Journal of Medicinal Chemistry

ARTICLE

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Discovery of *N*-(3-(1-Methyl-1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indol-6-yl) thiophene-2-carboximidamide as a Selective Inhibitor of Human Neuronal Nitric Oxide Synthase (nNOS) for the Treatment of Pain

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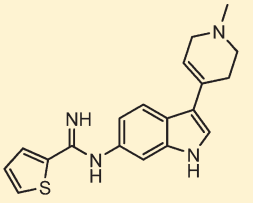
ABSTRACT: 3,6-Disubstituted indole derivatives were designed, synthesized, and evaluated as inhibitors of human nitric oxide synthase (NOS). Bulky amine containing substitution on the 3-position of the indole ring such as an azabicyclic system showed better selectivity over 5- and 6-membered cyclic amine substitutions. Compound (−)-**19** showed the best selectivity for neuronal NOS over endothelial NOS (90-fold) and inducible NOS (309-fold) among the current series. Compounds **16** and (−)-**19** were shown to be either inactive or very weak inhibitors of human cytochrome P450 enzymes, indicating a low potential for drug−drug interactions. Compound **16** was shown to reverse thermal hyperalgesia in vivo in the Chung model of neuropathic pain. Compound **16** was also devoid of any significant vasoconstrictive effect in human coronary arteries, associated with the inhibition of human eNOS. These results suggest that **16** may be a useful tool for evaluating the potential role of selective nNOS inhibitors in the treatment of pain such as migraine and CTTH.



Selective human nNOS inhibitor (**16**)

IC_{50} (μM): nNOS = 0.8, eNOS = 26.5, iNOS = 12.3

INTRODUCTION

Nitric oxide (NO) is one of the most studied cell-signaling molecules, believed to be involved in a number of physiological processes such as inflammation, regulation of blood pressure, opioid tolerance, immunity, and neurotransmission.[1,2] Nitric oxide synthase (NOS) converts L-arginine to L-citrulline and produces NO, utilizing NADPH and O_2 as substrates and flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6*R*)-2-amino-6-[(1*R*,2*S*)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1*H*)-one (BH4), and heme as cofactors.[3,4] NOS consist of three isoforms: neuronal NOS (nNOS or NOS1), a constitutive form thought to play a role in neurotransmission; endothelial NOS (eNOS or NOS3), which is also constitutive and plays a role in regulating vascular tone and platelet aggregation; inducible NOS (iNOS or NOS2), which is generally produced during bacterial infection, tumor cell cytolysis, and inflammation.

Excessive NO or production by individual isoforms such as nNOS and iNOS plays an important role in the development of several disorders including septic shock, stroke, neurodegenerative disorders (Parkinson's, ALS and MS), and pain (migraine, CTTH, visceral, and neuropathic).[5−11] For example, **1**, a nonselective NOS inhibitor, was able to show 67% antimigraine effect compared to placebo in a randomized double-blind clinical study with 15 migraine patients.[12] A similar randomized double-blind, crossover clinical study was conducted on 16 patients with CTTH using **1** (6 mg/kg). Compound **1** significantly reduced the headache pain intensity on the visual analogue scale (2 h after the treatment) when compared to placebo, however the effect was less pronounced when compared to the migraine study.[13] Compounds **2** and **3** are the most extensively studied nonpeptidic NOS inhibitors and provided the foundation for elucidating the pharmacological role of selective nNOS inhibitors in several disorders including nociception, opioid-induced side effects, antidepressant, and anxiolytic properties (Figure 1).[14−16] Compound **4** is also a selective nNOS inhibitor has been shown to provide greater neuroprotection (44%) than **2** (22%) or **3** (8%) in a gerbil model of global cerebral ischemia.[17] The selective inhibition of nNOS or iNOS over the eNOS isoform is necessary in order to avoid the cardiovascular liabilities associated with eNOS.[18,19] Numerous attempts have been made toward the design and synthesis of selective NOS inhibitors targeting both the L-arginine and BH4 binding sites.[20−22] The early NOS inhibitor design, which is based on L-arginine (substrate), produced only nonselective NOS inhibitors. However, subsequent designed inhibitors based on the available crystal structures of NOS enzymes[23] that targeted the arginine binding site or BH4 cofactor site as well as dimerization inhibitors have been shown to be more potent and selective among NOS isoforms.[20−22]

The simplified pharmacophore model adapted by our group for competitively inhibiting NOS at the substrate binding site

Received: August 8, 2011
Published: September 16, 2011

dx.doi.org/10.1021/jm201063u | *J. Med. Chem.* 2011, 54, 7408–7416

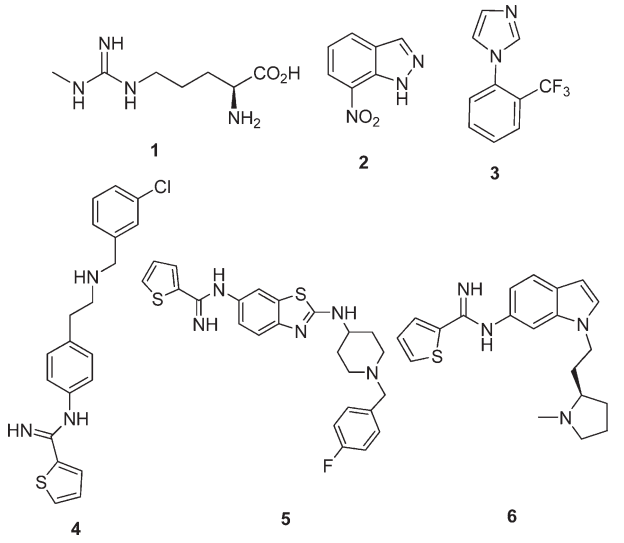
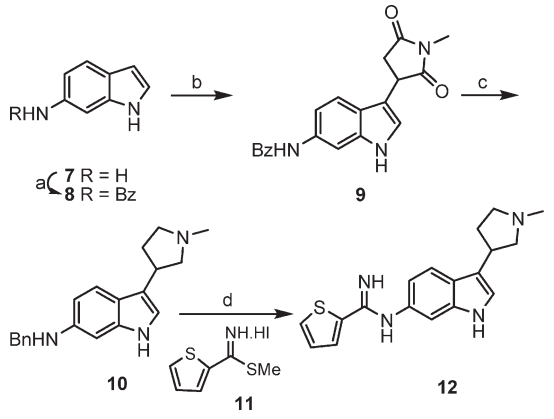


Figure 1. Most studied literature examples (**1**−**3**) and thiophene amidine compounds (**4**−**6**) with basic amine side chain.

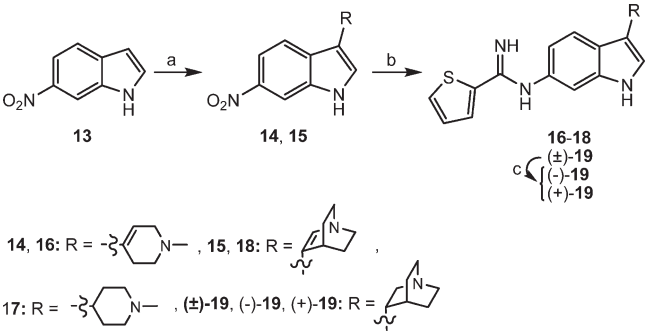
Scheme 1[a]



[a] (a) BzCl, Et_3N, THF, rt; (b) *N*-methylmaleimide, AcOH, reflux; (c) $LiAlH_4$, THF, rt; (d) (i) $Pd(OH)_2$, EtOH, rt, (ii) **11**, EtOH, rt.

contains a guanidine isosteric group and a basic amine group both attached to a central aryl scaffold.[24] The amidine group makes an important bidentate interaction with the conserved glutamic acid residue to achieve the necessary potency, whereas the basic amine (**4**−**6**) is shown to contribute to nNOS isoform selectivity.[24−28] However, this model is somewhat simplified because several other factors are likely important for achieving isoform selectivity.[22,23] Our design strategy is based on an indole core, which has been an important structural component in many pharmaceutical agents and also referred to as "privileged structure" (capable of binding to many receptors).[29] In our previous reports with 2-aminobenzothiazoles (**5**)[24] and disubstituted indole derivatives (**6**),[26,27] we were able to show that the selectivity for nNOS isoform over eNOS and iNOS isoforms can be increased by attaching a bulky or cyclic amine side chain to the central aryl scaffold. On the basis of these results, in our present strategy, selected 5- and 6-membered and bicyclic amine side chains were introduced onto the 3-position of the indole ring while keeping the thiophene amidine group fixed at 6-position.

Scheme 2[a]



[a] (a) *N*-Methyl-4-piperidone, pyrrolidine, EtOH, reflux or quinuclidin-3-one·HCl, KOH, MeOH:H_2O, reflux; (b) (i) H_2N-NH_2·H_2O, Raney-Ni, MeOH, reflux or Pd-C/H_2, EtOH, rt; (ii) **11**, EtOH, rt; (c) SFC chiral chromatographic separation.

As part of our ongoing efforts to find small molecule selective nNOS inhibitors,[24,26−28] herein we report the synthesis and biological activity evaluations of 3,6-disubstituted indole derivatives that led to the identification of **16** as a lead candidate among the series. The results disclosed in this report identify **16** as a candidate to investigate the role of selective nNOS inhibitors for the treatment of neuropathic pain and potentially primary headaches such as migraine and CTTH.

RESULTS AND DISCUSSION

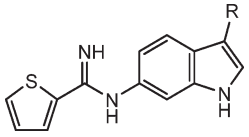
Chemistry. 3,6-Disubstituted indole derivatives were prepared as shown in Schemes 1 and 2. The substitution on 3-position of the indole ring was introduced by a key carbon−carbon bond formation reaction between the protected 6-amino indole **8** and *N*-methylmaleimide under acidic conditions (Scheme 1).[30] Reduction of both the 1-methyl-2,5-dioxopyrrolidine ring and *N*-benzoyl group was carried out with $LiAlH_4$ at room temperature to obtain compound **10**. Deprotection of the benzyl group under hydrogenation conditions followed by coupling with the thiophene-2-carbimidothioate **11**[31] provided the final compound **12**.

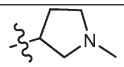
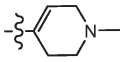
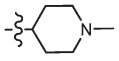
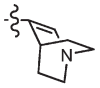
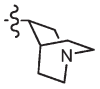
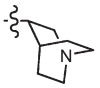
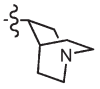
Condensation of 6-nitro-1*H*-indole (**13**) with either *N*-methyl-4-piperidone or quinuclidin-3-one hydrochloride was carried out under basic conditions to obtain the 3-substituted indole derivatives **14** and **15**, respectively (Scheme 2).[32,33] Reduction of the nitro group in **14** was performed under hydrogenation conditions with hydrazine hydrate. A mixture of two intermediate amines (5:2) was observed due to the partial reduction of the 1-methyl-1,2,3,6-tetrahydropyridine ring attributable to the prolonged reaction conditions. The mixture of amines was coupled to the thiophene-2-carbimidothioate **11** to obtain the final compounds **16** and **17** in 50% and 20%, respectively, which were separated by column chromatography on silica gel.

The reduction of nitro group in compound **15** was performed under hydrogenation conditions with hydrazine hydrate to obtain the amine intermediate, which was then coupled to the thiophene-2-carbimidothioate **11** to obtain the final compound **18** (Scheme 2). To avoid the reduction of the 1-azabicyclo-[2.2.2]oct-2-ene ring system, the reaction was heated to reflux for a maximum of 5 min in a preheated oil bath. The consumption of the starting nitro compound **15** was evident by the disappearance of the yellow color of the solution. Reduction of both the nitro

dx.doi.org/10.1021/jm201063u |*J. Med. Chem.* 2011, 54, 7408–7416

Table 1. Inhibition of Human NOS Enzymes by 3,6-Disubstituted Indole Derivatives



Compound	R	Human NOS IC_{50} (μM)[a]			Selectivity	
		nNOS	eNOS	iNOS	eNOS/nNOS	iNOS/nNOS
12		0.2 (0.11-0.34)	7.1 (4.95-10.1)	2.6 (1.8-3.8)	36	13
16		0.8 (0.5-1.4)	26.5 (19.5-35.9)	12.3 (7.3-20.8)	33	15
17		0.12 (0.1-0.2)	10.7 (7.7-14.8)	9.3 (4.3-20.1)	89	78
18		1.5 (1.0-2.4)	46.2 (29.7-71.8)	8.4 (5.2-13.3)	31	6
(±)-19		1.23 (0.76-1.9)	33.3 (12.9-85.1)	18.4 (9.4-35.6)	27	15
(-)-19		0.45 (0.23-0.88)	40.6 (25.9-63.5)	139 (90.6-212.1)	90	309
(+)-19		0.53 (0.28-1.0)	23.3 (17.4-31.1)	52.1 (24.8-109.1)	44	98
1[b]		0.95 (0.63-1.4)	0.65 (0.45-0.94)	1.8 (0.47-6.7)	0.7	2
L-NAME[b]		0.69 (0.53-0.90)	0.68 (0.27-1.72)	0.83 (0.57-1.21)	1	1.2
6[c]		0.32 (0.12-0.7)	16 (6.6-38.2)	72 (19.7-267)	50	225

[a] Values reported in parentheses are 95% confidence intervals. In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline. [b] Compound **1** and L-NAME are known nonselective NOS inhibitors; tested for comparison. [C] Compound **6** is previously reported selective nNOS inhibitor, included for comparison.

group as well as the 1-azabicyclo[2.2.2]oct-2-ene ring system was carried out under standard hydrogenation conditions with palladium on carbon to provide the amine intermediate, which was reacted with thiophene-2-carbimidothioate **11** to obtain the final compound **19** as a mixture of enantiomers (Scheme 2). The racemic mixture **19** was separated using semipreparative chiral HPLC techniques to obtain the pure enantiomers (−)-**19** and (+)-**19**. The absolute stereochemistry of the separated enantiomers was not determined at this time.

Structure−Activity Relationship (SAR) Studies. All compounds were converted into their corresponding dihydrochloride salts to improve their water solubility. Table 1 displays the in vitro activity of 3,6-disubstituted indole derivatives, determined as IC_{50} values against all three human NOS isoforms. Human NOS inhibitory activities were determined by measuring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline using a radiometric assay. The current design strategy of attaching a mono- and bicyclic basic amine at 3-position of the indole ring is adapted from our previous results with 2-aminobenzothiazole and substituted indole derivatives, where bulky and cyclic amine substitutions provided better potency and selectivity for the nNOS isoform.[24,26,27] It was observed that a bulky substitution such as an azabicyclic system in **18**, (±)-**19**, (−)-**19**, and (+)-**19** showed better selectivity over 5- and 6-membered cyclic amine substitution in **12**, **16**, and **17** and is consistent with our previous observation with 2-aminobenzothiazole and substituted indole derivatives.[24,26,27] At the same time, the 6-membered cyclic amine in **17** improved the potency and selectivity relative to 5-membered cyclic amine substitution in **12**. Compound (−)-**19** with an azabicyclic amine showed the best selectivity for human

Table 2. Inhibition of Human Cytochrome P450 Enzymes by 16 and (−)-19

		IC_{50} (μM)	
CYP subtype	substrate	**16**	(−)-**19**
CYP1A2	CEC	>100	NA[a]
CYP2C9	MFC	~72.7	NA[a]
CYP2C19	CEC	40.1	~49.6
CYP2D6	AMMC	~63.2	>100
CYP3A4	BFC	74.8	~100
CYP3A4	BQ	>100	>100

[a] NA: no inhibition observed over the concentration range tested.

nNOS over eNOS (90-fold) and iNOS (309-fold) among the current series. On the basis of their potency and selectivity, compounds **16** and (−)-**19** were selected for further evaluation in various in vitro and in vivo assays.

Cytochrome P450 Enzyme Inhibition Studies. Cytochrome P450 enzyme inhibition studies were performed to assess the potential for metabolism-based drug−drug interactions (Table 2), which is an inevitable hurdle during the drug development process.[34] This will also rule out the inhibitory activity with cytochrome P450 enzymes, where compounds that bind to heme iron (for example, imidazole-containing compounds)[35] have shown to be potent inhibitors of cytochrome P450 enzymes that are closely related to NOS. Hence, compounds **16** and (−)-**19** were tested in a range (0.0457−100 μM) of concentrations against the five major human cytochrome P450 enzymes and the IC_{50} values were reported in Table 2. Both compounds showed very little drug−drug interaction potential, as the compounds are not active or very weak inhibitors of the five major human cytochrome P450 enzymes.

The Chung or Spinal Nerve Ligation (SNL) Model Studies. On the basis of NOS inhibitory and selectivity data and in vitro CYP inhibition data, **16** was selected for further profiling in an in vivo model of neuropathic pain (Figure 2).[36] The Chung or spinal nerve ligation (SNL) model involves ligation of both the L_5 and L_6 spinal nerves of one side of the rat, which will produce thermal and mechanical allodynia of the affected foot. Withdrawal latency to application of radiant heat to the paw was tested. Intraperitoneal administration of **16** at 10 mg/kg and 60 mg/kg resulted in reversal of thermal hyperalgesia in a dose-dependent manner. A reversal of thermal hyperalgesia was observed between 60 and 120 min, with a maximum effect of 67% reversal at 90 min following a single dose of 60 mg/kg (intraperitoneal).

eNOS Mediated Vasoconstriction Effect on Isolated Human Resistance Arteries. Compound **16** was assessed for the contractile response (inhibition of acetylcholine-mediated vasorelaxation) on isolated human resistance arteries to assess the undesirable cardiovascular effect associated with the inhibition of eNOS in comparison to a known nonselective NOS inhibitor, L-nitro arginine methyl ester (L-NAME) (Figures 3 and 4) and a negative (vehicle) control.[18,19] The arteries were preconstricted with U46619, a thromboxane A2 (TxA2) mimetic agent, and then exposed to acetylcholine (ACh), an endothelium, and nitric oxide dependent vasodilator. The response to ACh provides the information on the activity of eNOS in an active human biological tissue and thereby any inhibitory effect of **16** on eNOS. Furthermore, if inhibition of

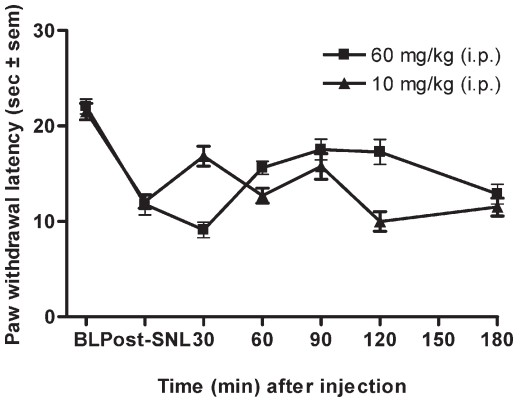


Figure 2. Compound **16** attenuates thermal hyperalgesia in the L_5/L_6 SNL model of neuropathic pain in a dose-dependent manner.

relaxation is due to the inhibition of eNOS, the addition of L-arginine (substrate for eNOS) would recover the relaxation due to ACh. ACh-mediated vasorelaxation effects of L-NAME (positive control) and **16** were tested (1 μM, 10 μM, 100 μM) in the absence and presence of L-arginine to determine whether any effects were due to the inhibition of human eNOS. L-NAME at all concentrations inhibited responses to ACh in a dose-dependent manner (Figure 3A). The inhibitory effect of L-NAME (100 μM) was reversed with the addition of L-arginine (1 mM), which is a substrate for eNOS (Figure 3B). No significant inhibition with **16** on the ability of the blood vessels to respond to ACh was observed at any tested concentration (Figure 4A). Furthermore, the addition of L-arginine did not significantly affect the responses to ACh in the presence of **16**, providing further evidence that **16** did not have an effect on agonist-mediated activation of human eNOS (Figure 4B). The data suggests that **16** would be devoid of any vasoconstrictive effect at concentrations that are physiologically relevant based on its human eNOS potency. The lack of vasoconstrictive effect of **16** is consistent with its weak inhibitory potency in the human eNOS enzyme (IC_{50} = 26.5 μM).

■ CONCLUSIONS

In conclusion, a series of selective 3,6-disubstitued indole derivatives were synthesized and shown to be selective inhibitors of human nNOS over eNOS and iNOS isoforms. A highly bulky amino-substituent in the 3-position of the indole ring such as an azabicyclic amine provided better selectivity over the smaller 5- or 6-membered cyclic amine substitution. Compounds **16** and (−)-**19** with better selectivity for human nNOS over eNOS were shown to be inactive or very weak inhibitors of human cytochrome P450 enzymes, indicating an unlikely potential for CYP-450 dependent drug−drug interactions. Intraperitoneal administration of **16** was also shown to reverse the thermal hyperalgesia in the SNL model of neuropathic pain. The lack of inhibition of ACh-mediated vasorelaxation in isolated human resistance arteries by **16** mitigates a cardiovascular effect associated with the inhibition of human eNOS. The significant results with **16** presented in this communication provide an opportunity to investigate the role that the nNOS enzyme plays in neuropathic pain as well as in other clinical indications of pain such as migraine and CTTH.

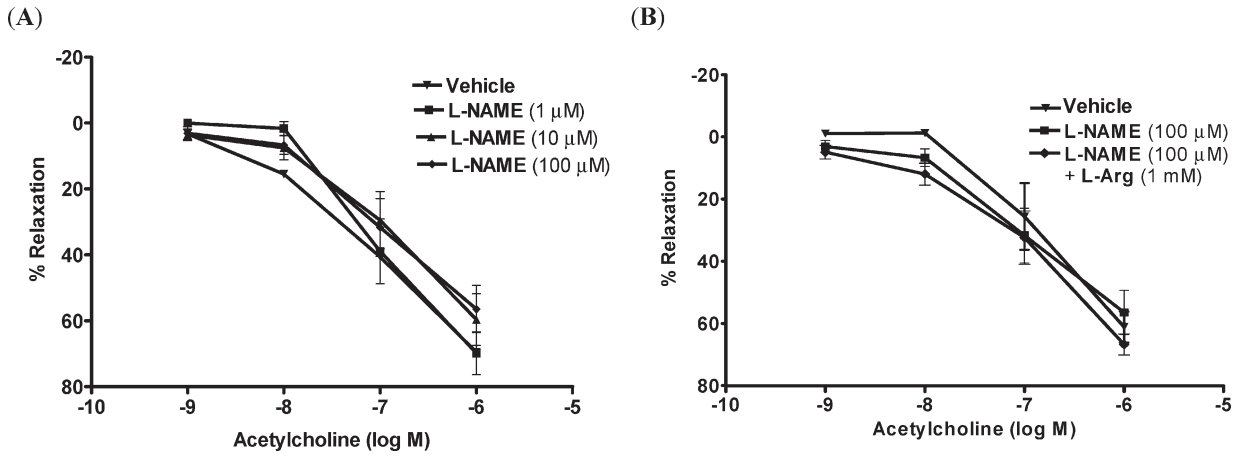


Figure 3. (A) Effect of L-NAME on the responses to ACh. (B) Effect of L-arginine (1 mM) on response to ACh in the presence of L-NAME.

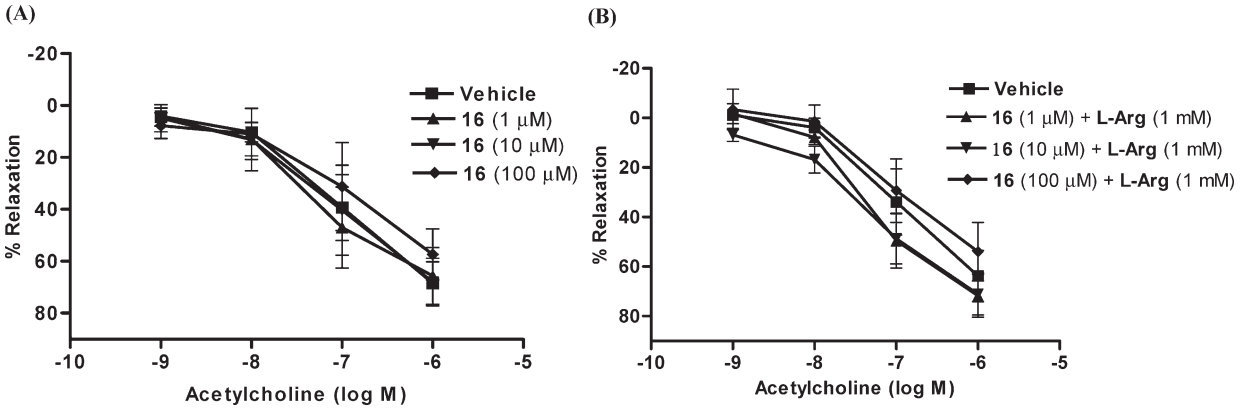


Figure 4. (A) Effect of **16** on the responses to ACh. (B) Effect of L-arginine (1 mM) on response to ACh in the presence of **16**.

EXPERIMENTAL SECTION

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 (Sigma-Aldrich, 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 obtained on a Bruker 300 MHz spectrometer. Low and high resolution mass spectra were obtained on an applied Biosystems/MDS Sciex QstarXL hybrid quadrupole/TOF instrument using electrospray ionization. Chemical purity was determined by Agilent 1100 HPLC system using Zorbax, SB-C18 reverse phase column, and the purity was determined to be ≥95% until unless specified. The chiral purity was determined by CHIRALPAK AD-H column and determined to be ≥99%. No attempts were made to optimize the yields.

***N*-(1*H*-Indol-6-yl)benzamide (8).** A solution of 1*H*-indol-6-amine (**7**) (2.0 g, 15.13 mmol) in dry THF (30 mL) was treated with Et_3N (6.32 mL, 45.39 mmol), followed by benzoyl chloride (1.84 mL, 15.88 mmol) at 0 °C. The reaction was brought to room temperature and stirred for 1 h. The reaction was diluted with water and product was extracted into ethyl acetate. The combined ethyl acetate layer was washed with brine, dried (Na_2SO_4), and solvent was evaporated to obtain the crude product. The crude was diluted with ethyl acetate (25 mL) followed by hexanes (150 mL), and the precipitate was filtered to obtain the title compound (3.55 g, 99%) as a solid. ^{1}H NMR ($CDCl_3$) δ 6.53 (t, 1H, J = 2.4 Hz), 6.95 (dd, 1H, J = 2.1, 8.4 Hz), 7.21 (t, 1H, J = 2.7 Hz), 7.48−7.60 (m, 4H), 7.88−7.92 (m, 3H), 8.27 (brs, 2H). ESI-MS (m/z, %): 259 (M + Na, 80), 237 (MH^+, 100).

***N*-(3-(1-Methyl-2,5-dioxopyrrolidin-3-yl)-1*H*-indol-6-yl)-benzamide (9).** A solution of *N*-(1*H*-indol-6-yl)benzamide (**8**) (3.5 g, 14.81 mmol) and *N*-methylmaleimide (4.07 g, 37.03 mmol) in glacial acetic acid (100 mL) was refluxed for 56 h. The reaction was brought to room temperature, and acetic acid was evaporated. The crude was taken into ethyl acetate, washed with satd $NaHCO_3$ solution and brine and dried (Na_2SO_4). Solvent was evaporated, and crude was purified by column chromatography (EtOAc:hexanes, 1:3 to 1:1) on silica gel to obtain the title compound (2.32 g, 45%) as a foam. ^{1}H NMR (DMSO-d_6) δ 2.80 (dd, 1H, J = 5.1, 18.0 Hz), 2.92 (s, 3H), 3.23 (dd, 1H, J = 9.3, 18.0 Hz), 4.34 (dd, 1H, J = 5.1, 9.3 Hz), 7.26−7.36 (m, 2H), 7.45−7.60 (m, 4H), 7.95−7.98 (m, 2H), 8.07 (s, 1H), 10.17 (s, 1H), 11.02 (s, 1H). ESI-MS (m/z, %): 370 (M + Na, 100), 348 (MH^+, 58).

***N*-Benzyl-3-(1-methylpyrrolidin-3-yl)-1*H*-indol-6-amine (10).** A solution of *N*-(3-(1-methyl-2,5-dioxopyrrolidin-3-yl)-1*H*-indol-6-yl)benzamide (**9**) (2.28 g, 6.56 mmol) in dry THF (30 mL) was treated with $LiAlH_4$ (2.49 g, 65.63 mmol) portionwise over a period of 45 min at 0 °C. The reaction was brought to room temperature and stirred for 48 h. The reaction was quenched with sodium sulfate decahydrate (8.0 g), followed by careful addition of water (9 mL) at 0 °C, and stirred for 30 min at room temperature. The reaction was diluted with ethyl acetate, filtered, and washed with ethyl acetate.

dx.doi.org/10.1021/jm201063u | *J. Med. Chem.* 2011, 54, 7408–7416

The combined ethyl acetate layer was evaporated, and the crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 5:95 to 1:9) on silica gel to obtain the title compound (0.44 g, 22%) as a foam. ^{1}H NMR (DMSO-d_6) δ 1.76−1.87 (m, 1H), 2.11−2.23 (m, 1H), 2.27 (s, 3H), 2.36 (t, 1H, J = 8.4 Hz), 2.42−2.48 (m, 1H), 2.64−2.71 (m, 1H), 2.90 (t, 1H, J = 8.1 Hz), 3.37−3.45 (m, 1H), 4.26 (d, 2H, J = 5.7 Hz), 5.88 (t, 1H, J = 6.0 Hz), 6.34 (d, 1H, J = 0.9 Hz), 6.45 (dd, 1H, J = 1.8, 8.4 Hz), 6.72 (d, 1H, J = 1.2 Hz), 7.17−7.38 (m, 6H), 10.11 (s, 1H). ESI-MS (m/z, %): 306 (MH^+, 100). ESI-HRMS calculated for $C_{20}H_{24}N_3$ (MH^+), 306.1964; observed, 306.1967.

_N_-(3-(1-Methylpyrrolidin-3-yl)-1_H_-indol-6-yl) thiophene-2-carboximidamide (12). A solution of _N_-benzyl-3-(1-methylpyrrolidin-3-yl)-1_H_-indol-6-amine (**10**) (0.42 g, 1.37 mmol) in absolute ethanol (5 mL) was treated with 20% $Pd(OH)_2$ on carbon (0.5 g), purged with hydrogen gas, and stirred under hydrogen (balloon pressure) for 48 h. The reaction was filtered through a pad of Celite and washed with ethanol. The combined ethanol layer was treated with methyl thiophene-2-carbimidothioate hydroiodide (**11**) (0.78 g, 2.75 mmol) at room temperature and stirred for 48 h. The reaction was basified with satd $NaHCO_3$ solution, and product was extracted into CH_2Cl_2. The combined CH_2Cl_2 layer was washed with brine 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) on silica gel to obtain the title compound (0.285 g, 64%) as a solid. ^{1}H NMR (DMSO-d_6) δ 1.86−1.95 (m, 1H), 2.18−2.27 (m, 1H), 2.45−2.59 (m, 2H), 2.68−2.76 (m, 1H), 2.96 (t, 1H, J = 8.1 Hz), 3.45−3.56 (m, 1H), 6.29 (brs, 2H), 6.54 (dd, 1H, J = 1.2, 8.1 Hz), 6.78 (s, 1H), 6.99 (d, 1H, J = 1.8 Hz), 7.09 (t, 1H, J = 4.2 Hz), 7.49 (d, 1H, J = 8.1 Hz), 7.58 (d, 1H, J = 4.8 Hz), 7.71 (d, 1H, J = 3.6 Hz), 10.51 (s, 1H). ESI-MS (m/z, %): 325 (MH^+, 38), 282 (31), 163 (100). ESI-HRMS calculated for $C_{18}H_{21}N_4S$ (MH^+), 325.1481; observed, 325.1495.

3-(1-Methyl-1,2,3,6-tetrahydro-pyridin-4-yl)-6-nitro-1_H_-indole (14). A solution of 6-nitro-1_H_-indole (**13**) (0.5 g, 3.08 mmol) in dry EtOH (5 mL) was treated with pyrrolidine (0.77 mL, 9.25 mmol) and _N_-methyl-4-piperidone (0.75 mL, 6.16 mmol) at room temperature, and the resulting solution was refluxed for 48 h. The reaction was brought to room temperature, further cooled to 0 °C, and the solid was filtered off. The solid was washed with ethanol and dried to obtain the title compound (0.567 g, 72%). ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 3H), 2.50−2.58 (m, 4H), 3.00−3.05 (m, 2H), 6.18 (s, 1H), 7.83 (s, 1H), 7.89 (dd, 1H, J = 2.1, 9.0 Hz), 7.97 (d, 1H, J = 9.0 Hz), 8.31 (d, 1H, J = 2.1 Hz), 11.88 (brs, 1H). ESI-MS (m/z, %): 258 (M^+, 100).

3-(6-Nitro-1_H_-indol-3-yl)-1-azabicyclo[2.2.2]oct-2-ene (15). A solution of 6-nitro-1_H_-indole (**13**) (1.0 g, 6.16 mmol) in MeOH:H_2O (20 mL, 1:1) was treated with KOH (1.73 g, 30.83 mmol), followed by 3-quinoclidine hydrochloride (1.99 g, 12.33 mmol) at room temperature, and the resulting dark-brown mixture was refluxed for 36 h. The reaction was brought to room temperature, filtered, and washed with MeOH:H_2O (3 × 5 mL, 1:1), followed by methanol (5 mL). The yellow solid was dried under vacuum to obtain the title compound (1.4 g, 84%). ^{1}H NMR (DMSO-d_6) δ 1.46−1.58 (m, 2H), 1.68−1.76 (m, 2H), 2.52−2.58 (m, 2H), 2.89−2.97 (m, 2H), 3.08−3.12 (m, 1H), 6.89 (d, 1H, J = 1.2 Hz), 7.91−7.97 (m, 3H), 8.32 (d, 1H, J = 1.2 Hz), 11.98 (s, 1H). ESI-MS (m/z, %): 270 (MH^+, 100).

_N_-[3-(1-Methyl-1,2,3,6-tetrahydro-pyridin-4-yl)-1_H_-indol-6-yl]-thiophene-2-carboxamidine (16) and _N_-[3-(1-Methyl-piperidin-4-yl)-1_H_-indol-6-yl]-thiophene-2-carboxamidine (17). A solution of 3-(1-methyl-1,2,3,6-tetrahydro-pyridin-4-yl)-6-nitro-1_H_-indole (**14**) (0.15 g, 0.58 mmol) in dry MeOH (5 mL) was treated with Raney nickel (∼0.05 g) and hydrazine hydrate (0.18 mL, 5.82 mmol) at room temperature, and the resulting mixture was refluxed for 3 h. The reaction was brought to room temperature, filtered though a pad of Celite, and washed with MeOH: H_2Cl_2 (1:1, 2 × 10 mL). The combined organic layer was evaporated, and crude was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 1:9) on silica gel to obtain the intermediate mixture of amines in 5:2 ratio.

A solution of the above intermediate mixture of amines in dry EtOH (10 mL) was treated with methyl thiophene-2-carbimidothioate hydroiodide (**11**) (0.33 g, 1.165 mmol) at room temperature and stirred for 24 h. The reaction was worked-up and purified as described for **12** to obtain the title compounds **16** (0.085 g, 50%) and **17** (0.04 g, 20%). Compound **16**, foam; ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 3H), 2.50−2.57 (m, 4H), 3.00−3.04 (m, 2H), 6.09 (s, 1H), 6.31 (brs, 1H), 6.59 (dd, 1H, J = 1.2, 8.4 Hz), 6.82 (s, 1H), 7.09 (dd, 1H, J = 3.6, 4.9 Hz), 7.24 (d, 1H, J = 2.1 Hz), 7.59 (d, 1H, J = 5.1 Hz), 7.70−7.73 (m, 2H), 10.85 (s, 1H). ESI-MS (m/z, %), 337 (M^+, 100). Compound **17**, foam; ^{1}H NMR (DMSO-d_6) δ 1.62−1.75 (m, 2H), 1.90−1.94 (m, 2H), 2.02−2.09 (m, 2H), 2.22 (s, 3H), 2.64−2.72 (m, 1H), 2.85−2.89 (m, 2H), 6.31 (brs, 1H), 6.53 (dd, 1H, J = 1.2, 8.2 Hz), 6.79 (s, 1H), 6.94 (d, 1H, J = 1.8 Hz), 7.09 (dd, 1H, J = 3.6, 4.9 Hz), 7.45 (d, 1H, J = 8.4 Hz), 7.59 (d, 1H, J = 4.2 Hz), 7.72 (d, 1H, J = 3.6 Hz), 10.53 (brs, 1H). ESI-MS (m/z, %), 339 (M^+, 100).

_N_-(3-(1-Azabicyclo[2.2.2]oct-2-en-3-yl)-1_H_-indol-6-yl)thiophene-2-carboximidamide (18). 3-(6-Nitro-1_H_-indol-3-yl)-1-azabicyclo[2.2.2]oct-2-ene (**15**) (0.4 g, 1.48 mmol) in dry methanol (10 mL) was treated with Raney nickel (∼0.05 g) and hydrazine hydrate (0.46 mL, 1.02 mmol). The resulting mixture was placed in a preheated oil bath and refluxed for 2 min or until yellow color disappears. The reaction was worked-up and purified as described for **16** and **17** to obtain the amine intermediate (3-(1-azabicyclo[2.2.2]oct-2-en-3-yl)-1_H_-indol-6-amine) (0.35 g, quantitative) as a foam. ^{1}H NMR (DMSO-d_6) δ 1.42−1.54 (m, 2H), 1.62−1.74 (m, 2H), 2.52−2.56 (m, 2H), 2.86−2.94 (m, 2H), 2.98−3.02 (m, 1H), 4.75 (s, 2H), 6.41 (dd, 1H, J = 2.1, 8.4 Hz), 6.53 (d, 1H, J = 1.8 Hz), 6.69 (s, 1H), 7.16 (d, 1H, J = 2.4 Hz), 7.35 (d, 1H, J = 8.4 Hz), 10.57 (s, 1H). ESI-MS (m/z, %): 240 (MH^+, 100).

Prepared from the above amine intermediate (3-(1-azabicyclo[2.2.2]-oct-2-en-3-yl)-1_H_-indol-6-amine) (0.33 g, 1.37 mmol) and methyl thiophene-2-carbimidothioate hydroiodide (**11**) (0.78 g, 2.75 mmol) as described for **12** to obtain the title compound (0.42 g, 81%) as a solid. ^{1}H NMR (DMSO-d_6) δ 1.44−1.56 (m, 2H), 1.66−1.78 (m, 2H), 2.52−2.58 (m, 2H), 2.89−2.98 (m, 2H), 3.04−3.10 (m, 1H), 6.29 (s, 2H), 6.61 (dd, 1H, J = 1.8, 8.7 Hz), 6.78 (s, 1H), 6.83 (s, 1H), 7.09 (t, 1H, J = 4.2 Hz), 7.39 (d, 1H, J = 2.4 Hz), 7.59 (d, 1H, J = 4.8 Hz), 7.63 (d, 1H, J = 8.4 Hz), 7.72 (d, 1H, J = 3.3 Hz), 10.97 (s, 1H). ESI-MS (m/z %): 349 (MH^+, 95), 161 (100). ESI-HRMS calculated for $C_{20}H_{21}N_4S$ (MH^+), 349.1481; observed, 349.1494.

_N_-(3-(Quinuclidin-3-yl)-1_H_-indol-6-yl)thiophene-2-carboximidamide (19). A solution of 3-(6-nitro-1_H_-indol-3-yl)-1-azabicyclo-[2.2.2]oct-2-ene (**15**) (0.4 g, 1.48 mmol) in dry ethanol (10 mL) was treated with Pd−C (∼0.05 g), purged with hydrogen gas, and stirred for 36 h at room temperature under hydrogen (balloon pressure). The reaction was filtered through a pad of Celite and was washed with ethanol. The combined ethanol layer was treated with methyl thiophene-2-carbimidothioate hydroiodide (**11**) (0.84 g, 2.97 mmol) at room temperature and stirred overnight (14 h). The reaction was worked-up and purified as described for **12** to obtain the title compound (0.4 g, 77%) as a solid. ^{1}H NMR (DMSO-d_6) δ 1.20−1.28 (m, 1H), 1.56−1.74 (m, 3H), 2.62−2.72 (m, 1H), 2.79−2.92 (m, 3H), 3.09−3.34 (m, 4H), 6.30 (s, 2H), 6.53 (d, 1H, J = 8.4 Hz), 6.79 (s, 1H), 7.07−7.10 (m, 2H), 7.38 (d, 1H, J = 8.4 Hz), 7.58 (d, 1H, J = 4.5 Hz), 7.72 (d, 1H, J = 3.0 Hz), 10.61 (s, 1H). ESI-MS (m/z, %): 351 (MH^+, 38), 176 (100). ESI-HRMS calculated for $C_{20}H_{23}N_4S$ (MH^+), 351.1637; observed, 351.1637.

_N_-(3-(Quinuclidin-3-yl)-1_H_-indol-6-yl)thiophene-2-carboximidamide ((-)-19 and (+)-19). _N_-(3-(Quinuclidin-3-yl)-1_H_-indol-6-yl)thiophene-2-carboximidamide (**19**) was separated using chiral

Table 3. Gradient System Used for Determining the Chemical Purity of All Final Compounds

time (min)	0.1% TFA in acetonitrile (%)	0.1% TFA in water (%)
0	0	100
5−7	30	70
7.5−10	50	50
12	30	70
14−15	0	100

HPLC (30% 2-propanol (0.1% DEA)/CO_2, 100 bar). Column: Chiralcel OD-H (2 cm × 20 cm) SN 06−6079. Flow rate 65 mL/min. Wavelength 220 nm. Injection volume 0.3−0.5 mL, 7.3 mg/mL methanol. First eluting enantiomer (−)-**19**: $[\alpha]_D$ = −11.88 (0.706 g/100 mL, MeOH). ^{1}H NMR (MeOH-d_4) δ 1.45−1.59 (m, 1H), 1.82−2.06 (m, 3H), 2.12 (brs, 1H), 2.92−2.99 (m, 1H), 3.06−3.16 (m, 4H), 3.44−3.49 (m, 2H), 6.74 (dd, 1H, J = 1.8, 8.4 Hz), 7.01 (d, 1H, J = 1.5 Hz), 7.13 (dd, 1H, J = 3.6, 5.1 Hz), 7.17 (s, 1H), 7.53 (d, 1H, J = 8.1 Hz), 7.58 (dd, 1H, J = 1.2, 5.1 Hz), 7.65 (dd, 1H, J = 0.9, 3.6 Hz). ESI-MS (m/z, %), 351 (MH^+, 60) 176 (100). ESI-HRMS calculated for $C_{20}H_{22}N_4S$ (MH^+), 351.1637; observed, 351.1645. Second eluting enantiomer (+)-**19**: $[\alpha]_D$ = +13.93 (0.933 g/100 mL, MeOH). ^{1}H NMR (MeOH-d_4) δ 1.29−1.48 (m, 1H), 1.78−1.96 (m, 3H), 2.07 (brs, 1H), 2.84−2.92 (m, 1H), 3.01−3.16 (m, 4H), 3.42−3.39 (m, 2H), 6.72 (dd, 1H, J = 1.8, 8.4 Hz), 6.99 (d, 1H, J = 1.5 Hz), 7.12 (dd, 1H, J = 3.6, 5.1 Hz), 7.14 (s, 1H), 7.51 (d, 1H, J = 8.1 Hz), 7.56 (dd, 1H, J = 1.2, 5.1 Hz), 7.63 (dd, 1H, J = 0.9, 3.6 Hz). ESI-MS (m/z, %), 351 (MH^+, 60) 176 (100). ESI-HRMS calculated for $C_{20}H_{22}N_4S$ (MH^+), 351.1637; observed, 351.1642.

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.

Chemical Purity. The chemical purity (Table 4) was determined by using Agilent Zorbax, SB-C18 (3.5 μM, 2.1 mm × 100 mm) column using gradient method (0.1% TFA in acetonitrile: 0.1% TFA in water, Table 3) with 0.5 mL/min flow rate at 254 nm. Injection volume: 5 μL (1 mg/mL in MeOH).

Chiral Purity. The chiral purity (Table 4) was determined by CHIRALPAK AD-H (column no. ADH0CE-LG122) column using isocratic method (ethanol:hexanes (contains 0.2% DEA), 3:7) with 1.5 mL/min flow rate at 254 nm. Injection volume: 10 μL (1 mg/mL in MeOH).

NOS Enzyme Assays. Recombinant human nNOS, eNOS, and iNOS were produced in Baculovirus-infected Sf9 cells. In a radiometric method, NOS activity is 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 dithiothreitol, 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 is added to the enzyme assay solution, followed by a preincubation time of 15 min at RT. The reaction is 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 is 150 μL in every well.

Table 4. The Chemical and Chiral Purity of All Final Compounds

compound	retention time (min)	chemical purity (%)	retention time (min)	chiral purity (%)
12	7.46	93.2	NA[a]	NA[a]
16	7.59	96.5	NA[a]	NA[a]
17	7.63	96.2	NA[a]	NA[a]
18	7.69	92.7	NA[a]	NA[a]
(±)-**19**	7.86	97.4	NA[a]	NA[a]
(−)-**19**	9.14	95.2	6.92	100
(+)-**19**	9.15	96.8	33.93	99.2

[a] NA: not applicable.

The reactions are 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 12000g for 10 min in the centrifuge. Then 70 μL Aliquot of the supernatant was added to 100 μL of scintillation fluid and the radio activity was counted in a liquid scintillation counter (1450 Microbeta Jet, Wallac). Specific NOS activity was 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 (**1**). All assays were performed in duplicate.

Cytochrome P450 Enzyme Inhibition Studies. The assay used microsomes (Supersomes, GENTEST Corp.), prepared from insect cells, expressing the various subtypes of the cytochrome P450 isozymes (CYP). The assay monitored the formation of a fluorescent metabolite following incubation of the microsomes with a specific fluorogenic CYP substrate. Reactions (0.2 mL) were performed in 96-well microtiter plates at 37 °C in the presence of an NADPH regenerating system ($NADP^+$, glucose-6-phosphate, glucose-6-phospate dehydrogenase, and $MgCl_2$). Inhibition of metabolic product formation by the test compound for each enzyme was tested in the absence or presence of varying concentrations of test compounds. An enzyme-selective inhibitor was also tested in the assay as a positive control. All determinations are performed in duplicate.

Efficacy in the Chung Model of Neuropathic Pain. Nerve ligation injury was performed according to the literature procedure.[36] 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 to recover in individual cages. This technique produced signs of neuropathic dysesthesias, including tactile allodynia, thermal hyperalgesia, and guarding of the affected paw, which began on day 1 of the surgery and peaked on day 16. After a period of recovery following the surgical intervention, rats showed enhanced sensitivity to painful and normally nonpainful stimuli.

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). Compound **16** (±L-arginine) was added at the selected concentration for a period of 50 min. In the presence of **16** (±L-arginine), all vessels were then submaximally vasoconstricted with U46619 prior to CCRCs to ACh (1×10^{-10} M to 1×10^{-5} M). Following wash-out with PSS to baseline, **16** (±L-arginine) was readded for 50 min at the next highest concentration. CCRCs to ACh following U46619 preconstriction were then repeated. In total, three concentrations of **16** and equivalent concentrations of L-NAME were studied in each artery ring (10^{-6} M, 10^{-5} M and 10^{-4} M) in the presence and absence of 10^{-3} M L-arginine.

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.

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ACKNOWLEDGMENT

We are grateful to Asinex Ltd (Moscow, Russia) for performing the human iNOS inhibition assays. We thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for providing an Industrial Research Fellowship to G.M.

ABBREVIATIONS USED

NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; CTTH, chronic tension-type headache; L-NAME, L-nitro arginine methyl ester; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; BH4, (6*R*)-2-amino-6-[(1*R*,2*S*)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1*H*)-one; SNL, spinal nerve ligation; ACh, acetylcholine

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