurry in water) and hydrazine hydrate (1.00 mL, 18.70 mmol). The resulting mixture was immersed in a preheated oil bath and refluxed for 15 min or until the solution became clear. Same workup as in (±)-**12**. The crude was purified on column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 2:98) to give *trans*-(±)-**34** as a light-brown solid (0.60 g, 92%). ^{1}H NMR ($CDCl_3$) δ 7.76 (s, 1H, NH), 7.28 (s, 1H), 7.16 (d, 1H, J = 8.4 Hz), 6.89 (d, 1H, J = 2.1 Hz), 6.64 (dd, 1H, J = 2.1, 8.4 Hz), 4.51−4.36 (m, 1H), 3.50 (s, 2H, NH), 2.76 (s, 3H), 2.16−2.05 (m, 2H), 1.88 (ddd, 1H, J = 5.4, 12.4, 25.1 Hz), 1.72−1.46 (m, 6H), 1.42 (s, 9H). EI-MS (m/z, %) 343 (M^+, 70), 212 (100).

*tert-Butyl 3-(5-Amino-1H-indol-3-yl)cyclohexyl(methyl)-carbamate (cis-(±)-**34**)*. Same procedure as for *trans*-(±)-**34** (0.34 g, 97%). ^{1}H NMR ($CDCl_3$) δ 7.72 (s, 1H, NH), 7.15 (d, 1H, J = 8.4 Hz), 6.95 (s, 1H), 6.88 (d, 1H, J = 2.4 Hz), 6.65 (dd, 1H, J = 2.1, 8.4 Hz), 4.26−4.13 (m, 1H), 3.52 (s, 2H, NH), 2.93−2.84 (m, 1H), 2.74 (s, 3H), 2.11−2.03 (m, 2H), 1.96−1.89 (m, 1H), 1.80−1.75 (m, 1H), 1.66−1.31 (m, 4H), 1.48 (s, 9H). EI-MS (m/z, %), 343 (M^+, 100).

*tert-Butyl 3-(5-Amino-1H-indol-3-yl)cyclohexyl(ethyl)carbamate (trans-(±)-**35**)*. To a solution of *trans*-(±)-**33** (0.70, g 1.81 mmol) in dry MeOH (15 mL) was added Raney nickel (0.1 g 50% as a slurry in water) and hydrazine hydrate (0.90 mL, 18.10 mmol). The resulting mixture was immersed in a preheated oil bath and refluxed for 15 min or until the solution became clear. Same workup as for (±)-**12** . The crude was purified on column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 2:98) to give *trans*-(±)-**35** as a brownish solid (0.64 g, quantitative). ^{1}H NMR ($CDCl_3$) δ 7.82 (s, 1H, NH), 7.33 (s, 1H),

7.26 (s, 1H), 7.15 (d, 1H, J = 8.4 Hz), 6.89 (d, 1H, J = 2.1 Hz), 6.64 (dd, 1H, J = 2.1, 8.4 Hz), 4.43 (s, 1H), 3.56−3.43 (m, 1H), 3.24−3.06 (m, 2H), 2.17−2.07 (m, 2H), 1.92−1.82 (m, 1H), 1.79−1.71 (m 1H), 1.69−1.53 (m, 3H), 1.45 (s, 9H), 1.12 (t, 3H, J = 6.8 Hz). EI-MS (m/z, %) 357 (M^+, 70), 212 (100).

*tert-Butyl 3-(5-Amino-1H-indol-3-yl)cyclohexyl(ethyl)carbamate (cis-(±)-**35**).* Same procedure as for *trans*-(±)-**35** (0.21 g, 96%). ^{1}H NMR ($CDCl_3$) δ7.725 (s, 1H), 7.15 (d, 1H, J = 8.7 Hz), 6.96 (s, 1H), 6.87 (d, 1H, J = 2.1 Hz), 6.65 (dd, 1H, J = 2.1, 8.7 Hz), 4.19−4.12 (m, 1H), 3.22−3.05 (m, 2H), 2.90−2.80 (m, 1H), 2.15−2.11 (m, 1H), 2.04−1.98 (m, 1H), 1.94−1.90 (m, 1H), 1.83−1.80 (m, 1H), 1.48 (s, 9H), 1.66−1.30 (m, 3H), 1.09 (t, 3H, J = 6.9 Hz). EI-MS (m/z, %) 357 (M^+, 100).

*tert-Butyl Methyl(3-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohexyl)carbamate (trans-(±)-**36**).* To a solution of *trans*-(±)-**34** (0.57 g, 1.66 mmol) in dry EtOH (25 mL) was added **15** (0.75 g, 3.32 mmol) and the reaction left to stir at rt for 48 h. Same workup as for (±)-**16**. The crude was purified on column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 2:98 then 5:95) to give *trans*-(±)-**36** as a yellow solid (0.62 g, 81%). ^{1}H NMR (DMSO-d_6) δ 10.68 (s, 1H, NH), 7.70 (d, 1H, J = 3.6 Hz), 7.58 (d, 1H, J = 4.5 Hz), 7.28 (d, 2H, J = 8.4 Hz), 7.09 (dd, 1H, J = 3.6, 5.1 Hz), 6.93 (s, 1H), 6.64 (dd, 1H, J = 1.8, 8.4 Hz), 6.22 (s, 2H, NH), 4.27−4.24 (m, 1H), 3.53−3.40 (m, 1H), 2.69 (s, 3H), 2.04−1.98 (m, 1H), 1.93−1.88 (m, 2H), 1.71−1.42 (m, 5H), 1.35 (s, 9H). ESI-MS (m/z, %) 453 (NaM^+, 100).

*tert-Butyl Methyl(3-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohexyl)carbamate (cis-(±)-**36**).* Same procedure as for *trans*-(±)-**36** (0.32 g, 75%). ^{1}H NMR (DMSO-d_6) δ 10.59 (s, 1H, NH), 7.70 (d, 1H, J = 3.3 Hz), 7.58 (d, 1H, J = 4.8 Hz), 7.26 (d, 1H, J = 8.4 Hz), 7.09 (dd, 1H, J = 3.6, 4.8 Hz), 7.04 (s, 1H), 6.98 (s, 1H), 6.62 (dd, 1H, J = 1.8, 8.4 Hz), 6.20 (s, 2H, NH), 4.09−3.78 (m, 1H), 2.87−2.79 (m, 1H), 2.69 (s, 3H), 2.00−1.84 (m, 5H), 1.68−1.46 (m, 5H), 1.38 (s, 9H). ESI-MS (m/z, %) 453 (NaM^+, 100).

*N-(3-(3-(Methylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (trans-(±)-**37**).* Compound *trans*-(±)-**36** (0.60 g, 0.13 mmol) was treated with 20% TFA solution (31 mL) in dichloromethane at 0 °C and the mixture left to stir for 2 h at 0 °C. The solution then was neutralized with 10% NH_4OH and the organic layer separated and evaporated. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:4) to give *trans*-(±)-**37** as a yellow solid (0.45 g, quantitative). ^{1}H NMR (DMSO-d_6) δ 10.59 (s, 1H, NH), 7.71 (d, 1H, J = 3.3 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.09 (dd, 1H, J = 3.6, 4.8 Hz), 7.02 (d, 2H, J = 10.4 Hz), 6.63 (d, 1H, J = 10.2 Hz), 6.20 (s, br, 2H, NH), 3.51−3.24 (m, 3H), 2.40 (s, 3H), 2.07−1.96 (m, 1H), 1.91−1.83 (m, 1H), 1.77−1.69 (m, 3H), 1.60−1.51 (m, 3H). ESI-MS (m/z, %) 353 (MH^+, 80), 322 (100). ESI-HRMS (MH^+) calcd for $C_{20}H_{25}N_4S$, 353.1794; found, 353.1812. HPLC purity 98.46%.

*N-(3-(3-(Methylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (cis-(±)-**37**).* Same procedure as for *trans*-(±)-**37** (0.22 g, quantitative). ^{1}H NMR (DMSO-d_6) δ, 9.61 (s, 1H), 8.58 (s, 2H, NH), 8.16 (d, 1H, J = 4.5 Hz), 8.12 (d, 1H, J = 3.6 Hz), 7.65 (s, 1H), 7.52 (d, 1H, J = 8.4 Hz), 7.39 (pseudo t, 1H, J = 4.5 Hz), 7.28 (d, 1H, J = 2.1 Hz), 7.10 (d, 1H, J = 8.4 Hz), 3.25−3.08 (m, 1H), 2.94−2.86 (m, 1H), 2.58 (s, 3H), 2.35−2.27 (m, 1H), 2.11−2.08 (m, 1H), 2.01−1.84 (m, 2H), 1.61−1.28 (m, 4H). ESI-MS (m/z, %) 353 (MH^+, 100). ESI-HRMS (MH^+) calcd for $C_{20}H_{25}N_4S$, 353.1794; found, 353.1792.

Compound *cis*-(±)-**37** was separated into its enantiomers on a chiral HPLC.

cis-(+)-**37** (less polar enantiomer). $[\alpha]_D$ = +23.77 (4.50 mg in 2 mL MeOH). ESI-MS (m/z, %) 353 (MH^+, 50), 322 (100). ESI-HRMS calcd for $C_{16}H_{25}N_4O_5$ (MH^+), 353.1819; found, 353.1807. ee 100% by HPLC. HPLC purity 99.2%.

trans-(−)-**37** (more polar enantiomer). $[\alpha]_D$ = −28.64 (4.80 mg in 2 mL MeOH). ESI-MS (m/z, %) 353 (MH^+, 50), 322 (100). ESI-HRMS (MH^+) calcd for $C_{16}H_{25}N_4O_5$, 353.1819; found, 353.1809. ee by 99% HPLC. HPLC purity 99.1%

*tert-Butyl Ethyl(3-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohexyl)carbamate (trans-(±)-**38**).* To a solution of *trans*-(±)-**35** (0.62 g, 1.73 mmol) in dry EtOH (25 mL) was added **15** (1.00 g, 3.47 mmol) and the reaction left to stir at rt for 48 h. Same workup as for (±)-**16**. The crude was purified on column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 2:98 then 5:95) to give *trans*-(±)-**38** as a yellow solid (0.80 g, quantitative). ^{1}H NMR (DMSO-d_6) δ 10.67 (s, 1H, NH), 7.70 (d, 1H, J = 3.9 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.29 (s, 1H), 7.26 (s, 1H), 7.09 (dd, 1H, J = 3.6, 5.1 Hz), 6.92 (s, 1H), 6.64 (dd, 1H, J = 1.8, 8.4 Hz), 6.21 (s, 2H), 4.26−4.19 (m, 1H), 3.53−3.42 (m, 1H), 3.20−3.05 (m, 2H), 2.04−1.84 (m, 3H), 1.68−1.44 (m, 5H), 1.36 (s, 9H), 1.04 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 467 (MH^+, 100).

*tert-Butyl Ethyl(3-(5-(thiophene-2-carboximidamido)-1H-indol-3-yl)cyclohexyl)carbamate (cis-(±)-**38**).* The same procedure as for *trans*-(±)-**38** (0.19 g, 78%). ^{1}H NMR (DMSO-d_6) δ 10.83 (s, 1H, NH), 7.88 (d, 1H, J = 2.1 Hz), 7.84 (d, 1H, J = 3.3 Hz), 7.37 (d, 1H, J = 8.7 Hz), 7.29 (s, 1H), 7.22 (dd, 1H, J = 4.5, 8.7 Hz), 7.13 (s, 1H), 6.83 (d, 1H, J = 8.4 Hz), 4.03−3.89 (m, 1H), 3.13 (q, 2H, J = 6.0 Hz), 2.89−2.77 (m, 1H), 1.94−1.80 (m, 3H), 1.74−1.57 (m, 2H), 1.56−1.46 (m, 3H), 1.39 (s, 9H), 1.04 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 467 (MH^+, 100).

*N-(3-(3-(Ethylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (trans-(±)-**39**).* Compound *trans*-(±)-**38** (0.75 g, 1.61 mmol) was treated with 20% TFA solution (31 mL) in dichloromethane at 0 °C and the mixture left to stir for 2 h at 0 °C. Same workup as for *trans*-(±)-**37**. The crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:4) to give *trans*-(±)-**39** as a yellow solid (0.50 g, 85%). ^{1}H NMR (DMSO-d_6) δ 10.54 (s, 1H, NH), 7.70 (d, 1H, J = 3.0 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.26 (d, 1H, J = 8.4 Hz), 7.09 (dd, 1H, J = 3.9, 5.1 Hz), 7.00−6.98 (m, 2H), 6.62 (d, 1H, J = 8.4 Hz), 6.19 (s, 2H, NH), 3.23−3.08 (m, 1H), 2.99−2.85 (m, 1H), 2.58 (q, 2H, J = 7.2 Hz), 1.97−1.89 (m, 2H), 1.82−1.58 (m, 3H), 1.51−1.44 (m, 3H), 1.05 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 367 (MH^+, 50%), 322 (100). ESI-HRMS (MH^+) calcd for $C_{21}H_{27}N_4S$, 367.1950; found, 367.1956. HPLC purity 91.02%

*N-(3-(3-(Ethylamino)cyclohexyl)-1H-indol-5-yl)thiophene-2-carboximidamide (cis-(±)-**39**).* Same procedure as for *trans*-(±)-**39** (0.50 g, 85%). ^{1}H NMR (DMSO-d_6) δ 10.62 (s, 1H, NH), 7.71 (d, 1H, J = 3.0 Hz), 7.59 (d, 1H, J = 5.1 Hz), 7.28 (d, 1H, J = 8.7 Hz), 7.10 (dd, 1H, J = 3.6, 5.1 Hz), 7.03−7.01 (m, 2H), 6.64 (d, 1H, J = 8.4 Hz), 6.22 (s, 2H, NH), 3.07−2.99 (m, 1H), 2.88−2.82 (m, 3H), 2.37−2.27 (m, 1H), 2.11−1.81 (m, 3H), 1.53−1.21 (m, 4H), 1.11 (t, 3H, J = 6.9 Hz). ESI-MS (m/z, %) 367 (MH^+, 50), 322 (100). ESI-HRMS calcd for $C_{21}H_{27}N_4S$ (MH^+), 367.1950; found, 367.1968. HPLC purity 96.69%

General Procedure for Conversion of the Free Base to the Corresponding Dihydrochloride Salt. A solution of the free base (1.0 equiv) in methanol was treated with 1 M HCl solution in diethyl ether (3.0 equiv) dropwise at room temperature. The resulting mixture was stirred for 10 min and concentrated to dryness. The product was dried under reduced pressure to obtain the dihydrochloride salt as a solid. The chemical purity of the dihydrochloride salts was similar to their corresponding free bases.

NOS Enzyme Assays. Recombinant human nNOS, eNOS, and iNOS were produced in Baculovirus-infected Sf9 cells. In a radiometric method, NOS activity was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. To measure eNOS and nNOS, 10 μL of enzyme was added to 100 μL of 40 mM HEPES, pH = 7.4, containing 2.4 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL BSA, 1 mM EDTA, 1 mM dithiothreitol, 1 μM FMN, 1 μM FAD, 10 μM tetrahydrobiopterin, 1 mM NADPH, and 1.2 μM CaM. To measure iNOS, 10 μL of enzyme was added to 100 μL of 100 mM HEPES, pH = 7.4, containing 1 mM $CaCl_2$, 1 mM EDTA, 1 mM dithiotheitol, 1 μM FMN, 1 μM FAD, 10 μM tetrahydrobiopterin, 120 μM NADPH, and 100 nM CaM.

To measure enzyme inhibition, a 15 μL solution of a test substance was added to the enzyme assay solution, followed by a preincubation time of 15 min at RT. The reaction was initiated by addition of 20 μL of L-arginine containing 0.25 μCi of [^{3}H] arginine/mL and 24 μM L-arginine. The total volume of the reaction mixture was 150 μL in every well. The reactions were carried out at 37 °C for 45 min. The reaction was stopped by adding 20 μL of ice-cold buffer containing 100 mM

HEPES, 3 mM EGTA, 3 mM EDTA, pH = 5.5. [^{3}H]L-citrulline was separated by DOWEX (ion-exchange resin DOWEX 50 W X 8−400, SIGMA), and the DOWEX was removed by spinning at 12000*g* for 10 min in the centrifuge. Then a 70 μL aliquot of the supernatant was added to 100 μL of scintillation fluid, and the radioactivity was counted in a liquid scintillation counter (1450 Microbeta Jet, Wallac). Specific NOS activity is reported as the difference between the activity recovered from the test solution and that observed in a control sample containing 240 mM of the inhibitor L-NMMA. All assays were performed in duplicate.

Efficacy in the Chung Model of Neuropathic Pain. Nerve ligation injury was performed according to the literature procedure. Rats were anesthetized with halothane, and the L_5 and L_6 spinal nerves were exposed, carefully isolated, and tightly ligated with 4−0 silk suture distal to the DRG. After ensuring homeostatic stability, the wounds were sutured and the animals allowed recovery in individual cages. This technique produces signs of neuropathic dysesthesias, including tactile allodynia, thermal hyperalgesia, and guarding of the affected paw, which begins on day 1 of the surgery and peaks on day 16. After a period of recovery following the surgical intervention, rats show enhanced sensitivity to painful and normally nonpainful stimuli.

hERG K^+ Channel Binding Assay. The assay was carried with human recombinant HEK-293 cells using [^{3}H]astemizole as a ligand (2 nM) with incubation at 22 °C for 75 min with reference to astemizole according to the literature procedure. The specific ligand binding to the receptor is defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabeled ligand. The results are expressed as a percent of control specific binding ((measured specific binding/control specific binding) × 100) obtained in the presence of the compounds (i.e., *cis*-(±)-**37**). The IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by nonlinear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting ($Y = D + [(A - D)/(1 + (C/C_{50})^{nH})]$, where Y = specific binding, D = minimum specific binding, A = maximum specific binding, C = compound concentration, C_{50} = IC_{50}, and nH = slope factor).

Contractile Effects on Human Resistance Arteries. Fresh specimens of human resistance arteries were obtained from surgical explant tissue with full informed consent and ethical permission from the donor. All test tissues, having been cut into ring segments of approximately 2 mm length, were attached by 40 μm diameter wire running through the lumen of the vessel to stainless steel heads in 10 mL myograph baths containing Krebs-bicarbonate physiological saline solution (PSS), aerated with 95% O_2 and 5% CO_2, and maintained at a temperature of 37 °C. Changes in tension were recorded using a Danish Myotech isometric transducer. The segments were allowed to equilibrate for at least 30 min and were washed with PSS every 15 min during the equilibration period. Segments were processed through a standardization procedure to reduce signal variability prior to pharmacological intervention. All segments were then exposed to KPSS (62.5 mM) three times to provide a reference means of contractility.

The pharmacology was conducted in the following order.

1. Test tissue was first challenged to provide a measure of maximum contractility.
2. Test tissue was washed with PSS and allowed to return to baseline.
3. Test tissue was then tested for endothelial integrity by precontracting the tissue with thromboxane mimetic U46619 (1×10^{-7} M) and then adding cumulative concentrations of a known endothelium-dependent dilator agonist (ACh; 1×10^{-10} M to 1×10^{-5} M). If the endothelium was intact, ACh produced relaxations.
4. Test tissue was rinsed and allowed to return to baseline.
5. The test article was tested in the presence and absence of L-arginine (10^{-3} M). *cis*-(±)-**37** (±L-arginine) was added at the selected concentration for a period of 50 min. In the presence of *cis*-(±)-**37** (±L-arginine), all vessels were then submaximally vasoconstricted with U46619 prior to CCRCs to ACh (1×10^{-10} M to 1×10^{-5} M). L-NMMA was studied in each artery ring (100 μM) in the presence and absence of L-arginine (10 mM) as positive control.

Responses were expressed as a % of the maximal contractile response to U46619 (as a negative % change for a vasodilatory response). The % relaxation (reversal) of U46619-mediated contractions in response to ACh was plotted as concentration versus response. Direct contractile effects were expressed as a % of the maximum contractile response to KPSS (62.5 mM). Best-fit curves were constructed using nonlinear regression.

NE Transporter (Antagonist Radioligand) Assay.[42] Evaluation of the affinity of compounds for the human norepinephrine transporter in transfected CHO cells determined in a radioligand binding assay.

Experimental protocol: Cell membrane homogenates (20 μg protein) were incubated for 120 min at 4 °C with 1 nM [^{3}H]nisoxetine in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, and 5 mM KCl.

Nonspecific binding was determined in the presence of 1 μM desipramine. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters were dried and then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding.

The standard reference compound was protriptyline, which was tested in each experiment at several concentrations to obtain a competition curve from which its IC50 is calculated.

High Throughput Broad Screen. In each experiment, the respective reference compound was tested concurrently with *cis*-(+)-**37**, and the data were compared with historical values determined. Results showing an inhibition (or stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effect of *cis*-(+)-**37**. The specific ligand binding to the receptor is defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabeled ligand. The results are expressed as a percent of control specific binding ((measured specific binding/control specific binding) × 100) and as a percent inhibition of control specific binding (100 − ((measured specific binding/control specific binding) × 100)) obtained in the presence of the test compound.

■ ASSOCIATED CONTENT

Supporting Information

Analytical and preparative HPLC methods including chiral methods for all final compounds. 1D ^{1}H NMR for *cis*-**24**/*trans*-**24**, *cis*-**26**/*trans*-**26**, and *cis*-**28**/*trans*-**28**. 1D and 2D ^{1}H NMR for *cis*-(±)-**30**, *trans*-(±)-**30**, *cis*-(±)-**32**, and *trans*-(±)-**32**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NE, norepinephrine; NERI, norepinephrine reuptake inhibitor; NET, norepinephrine transporter; CNS, central nervous system; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NMMA, *N*-methyl-L-arginine; SAR, structure−activity relationship; CYP450, cytochrome P450; hERG, human ether-a-go-go related gene; QTc, heartrate-corrected QT interval; SNL, spinal nerve ligation; PSA, polar surface area; PK, pharmacokinetics; ACh, acetylcholine

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