Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Discovery of *cis*-*N*-(1-(4-(Methylamino)cyclohexyl)indolin-6-yl)thiophene-2-carboximidamide: A 1,6-Disubstituted Indoline Derivative as a Highly Selective Inhibitor of Human Neuronal Nitric Oxide Synthase (nNOS) without Any Cardiovascular Liabilities

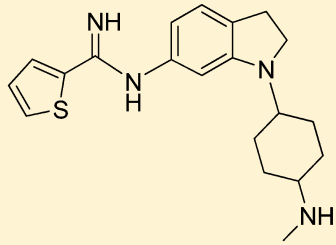
Subhash C. Annedi,*[,†] Jailall Ramnauth,[†] Shawn P. Maddaford,[†] Paul Renton,[†] Suman Rakhit,[†] Gabriela Mladenova,[†] Peter Dove,[†] Sarah Silverman,[†] John S. Andrews,[†] Milena D. Felice,[‡] and Frank Porreca[‡]

[†]NeurAxon Inc., 2395 Speakman Drive, Suite 1001, Mississauga, Ontario L5K 1B3, Canada

[‡]Department of Pharmacology, University of Arizona, 1501 North Campbell Avenue, Tucson, Arizona 85724, United States

Supporting Information

ABSTRACT: A series of 1,6-disubstituted indoline derivatives were synthesized and evaluated as inhibitors of human nitric oxide synthase (NOS) designed to mitigate the cardiovascular liabilities associated with previously reported tetrahydroquinoline-based selective neuronal NOS inhibitors due to higher lipophilicity (*J. Med. Chem.* **2011**, *54*, 5562−5575). This new series produced similar potency and selectivity among the NOS isoforms and was devoid of any cardiovascular liabilities associated with QT prolongation due to hERG activity or endothelial NOS mediated vasoconstriction effect. The SAR studies led to the identification of *cis*-**45**, which was shown to reverse thermal hyperalgesia in vivo in the spinal nerve ligation model of neuropathic pain with excellent safety profile (off-target activities at 80 CNS related receptors/ion channels/transporters). The results presented in this report make *cis*-**45** as an ideal tool for evaluating the potential role of selective nNOS inhibitors in CNS related disorders where excess NO produced by nNOS is thought to play a crucial role.



Highly selective inhibitor of human nNOS (*cis*-**45**) without any cardiovascular liabilities
IC_{50} (μM): nNOS = 0.37, eNOS = 195, iNOS = 83

INTRODUCTION

Nitric oxide (NO) is an inorganic free radical gas that is involved in a wide range of physiological functions and pathophysiological states.[1,2] NO is produced by a family of three heme binding enzymes called nitric oxide synthase (NOS) via the five electron oxidation of L-arginine to L-citrulline.[3,4] Although the three NOS isoforms are similar in their mechanism, they are produced by different genes, with different functions. Neuronal or brain NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are constitutively expressed and thought to play a role in neurotransmission and smooth muscle relaxation, respectively. The third isoform is an inducible NOS (iNOS or NOS2) that is expressed during bacterial infection, tumor cell cytolysis and inflammation.

The NOS enzymes are homodimeric proteins consisting of a *C*-terminal reductase domain and an *N*-terminal catalytic oxygenase domain connected by a calmodulin-binding linker. The *C*-terminal reductase domain transfers electrons from NADPH through two prosthetic groups, flavinadenine dinucleotide (FAD) and flavine mononucleotide (FMN), to the *N*-terminal oxygenase domain, which binds L-arginine, (6*R*)-2-amino-6-[(1*R*,2*S*)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1*H*)-one (BH4) and iron protoporphyrin (heme). Both the nNOS and eNOS isoforms are regulated by their dependence on Ca^{2+}/calmodulin concentration, while the iNOS isoform is not dependent on Ca^{2+}/calmodulin concentration because it already has a tightly bound calmodulin. The reduction of BH4 and heme iron allows the activation of O_2, which is followed by the hydroxylation of L-arginine to N^{ω}-hydroxy-L-arginine and then conversion to L-citrulline, ultimately releasing NO.

When it is highly regulated, the production of NO is considered beneficial to the host organism; however, excess NO or overproduction has been implicated in numerous disease states.[5] In particular, excess NO produced by nNOS has been shown to play an important role in the development of several disorders including septic shock, stroke, pain (migraine, chronic tension-type headache, visceral, and neuropathic) and neurodegenerative disorders (Parkinson's disease, MS, and Alzheimer's disease).[6,7] To gain the potential therapeutic benefits associated with nNOS inhibition, it is critical that the selective inhibition of nNOS is achieved to preserve the important functions of the other NOS isoforms such as eNOS.

Received: November 21, 2011
Published: December 16, 2011

For example, eNOS plays a crucial role in the regulation of blood pressure, and the inhibition of eNOS by a nonselective NOS inhibitor such as **1** (Figure 1) has been shown to cause

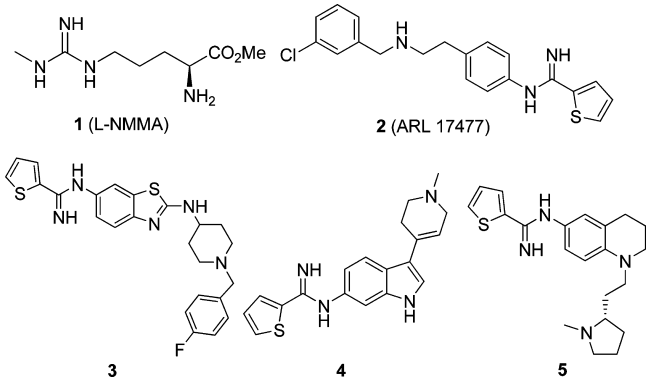


Figure 1. Compound **1** is one of the most studied early nonselective NOS inhibitor. Literature examples of selective nNOS inhibitors with basic amine side chain (**2**–**5**).

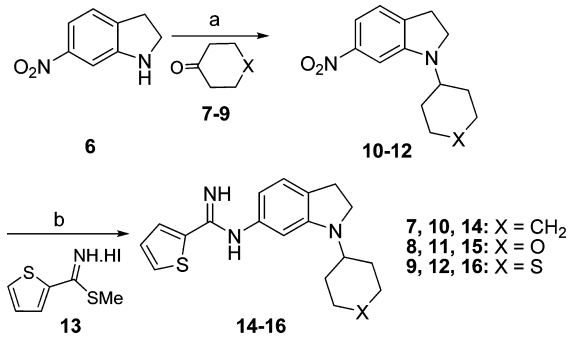
vasoconstriction in humans.[8,9] Investigation into the synthesis and chemistry of novel isoform selective inhibitors of NOS has been an ongoing challenge over the past two decades even though the general pharmacophore requirements are well established.[10] Early NOS inhibitors designed by mimicking the natural substrate, L-arginine (the most common mode), produced only nonselective NOS inhibitors. However, later generations of peptidic and nonpeptidic NOS inhibitors targeting the arginine binding site or BH4 site as well as dimerization inhibitors have been shown to be more potent and selective among NOS isoforms.[11−13]

The simplified pharmacophore model adapted by our group for competitively inhibiting NOS at the substrate binding site contains a guanidine isosteric group and a basic amine group both attached to a central aryl scaffold as described earlier.[14] By varying the guanidine isostere, the central linker, or the basic amine group, potent and selective nNOS inhibitors have been obtained (for example: **3**–**5**, Figure 1).[14−19] We recently reported a series of tetrahydroquinoline-based selective nNOS inhibitors with druglike properties and was shown to be active in two different pain models with reference compound **5** (Figure 1).[18] However **5** was associated with cardiovascular liabilities due to moderate inhibition at human ether-a-go-go-related gene (hERG K^+) and vasoconstrictive effect through an unknown mechanism, both attributable to its higher lipophilicity (vide infra).[20,21] To mitigate the cardiovascular liabilities associated with **5**, indoline-based compounds with smaller ring size with lower lipophilicity were designed, synthesized, and evaluated as NOS inhibitors. Herein, we now describe the synthesis and structure−activity relationship (SAR) of 1,6-disubstituted indoline-based selective nNOS inhibitors that led to the identification of *cis*-**45**, which has lower lipophilicity with better side effect profile when compared to reference compound **5**.

RESULTS AND DISCUSSION

Chemistry. Synthesis of all target compounds was carried out as described in Schemes 1 through 5. Reductive amination of 6-nitroindoline (**6**) with various ketones (**7**–**9**) was carried out with sodium triacetoxy borohydride to obtain compounds **10**–**12** (Scheme 1).[22] Reduction of the nitro group under

Scheme 1[a]



[a]Reagents and conditions: (a) **7**–**9**, $NaBH(OAc)_3$, AcOH, DCE, rt; (b) (i) Raney-Ni, hydrazine hydrate, MeOH, reflux, (ii) **13**, EtOH, rt.

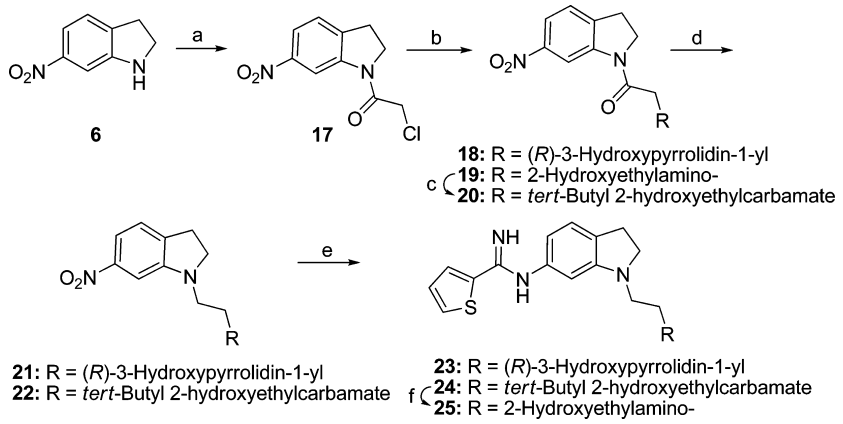
transfer hydrogenation conditions, followed by coupling with thiophene-2-carbimidothioate **13**,[23] gave the target compounds **14**–**16**. The *N*-alkylation of **6** with 2-chloroacetyl chloride[24] provided chloro intermediate **17**, which was substituted with two different amines under basic conditions to obtain **18** and **19**, respectively (Scheme 2). The secondary amine in **19** was protected as *tert*-butyl carbamate to obtain **20**. The amide group in **18** and **20** was reduced into the corresponding amine using borane in THF to obtain **21** and **22**, respectively. Reduction of nitro group in **21** and **22** into the corresponding amine followed by the coupling to thiophene-2-carbimidothioate **13** gave **23** and **24**, respectively. The deprotection of Boc- protecting group in **24** was carried out under strong acidic conditions to obtain the target compound **25**.

The reductive amination of 6-bromoindoline (**26**), 6-nitroindoline (**6**), and 5-fluoro-6-nitroindoline (**27**)[25] with *tert*-butyl 3-oxopyrrolidine-1-carboxylate (**28**) and *tert*-butyl 4-oxopiperidine-1-carboxylate (**29**) gave the coupled compounds **30**–**32**, respectively (Scheme 3). The bromo- group in **30** was converted into the corresponding amine using standard Buchwald−Hartwig amination conditions,[26] followed by coupling with thiophene-2-carbimidothioate **13** to give **33**. The reduction of the nitro- group in **31** and **32** into the corresponding amine was carried under transfer hydrogenation conditions, followed by coupling with thiophene-2-carbimidothioate **13** to give **34** and **35**, respectively. The Boc-protecting group in **33**–**35** was removed under strong acidic conditions to obtain the target compounds **36**–**38**, respectively. At this time no attempts were made to separate (±)-**36** into its enantiomers, and the compound was tested as a racemic mixture in the human NOS assay.

The reductive amination of 6-nitro indoline (**6**) with 1,4-dioxaspiro[4.5]decan-8-one (**39**) as described above provided **40** (Scheme 4). The deprotection of the acetal group was carried out under acidic conditions to obtain **41**, followed by the reductive amination with methyl amine hydrochloride to provide two diastereomers *cis*-**42** and *trans*-**42** in 1:1 ratio, which were partially separated by regular silica gel column chromatography (the stereochemistry was conformed with final compounds after the sequence was completed). The protection of the secondary amine in *cis*-**42** and *trans*-**42** was achieved under basic conditions to obtain *cis*-**43** and *trans*-**43**, respectively. The nitro- group reduction in *cis*-**43** and *trans*-**43** to the corresponding amine followed by coupling with thiophene-2-carbimidothioate **13** gave *cis*-**44** and *trans*-**44**, respectively. Finally, the Boc-deprotection was carried out

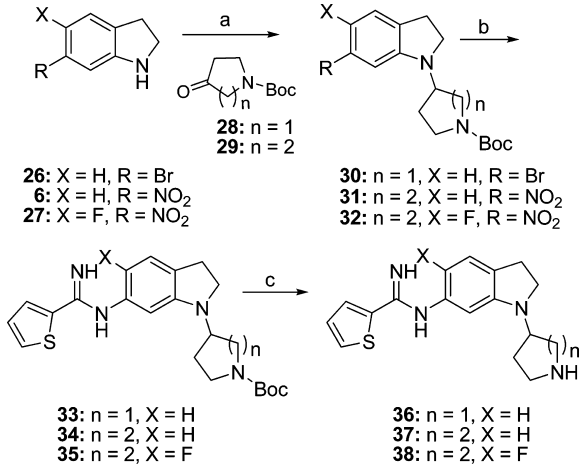
dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943−955

Scheme 2[a]



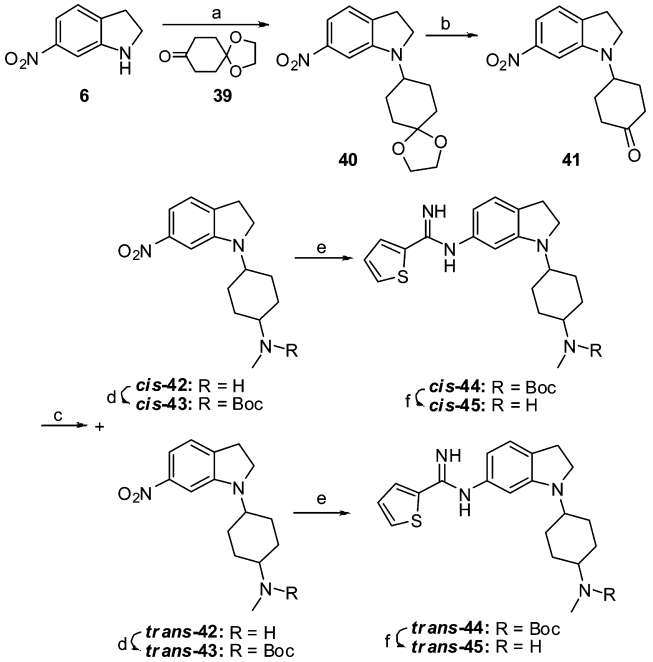
[a]Reagents and conditions: (a) 2-chloroacetyl chloride, toluene, 110 °C; (b) amine, K_2CO_3, acetonitrile:water (9:1), 85−100 °C; (c) Boc_2O, Et_3N, dioxane, rt; (d) 1 M BH_3 in THF, rt; (e) (i) Raney-Ni, hydrazine hydrate, MeOH, reflux, (ii) **13**, EtOH, rt; (f) 3 N HCl, MeOH, 90 °C.

Scheme 3[a]



[a]Reagents and conditions: (a) **28**, **29**, $NaBH(OAc)_3$, AcOH, DCE, rt; (b) (i) $Pd_2(dba)_3$, $PtBu_3$, LiHMDS, THF, reflux or Raney-Ni, hydrazine hydrate, MeOH, reflux, (ii) **13**, EtOH, rt; (c) 3 N HCl, MeOH, reflux.

Scheme 4[a]



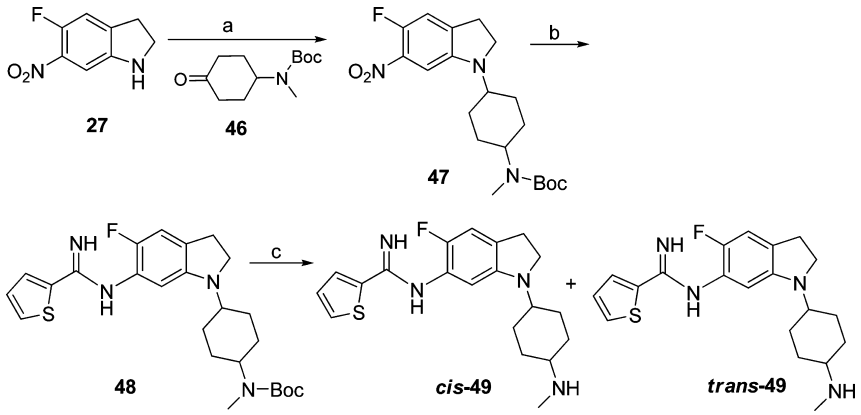
[a]Reagents and conditions: (a) **39**, $NaBH(OAc)_3$, AcOH, DCE, rt; (b) HCl, acetone; (c) CH_3NH_2·HCl, $NaBH(OAc)_3$, AcOH, DCE, rt; (d) Boc_2O, Et_3N, dioxane, rt; (e) (i) Raney-Ni, hydrazine hydrate, MeOH, reflux, (ii) **13**, EtOH, rt; (f) TFA, CH_2Cl_2, rt.

under mild acidic conditions using TFA to obtain the final compounds *cis*-**45** and *trans*-**45**. Both *cis*-**45** and *trans*-**45** differ by the *cis*- and *trans*- substitution at the 1,4 positions of the cyclohexyl ring. It is difficult to observe a clear NOE between these positions due to a certain degree of interconversion between boat and chair conformations, although it was expected that the *trans*- conformation would permit the 1,4 substituents to be in a more stable, equatorial position to stabilize the chair conformation (Supporting Information). In this conformation, the protons in the 2,3 (and 5,6) positions would be predominantly in equatorial and axial positions and experiencing the maximum affect of chemical shift anisotropy from the carbon−carbon bonds (Supporting Information). Therefore the *trans*- conformation have the largest chemical shift range (0.7 ppm) for proton 2,3 (and 5,6) as opposed to the *cis*- compound (0.4 ppm).[27] To avoid the sequential transformations as described in Scheme 4, the *tert*-butyl methyl(4-oxocyclohexyl)carbamate (**46**) side chain was coupled to 5-fluoro-6-nitro indoline (**27**) as described above to provide **47** as a nonseparable mixture of diastereomers (*trans*:*cis*, 1.5:1) (Scheme 5). Nitro- group reduction followed by coupling to thiophene-2-carbimidothioate **13** provided **48** as a nonseparable mixture of diastereomers. The deprotection of the Boc- group under acidic conditions provided the final compounds *cis*-**49** and *trans*-**49** (*cis*:*trans*, 1:1.5), which were separated by regular silica gel column chromatography. The stereochemistry of *cis*-**49** and *trans*-**49** was determined as described for *cis*-**45** and *trans*-**45**.

Structure−Activity Relationship (SAR) Studies. All compounds were converted into their corresponding dihydrochloride salts and inhibitory activities were measured against all three human NOS isoforms (Table 1). Recombinant human nNOS, eNOS, and iNOS were produced in baculovirus-260 infected Sf9 cells. In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943−955

Scheme 5[a]



[a]Reagents and conditions: (a) **46**, $NaBH(OAc)_3$, AcOH, DCE, rt; (b) (i) Pd−C/H_2, EtOH, rt, (ii) **13**, EtOH, rt; (c) 3 M HCl, MeOH, reflux.

L-citrulline. The enzymatic reaction was carried out in the presence or absence of varying concentrations of the inhibitor in water. Inhibition of enzyme activity by the inhibitor was measured by dividing the enzymatic conversion in the presence of inhibitor divided by the enzymatic conversion in the absence of inhibitor. IC_{50} value is the concentration of compound that gives rise to 50% inhibition.

In general, in vivo toxicity and in vitro receptor promiscuity are both associated with lipophilicity and are most likely to arise when clogP is greater than 3, particularly compounds with a basic amine.[28] The current design strategy of selecting the indoline core as a central aromatic linker is based on their lower lipophilicity (*cis*-**45**: clogP = 3.14), when compared to previously reported tetrahydroquinoline-based selective nNOS inhibitors represented by reference compound **5** (clogP = 4.44).[18] Attaching a bulky or cyclic basic amine to the 1-position of the indoline ring is adapted from our previous results with substituted indole and tetrahydroquinoline compounds.[15−18] To test this hypothesis with the new indoline core as a central aromatic linker, **14**−**16** were prepared without a basic amine. Even though **14**−**16** contain the bulky cyclic side chains, they showed 4−23-fold weaker potency for the nNOS isoform when compared to the compounds that contain basic amine side chains such as (±)-**36** and **37**.

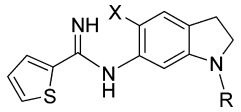
Compounds **23** and **25** with flexible side chains were substituted with hydroxy- and hydroxy ethyl- groups, respectively, to increase the steric bulk to maximize the selectivity for nNOS over eNOS and to reduce the Log *P* values (vide infra). Compounds **23** and **25** showed increased potency across all NOS isoforms, particularly for eNOS (**23**: IC_{50} = 7.12 μM; **25**: IC_{50} = 3.36 μM, most potent compound for nNOS and eNOS isoforms) among the current series with good selectivity for nNOS over eNOS (eNOS/nNOS = 71 for **23**, 48 for **25**). The cyclic amine side chains showed very good potency for nNOS and selectivity over eNOS (eNOS/nNOS = 40 for (±)-**36** and 183 for **37**). Moving the basic amine out of the cyclic ring (*cis*-**45** and *trans*-**45**) not only increase the side chain length by an extra carbon but also change the orientation and position of the basic amine, which is also considered as an important contributing factor for the human nNOS selectivity. *cis*-**45** and *trans*-**45** showed very good selectivity for nNOS over eNOS (527, 86 fold for *cis*-**45** and *trans*-**45**, respectively). There is a clear difference between the diastereomers as both the potency for nNOS and the selectivity for nNOS over eNOS reside in the *cis*- isomer.

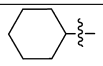
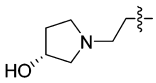
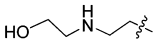
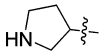
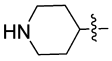
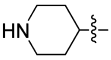
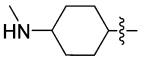
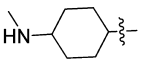
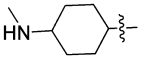
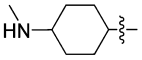
The polar and acidic residues in the nNOS active site require the inhibitor to contain polar and positively charged functional group such as a basic amine (prevailing as protonated at physiological pH), which is not an ideal combination due to blood−brain barrier and cell membrane permeability issues. In an effort to increase the nonprotonated species, it was envisioned that a fluorine substitution at the ortho- position (to the amidine group) should be able to lower the basicity of the amidine group while retaining the nNOS activity and selectivity over eNOS.[29] Accordingly, **38**, *cis*-**49**, and *trans*-**49** were prepared with a fluorine substitution ortho- to the amidine group in order to compare their nNOS activity and selectivity over eNOS with that of **37**, *cis*-**45**, and *trans*-**45**. The fluoro derivative **38** showed weaker potency across all isoforms (35-fold for nNOS, 2.5-fold for eNOS, and 201-fold for iNOS) when compared to **37** without a fluorine substitution. This may be due to restricted flexibility with the fluorine substitution, which may be preventing a favorable binding orientation as in case of **37** without fluorine substitution. Similar results were observed with *cis*-**49** and *trans*-**49**, where both the compounds showed weaker potencies across all NOS isoforms when compared to the nonfluorinated compounds *cis*-**45** and *trans*-**45** and is consistent with our previous observation with tetrahydroquinoline-based nNOS inhibitors.[18] Compound **25** was the most potent compound (nNOS IC_{50} = 0.07 μM) among the current series with good selectivity (∼50-fold) over both eNOS and iNOS isoforms, whereas *cis*-**45** was the most selective for nNOS isoform over eNOS (eNOS/nNOS = 527-fold) and iNOS (iNOS/nNOS = 224-fold) isoforms. On the basis of the potency at the nNOS isoform and selectivity over eNOS, *cis*-**45** was considered for further evaluation in a variety of in vitro and in vivo assays.

The Chung or Spinal Nerve Ligation (SNL) Model Study with *cis*-45. To investigate the potential therapeutic use of these selective nNOS inhibitors, *cis*-**45** was evaluated in an in vivo model of neuropathic pain (Figure 2).[30] 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 produces thermal and mechanical allodynia of the affected paw. Withdrawal latency following the application of radiant heat to the paw was tested. Intraperitoneal administration of *cis*-**45** at 30 mg/kg resulted in a partial reversal of thermal hyperalgesia with a maximum reversal effect of 66% observed at 30 min. The poor in vivo activity of *cis*-**45** may be attributed to its 5-fold weaker inhibitory activity in rat nNOS isoform (IC_{50} = 1.93

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943−955

Table 1. Inhibition of Human NOS Enzymes by 1, 6-Disubstituted Indoline Derivatives[g]



Compound No.	X	R	Human NOS IC_{50} (μM)[a]			Selectivity	
			nNOS	eNOS	iNOS	eNOS/nNOS	iNOS/nNOS
14	H		4.07 (0.82-20.0)	109 (69.8-170.8)	>100[b]	27	NC[c]
15	H		3.26 (2.37-4.49)	84.8 (42.8-168.1)	>100[b]	26	NC[c]
16	H		2.07 (1.30-3.27)	21.5 (2.27-202.8)	1.12 (0.60-2.07)	10	0.5
23	H		0.11 (0.07-0.15)	7.12 (4.85-10.43)	6.79 (5.70-8.09)	65	62
25	H		0.07 (0.05-0.09)	3.36 (2.12-5.31)	3.41 (2.38-4.16)	48	49
(±)-**36**	H		0.18 (0.13-0.26)	7.17 (3.63-14.12)	8.94 (7.46-10.7)	40	50
37	H		0.54 (0.44-0.67)	99.0 (66.0-163)	2.39 (1.79-3.19)	183	4
38	F		18.8 (10.7-32.8)	243 (189-310)	481 (323-714)	13	26
cis-**45**	H		0.37 (0.07-1.93) 1.93 (1.29-2.87)[d]	195 (146-259) 4.16 (3.04-5.68)[d]	83 (32.3-212) 2.34 (1.42-3.86)[d]	527 2	224 1
trans-**45**	H		2.12 (1.05-4.24)	237 (182-308)	189 (118-301)	112	89
cis-**49**	F		5.82 (3.59-9.42)	220 (141-342)	~ 897	38	154
trans-**49**	F		5.75 (2.21-14.8)	231 (171-311)	522 (377-722)	41	91
1[e]			0.95 (0.63-1.4)	0.65 (0.45-0.94)	1.8 (0.47-6.7)	0.7	2
5[f]			0.098 (0.05-0.19)	45.6 (8.6-242)	25.7 (17.0-39.0)	465	92

[a]In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline. [b]>100, Not active at the maximum tested concentration of 100 μM. [c]NC, Not calculable. [d]Inhibitory values are for rat NOS isoforms. [e]Compound **1** is a known nonselective NOS inhibitor; tested for comparison. [f]Compound **5** is a recently reported tetrahydroquinoline-based selective nNOS inhibitor; included for comparison. [g]Values reported in parentheses are 95% confidence intervals.

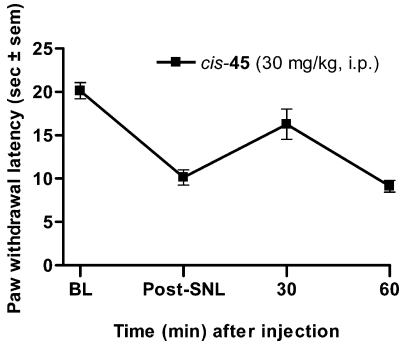


Figure 2. Compound *cis*-**45** attenuates thermal hyperalgesia in the L_5/L_6 SNL model of neuropathic pain. The mean values reported are from six animals.

μM) compared to its human nNOS isoform (IC_{50} = 0.37 μM). In addition *cis*-**45** was shown to be nonselective among the rat NOS isoforms (eNOS/nNOS = 2, iNOS/nNOS = 1), indicating a significant inhibitory activity difference between human and rat NOS enzymes, which is consistent with our previous observation with 2-aminobenzothiazole compounds.[14]

hERG K^+ Channel Inhibition Studies with *cis*-45. Antiarrhythmic drugs that induce QT prolongation by inhibiting the hERG K^+ channel has been linked to drug induced sudden cardiac death and are now the second leading cause for withdrawing approved drugs from the market.[31] Reference compound **5**, when tested in human recombinant HEK-293 cell lines for its ability to inhibit the hERG K^+ channel, showed moderate inhibitory activity (IC_{50} = 4.7 μM),[32] which poses a significant hurdle for the rapid drug development process and also receive an increased regulatory

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943−955

attention.[33] The hERG activity of **5** was assumed to be associated with the higher lipophilicity (clogP = 4.44) of the molecule. Reducing the lipophilicity of the molecule to control the hERG activity is one of the well-known phenomenon reported in the literature.[34] On the basis of the literature, a discrete structural modification from tetrahydroquinoline to indoline was designed, which will not only change the physicochemical properties but also reduce the lipophilicity (*cis*-**45**: clogP = 3.15 vs 4.44 for **5**) by one log unit due to its smaller ring size. To compare the hERG activity (conventional patch-clamp assay) of the lead candidate *cis*-**45** with reference **5**, *cis*-**45** was tested in human recombinant HEK-293 cell lines for its ability to inhibit the hERG K^+ channel. Compound *cis*-**45** showed ~3-fold weaker functional activity against hERG K^+ channel with IC_{50} of 13 μM.[32]

eNOS Mediated Vasoconstriction Effect on Isolated Human Resistance Arteries with *cis*-45. *cis*-**45** was assessed for the contractile response (inhibition of acetylcholine (ACh)-mediated vasorelaxation) on isolated human resistance arteries to assess the undesirable cardiovascular effect associated with the inhibition of eNOS in absence and presence of L-arginine (substrate for eNOS) with positive (**1**), negative (vehicle), and reference compound **5** (Figures 3, 4, and 5). The arteries were

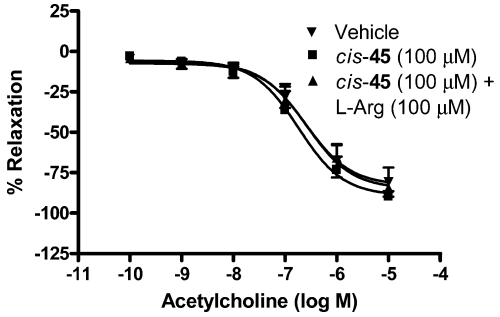


Figure 3. Effect of *cis*-**45** on the responses to ACh in absence and presence of L-arginine. No significant vasoconstriction effect was observed with *cis*-**45**.

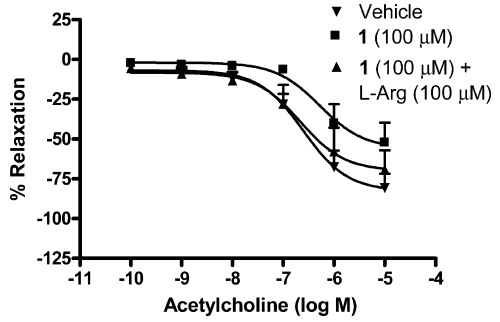


Figure 4. Effect of **1** on the responses to ACh in absence and presence of L-arginine. Significant vasoconstriction effect was observed with **1** (due to eNOS inhibition), which is reversed with the addition of L-arginine (substrate for eNOS), indicating that the effect is due to the eNOS inhibition.

preconstricted with U46619, a thromboxane A2 (TxA2) mimetic agent, and then exposed to ACh, an endothelium and nitric oxide dependent vasodilator. The response to ACh provides information on the activity of eNOS in an active human biological tissue and thereby any inhibitory effect of *cis*-**45** on eNOS. Furthermore, if inhibition of relaxation is due to the inhibition of eNOS, the addition of L-arginine (substrate for

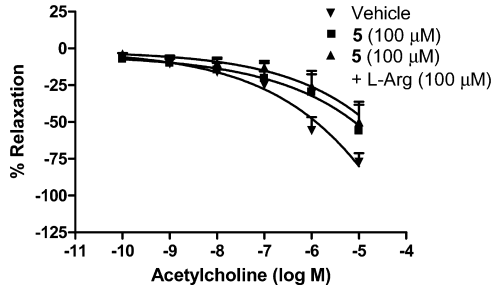


Figure 5. Effect of **5** on the responses to ACh in absence and presence of L-arginine. Significant vasoconstriction effect was observed with **5** (due to an unknown mechanism), which is not reversed with the addition of L-arginine (substrate for eNOS), indicating that the effect is not due to the eNOS inhibition.

eNOS) would recover the relaxation due to ACh. No significant inhibition with *cis*-**45** on the ability of the blood vessels to respond to ACh was observed at 100 μM concentration (Figure 3). Furthermore, the addition of L-arginine did not significantly affect the response to ACh in the presence of *cis*-**45**, providing further evidence that *cis*-**45** did not have an effect on agonist-mediated activation of human eNOS. The data suggests that *cis*-**45** would be devoid of any vasoconstrictive effect at concentrations that are physiologically relevant based on its human eNOS potency (IC_{50} = 195 μM).

In contrast, **1** and **5** both caused vasoconstriction at 100 μM concentration (Figure 4 and 5). The vasoconstriction effect caused by a nonselective NOS inhibitor **1** (due to eNOS inhibition) was reversed with the addition of L-arginine (100 μM) (Figure 4), whereas vasoconstriction effect caused by selective nNOS inhibitor **5** was not reversed with the addition of L-arginine (100 μM) (Figure 5), which indicates that the vasoconstriction effect is not due to the eNOS inhibition, but through an unknown mechanism, once again attributable to its higher lipophilicity (vide supra). Even though **5** is very selective for nNOS over eNOS (465) with weak inhibitory activity at eNOS isoform (IC_{50} = 45.6 μM), it still possess a cardiovascular liability and was hindered from further development. Where as *cis*-**45**, with its lower lipophilicity mitigates the cardiovascular liabilities and further evaluated in various in vitro assays to determine the side activity.

High Throughput Profile of *cis*-45. To identify off-target activities, *cis*-**45** was tested in 80 validated in vitro pharmacological assays (40 nonpeptidic receptors, 30 peptidic receptors, 2 nuclear receptors, 5 ion channels, and 3 transporters) that cover a broad range of CNS related targets.[35] This high throughput profile is used to identify the potential limitations and liabilities of the selection candidate associated with off-target activities to minimize the risks and overall cost associated with the drug development process. This process is also a rapid and cost-effective way of prioritizing the most promising compounds such as *cis*-**45** for further evaluation in various preclinical toxicology studies during the selection process. Accordingly, *cis*-**45** was tested at a single concentration of 10 μM in 80 different pharmacological assays to identify the off-target activities (Table 1 from Supporting Information). *cis*-**45** was found to be selective for most of the targets tested (<50% inhibition at 68 out of 80 receptors). *cis*-**45** showed weak inhibition at: opioid receptors μ (52%) and κ (69%), σ receptor (σ, 72%), α-2 adrenergic receptor (α_2, 58%), and serotonin transporters 5-HT_{1B} (60%) and 5-HT_{5a} (59%). *cis*-**45** showed strong inhibition at human muscarinic receptors M_1

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943–955

(80%), M_2 (80%), M_3 (85%), M_4 (95%), and M_5 (90%) and human norepinephrine transporter (86%). As a follow-up to the high throughput profile, the IC_{50} for norepinephrine transporter was calculated as 4.4 μM, which is considered to be very weak to have any effect at concentrations that are physiologically relevant. Overall, *cis*-**45** possesses an excellent in vitro safety profile.

CONCLUSIONS

In conclusion, a series of 1,6-disubstituted indoline derivatives were synthesized and shown to be selective inhibitors of human nNOS over eNOS and iNOS isoforms. Compounds with bulky cyclic side chain (**14**−**16**) without a basic amine showed up to 20-fold weaker potency when compared to a similar compound with basic amine side chain (**37**). This indicates that the presence of a basic amine is an important contributing factor to obtain submicromolar inhibitory potency for the nNOS isoform. A bulky cyclic amino- substituent at the 1-position of the indoline ring such as 4-(methylamino)cyclohexyl- group (*cis*-**45** and *trans*-**45**) provided better selectivity over pyrrolidine ((±)-**36**) or piperidine (**37**) based cyclic amine substitutions. Substitution of fluorine ortho- to the thiophene amidine group (5-position of the indoline ring) dramatically reduced the potency across all NOS isoforms, indicating an unfavorable orientation in the enzyme active site in this particular 1,6-disubstituted indoline derivatives. Intraperitoneal administration of *cis*-**45** was shown to reverse the thermal hyperalgesia in the SNL model of neuropathic pain. At the same time, *cis*-**45** is devoid of any cardiovascular liabilities associated with QT prolongation with hERG K^+ channel binding or vasoconstriction on human resistance arteries associated with the inhibition of human eNOS when compared to reference compound **5**. *cis*-**45** does not have any off-target activities (80 receptors) associated with the side effect profile, making it an ideal candidate to carry forward through the preclinical development process such as toxicological evaluations. The significant in vitro safety results presented in this communication with *cis*-**45** provide an opportunity to investigate the role that the nNOS enzyme plays in CNS related disorders such as migraine and chronic tension type headache (CTTH).

EXPERIMENTAL SECTION

General. All 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 MHz spectrometer. 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 Agilent Zorbax, SB-C18, and Waters XTerra RP8.5 μM reverse phase columns, and the purity was determined to be ≥95% for all final compounds. No attempts were made to optimize the yields.

1-Cyclohexyl-6-nitroindoline (10). A solution of 6-nitroindoline (**6**) (0.5 g, 3.05 mmol) in dry 1,2-dichloroethane (10 mL) was treated with cyclohexanone (**7**) (0.63 mL, 6.09 mmol), followed by acetic acid (0.43 mL, 7.61 mmol) and sodium triacetoxyborohydride (0.96 g, 4.57 mmol) at room temperature, and the resulting mixture was stirred for 3 h. The reaction was basified with 1 N NaOH 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 (EtOAc:hexanes, 1:9) on silica gel to obtain the title compound (0.72 g, 96%) as a dark-brown solid. ^{1}H NMR ($CDCl_3$) δ 7.46 (dd, 1H, J = 2.1, 8.1 Hz), 7.09−7.04 (m, 2H), 3.52 (t, 2H, J = 8.4 Hz), 3.42−3.34 (m, 1H), 3.01 (t, 2H, J = 8.4 Hz), 1.85−1.70 (m, 5H), 1.45−1.30 (m, 4H), 1.19−1.11 (m, 1H). ESI-MS (m/z, %): 247 (MH^+, 100), 165 (61), 119 (39).

6-Nitro-1-(tetrahydro-2*H*-pyran-4-yl)indoline (11). Prepared from 6-nitroindoline (**6**) (0.5 g, 3.05 mmol) and dihydro-2*H*-pyran-4(3*H*)-one (**8**) (0.562 mL, 6.09 mmol) as described for **10** to obtain the title compound (0.75 g, 99%) as a dark-red solid. ^{1}H NMR ($CDCl_3$) δ 7.49 (dd, 1H, J = 1.8, 7.9 Hz), 7.12−7.07 (m, 2H), 4.12−4.07 (m, 2H), 3.68−3.60 (m, 1H), 3.56−3.48 (m, 4H), 3.04 (t, 2H, J = 8.4 Hz), 1.81−1.73 (m, 4H). ESI-MS (m/z, %): 271 (M + Na, 100), 249 (MH^+, 76).

6-Nitro-1-(tetrahydro-2*H*-thiopyran-4-yl)indoline (12). Prepared from 6-nitroindoline (**6**) (0.6 g, 3.65 mmol) and dihydro-2*H*-thiopyran-4(3*H*)-one (**9**) (0.84 g, 7.31 mmol) as described for **10** to obtain the title compound (0.95 g, 98%) as an orange solid. ^{1}H NMR ($CDCl_3$) δ 7.48 (dd, 1H, J = 2.1, 7.8 Hz), 7.09−7.05 (m, 2H), 3.53 (t, 2H, J = 8.7 Hz), 3.43−3.34 (m, 1H), 3.03 (t, 2H, J = 8.7 Hz), 2.91−2.71 (m, 4H), 2.20−2.04 (m, 2H), 1.89−1.68 (m, 2H). ESI-MS (m/z, %): 287 (M + Na, 95), 265 (MH^+, 100).

***N*-(1-Cyclohexylindolin-6-yl)thiophene-2-carboximidamide (14).** A suspension of 1-cyclohexyl-6-nitroindoline (**10**) (0.6 g, 2.43 mmol) in dry methanol (10 mL) was treated with hydrazine hydrate (0.88 mL, 24.36 mmol) followed by Raney-Ni (0.1 g, 2.43 mmol) at room temperature. The resulting suspension was refluxed for 5 min in a preheated oil bath. The reaction was brought to room temperature, filtered through Celite bed, and washed with methanol. The combined methanol layer was evaporated and crude was purified by flash column chromatography (EtOAc:hexanes, 1:3) to obtain 1-cyclohexylindolin-6-amine as a solid.

A solution of above 1-cyclohexylindolin-6-amine in dry ethanol (10 mL) was treated with methyl thiophene-2-carbimidothioate hydroiodide (**13**) (1.37 g, 4.86 mmol) at room temperature, and the resulting mixture was stirred at same temperature for overnight (18 h). The reaction was diluted 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 (EtOAc:hexanes, 1:2) to obtain the title compound (0.5 g, 73.8%, over two steps) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.69 (d, 1H, J = 2.7 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.07 (dd, 1H, J = 3.9, 4.9 Hz), 6.90 (d, 1H, J = 7.5 Hz), 6.23 (s, 2H), 5.98 (d, 1H, J = 7.5 Hz), 5.90 (s, 1H), 3.35−3.29 (m, 3H, merged with water peak), 2.80 (t, 2H, J = 8.4 Hz), 1.73−1.59 (m, 5H), 1.36−1.26 (m, 4H), 1.17−1.07 (m, 1H). ESI-MS (m/z, %): 326 (MH^+, 100). ESI-HRMS calculated for $C_{19}H_{24}N_3S$ (MH^+), 326.1685; observed, 326.1678. HPLC purity: 97.82%.

***N*-(1-(Tetrahydro-2*H*-pyran-4-yl)indolin-6-yl)thiophene-2-carboximidamide (15).** Prepared from 6-nitro-1-(tetrahydro-2*H*-pyran-4-yl)indoline (**11**) (0.5 g, 2.01 mmol) as described for **14** to obtain the title compound (0.55 g, 92%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.69 (d, 1H, J = 3.3 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.07 (t, 1H, J = 3.9 Hz), 6.92 (d, 1H, J = 7.8 Hz), 6.23 (brs, 2H), 6.02−6.00 (m, 2H), 3.92−3.88 (m, 2H), 3.64−3.57 (m, 1H), 3.45−3.38 (m, 4H), 2.81 (t, 2H, J = 8.1 Hz), 1.64−1.60 (m, 4H). ESI-MS (m/z, %): 328 (MH^+, 100). ESI-HRMS calculated for $C_{18}H_{21}N_3OS$ (MH^+), 328.1478; observed, 328.1471. HPLC purity: 96.66%.

***N*-(1-(Tetrahydro-2*H*-thiopyran-4-yl)indolin-6-yl)thiophene-2-carboximidamide (16).** Prepared from 6-nitro-1-(tetrahydro-2*H*-thiopyran-4-yl)indoline (**12**) (0.5 g, 1.89 mmol) as described for **14** to obtain the title compound (0.54 g, 92%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.69 (d, 1H, J = 2.7 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.08 (t, 1H, J = 4.8 Hz), 6.91 (d, 1H, J = 7.5 Hz), 6.23 (brs, 2H), 6.00 (dd, 1H, J = 1.5, 7.5 Hz), 5.96 (s, 1H), 3.40−3.29 (m, 3H), 2.84−2.77 (m, 4H), 2.66−2.61 (m, 2H), 2.03−1.98 (m, 2H), 1.69−1.64 (m, 2H). ESI-MS (m/z, %): 344 (MH^+, 100). ESI-HRMS calculated for $C_{18}H_{22}N_3S_2$ (MH^+), 344.1249; observed, 344.1234. HPLC purity: 98.26%.

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943−955

2-Chloro-1-(6-nitroindolin-1-yl)ethanone (17). A suspension of 6-nitroindoline (**6**) (1.0 g, 6.09 mmol) in toluene (20 mL) was treated with chloroacetyl chloride (0.97 mL, 12.18 mmol) dropwise. The mixture was heated at 110 °C for 15 min, cooled to room temperature, and treated with saturated sodium bicarbonate solution. The product was extracted into ethyl acetate, washed with brine, and dried (Na_2SO_4). The crude was purified by flash column chromatography (EtOAc:hexanes, 5:95 to 1:1, then 2 M NH_3 MeOH: CH_2Cl_2, 1:9) on silica gel to obtain the title compound (1.22 g, 83%) as a light-brown foam. ^{1}H NMR ($CDCl_3$) δ 9.00 (s, 1H), 7.96 (dd, 1H, J = 2.1, 8.1 Hz), 7.32 (d, 1H, J = 8.1 Hz), 4.31 (t, 2H, J = 8.4 Hz), 4.18 (s, 2H), 3.35 (t, 2H, J = 8.7 Hz). ESI-MS (m/z, %): 263 (44, M + Na), 241 (MH^+, 100).

(*R*)-2-(3-Hydroxypyrrolidin-1-yl)-1-(6-nitroindolin-1-yl)-ethanone (18). A mixture of 2-chloro-1-(6-nitroindolin-1-yl)-ethanone (**17**) (1.1 g, 4.57 mmol), potassium carbonate (1.89 g, 13.71 mmol), and (*R*)-pyrrolidin-3-ol (0.55 mL, 6.86 mmol) in acetonitrile (20 mL) and H_2O (2 mL) was heated at 85 °C for 2 h. The dark mixture was concentrated and treated with H_2O under rapid stirring. The solid was filtered, washed with H_2O, and dried under reduced pressure to obtain the title compound (1.25 g, 94%) as a brown solid. ^{1}H NMR (DMSO-d_6) δ 8.80 (d, 1H, J = 1.5 Hz), 7.91 (dd, 1H, J = 2.1, 8.1 Hz), 7.48 (d, 1H, J = 8.4 Hz), 4.73 (brd, 1H, J = 4.5 Hz), 4.28–4.15 (m, 3H), 3.42 (brs, 2H), 3.25 (t, 2H, J = 8.7 Hz), 2.85 (dd, 1H, J = 6.0, 9.3 Hz), 2.72 (dd, 1H, J = 7.8, 15.6 Hz), 2.62–2.55 (m, 1H), 2.50–2.44 (m, 1H, overlap with DMSO peak), 2.05–1.93 (m, 1H), 1.60–1.52 (m, 1H). ESI-MS (m/z, %): 292 (MH^+, 100%).

2-(2-Hydroxyethylamino)-1-(6-nitroindolin-1-yl)ethanone (19). A suspension of 2-chloro-1-(6-nitroindolin-1-yl)ethanone (**17**) (1.5 g, 6.23 mmol), potassium carbonate (4.31 g, 31.2 mmol), and 2-aminoethanol (1.5 mL, 24.93 mmol) in a mixture of acetonitrile (20 mL) and water (4.00 mL) was heated at 55 °C for 2 h and then 100 °C for 30 min. The mixture was allowed to cool to room temperature, transferred to a separatory funnel containing EtOAc and H_2O, and then extracted. The organic layer was separated, dried (Na_2SO_4), filtered, and concentrated to give a brown residue. This residue was purified by flash column chromatography (MeOH:CH_2Cl_2, 5:95 to 2 M NH_3 in MeOH:CH_2Cl_2, 1:9) on silica gel to obtain the title compound (0.75 g, 45.4%) as a light-brown solid. ^{1}H NMR (DMSO-d_6) δ 8.81 (d, 1H, J = 2.1 Hz), 7.91 (dd, 1H, J = 2.1, 8.1 Hz), 7.49 (d, 1H, J = 8.1 Hz), 4.59–4.48 (m, 1H), 4.18 (t, 2H, J = 8.4 Hz), 3.53 (s, 2H), 3.49–3.47 (m, 2H), 3.27 (t, 2H, J = 8.4 Hz), 2.64 (t, 2H, J = 5.7 Hz), 2.16 (brs, 1H). ESI-MS (m/z, %): 266 (MH^+, 100%).

***tert*-Butyl 2-Hydroxyethyl(2-(6-nitroindolin-1-yl)-2-oxoethyl)carbamate (20).** A mixture of 2-(2-hydroxyethylamino)-1-(6-nitroindolin-1-yl)ethanone (**19**) (0.72 g, 2.71 mmol), di-*tert*-butyl dicarbonate (0.62 g, 2.85 mmol), and triethylamine (0.75 mL, 5.43 mmol) in dioxane (30 mL) was stirred at room temperature for 17 h. The solution was concentrated and subjected to flash chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 5:95) on silica gel to obtain the title compound (0.99 g, 100%) as a yellow residue. ^{1}H NMR (DMSO-d_6) δ 8.79 (brs, 1H), 7.93 (d, 1H, J = 7.8 Hz), 7.51 (d, 1H, J = 8.1 Hz), 4.69–4.64 (m, 1H), 4.21–4.15 (m, 4H), 3.57 (s, 2H), 3.55–3.49 (m, 2H), 3.35–3.23 (m, 2H, overlap with H_2O peak), 1.42 and 1.32 (2s, 9H). ESI-MS (m/z, %): 388 (MNa^+, 50%), 366 (MH^+, 35%), 266 (100%).

(*R*)-1-(2-(6-Nitroindolin-1-yl)ethyl)pyrrolidin-3-ol (21). A suspension of (*R*)-2-(3-hydroxypyrrolidin-1-yl)-1-(6-nitroindolin-1-yl)-ethanone (**18**) (1.22 g, 4.19 mmol) in THF (20 mL) at 0 °C was treated with 1 M borane in THF (20.94 mL, 20.94 mmol). The resulting yellow solution was allowed to warm to room temperature and stirred for 17 h. The yellow solution was cooled to 0 °C and quenched with MeOH (20 mL) dropwise with caution (very slowly at first). The resulting orange solution was concentrated, redissolved in MeOH (30 mL), and concentrated to dryness. This residue was purified by flash column chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 5:95) on silica gel to obtain the title compound (1.16 g, quantitative) as an orange–red residue.

***tert*-Butyl 2-Hydroxyethyl(2-(6-nitroindolin-1-yl)ethyl)-carbamate (22).** Prepared from *tert*-butyl 2-hydroxyethyl(2-(6-nitroindolin-1-yl)-2-oxoethyl)carbamate (**20**) (0.97 g, 2.65 mmol) as described for **21** to obtain the title compound (0.9 g, 96%) as an orange–red residue. ^{1}H NMR (DMSO-d_6) δ 7.41–7.37 (m, 1H), 7.21–7.16 (m, 1H), 7.12–7.10 (m, 1H), 4.71 (t, 1H, J = 5.4 Hz), 3.58 (t, 2H, J = 8.4 Hz), 3.49–3.46 (m, 2H), 3.40–3.35 (m, 4H), 3.29–3.23 (m, 2H), 3.04–2.95 (m, 2H), 1.35 and 1.31 (2s, 9H). ESI-MS (m/z, %): 374 (MNa^+, 75%), 352 (MH^+, 25%), 296 (95%), 252 (100%).

(*R*)-*N*-(1-(2-(3-Hydroxypyrrolidin-1-yl)ethyl)indolin-6-yl)-thiophene-2-carboximidamide (23). Prepared from (*R*)-1-(2-(6-nitroindolin-1-yl)ethyl)pyrrolidin-3-ol (**21**) (1.24 g, 4.47 mmol) as described for **14** to obtain the title compound (0.113 g, 7%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.70 (dd, 1H, J = 0.9, 3.6 Hz), 7.58 (dd, 1H, J = 0.6, 5.1 Hz), 7.07 (dd, 1H, J = 3.6, 5.1 Hz), 6.93 (d, 1H, J = 7.5 Hz), 6.26 (brs, 2H), 6.03 (brd, 1H, J = 8.7 Hz), 5.97 (brs, 1H), 4.67 (d, 1H, J = 4.8 Hz), 4.19–4.13 (m, 1H), 3.42–3.28 (m, 2H), 3.11 (t, 2H, J = 6.9 Hz), 2.83 (brt, 2H, J = 7.8 Hz), 2.73 (dd, 1H, J = 6.0, 9.3 Hz), 2.64–2.53 (m, 3H), 2.47–2.42 (m, 1H), 2.34 (dd, 1H, J = 3.9, 9.6 Hz), 2.01–1.89 (m, 1H), 1.56–1.46 (m, 1H). ESI-MS (m/z, %): 357 (MH^+, 100%). ESI-HRMS calculated for $C_{19}H_{25}N_4OS$ (MH^+), 357.1743; observed, 357.1742. HPLC purity: 97.88%.

***tert*-Butyl 2-Hydroxyethyl(2-(6-(thiophene-2-carboximidamido)indolin-1-yl)ethyl)carbamate (24).** Prepared from *tert*-butyl 2-hydroxyethyl(2-(6-nitroindolin-1-yl)ethyl)carbamate (**22**) (0.89 g, 2.53 mmol) as described for **14** to obtain the title compound (0.57 g, 53%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 7.69 (d, 1H, J = 3.3 Hz), 7.58 (d, 1H, J = 4.8 Hz), 7.08 (pseudo t, 1H, J = 3.9 Hz), 6.92 (d, 1H, J = 7.8 Hz), 6.25 (brs, 2H), 6.06–5.99 (m, 2H), 4.68 (t, 1H, J = 5.4 Hz), 3.47–3.32 (m, 6H, overlap with H_2O peak), 3.25–3.12 (m, 4H), 2.85 (t, 2H, J = 7.5 Hz), 1.37 (s, 9H). ESI-MS (m/z, %): 431 (MH^+, 100%).

***N*-(1-(2-(2-Hydroxyethylamino)ethyl)indolin-6-yl)thiophene-2-carboximidamide (25).** A solution of *tert*-butyl 2-hydroxyethyl(2-(6-(thiophene-2-carboximidamido)indolin-1-yl)ethyl)carbamate (**24**) (0.55 g, 1.27 mmol) in MeOH (10 mL) was treated with 3 N HCl (4.26 mL, 12.77 mmol) and heated at 90 °C for 30 min. The yellow solution was concentrated, diluted with H_2O (20 mL), and washed with CH_2Cl_2. The aqueous layer was concentrated and dried to obtain dihydrochloride salt of the title compound (0.45 g, 87%) as a brown solid. ^{1}H NMR (DMSO-d_6) δ 11.50 (brs, 1H), 9.81 (s, 1H), 9.15 (brs, 2H), 8.76 (brs, 1H), 8.16 (d, 2H, J = 4.8 Hz), 7.37 (pseudo t, 1H, J = 4.2 Hz), 7.18 (d, 1H, J = 7.5 Hz), 6.69 (s, 1H), 6.62 (d, 1H, J = 7.5 Hz), 3.70 (t, 2H, J = 5.1 Hz), 3.52–3.46 (m, 4H), 3.16 (brs, 2H), 3.02–2.95 (m, 4H). ESI-MS (m/z, %): 331 (MH^+, free base, 100%). ESI-HRMS calculated for $C_{17}H_{23}N_4OS$ (MH^+, free base), 331.1587; observed, 331.1587. HPLC purity: 96.43%.

***tert*-Butyl 3-(6-Bromoindolin-1-yl)pyrrolidine-1-carboxylate (30).** Prepared from 6-bromoindiline (**26**) (5.0 g, 25.2 mmol) and *tert*-butyl 3-oxopyrrolidine-1-carboxylate (**28**) (5.84 g, 31.6 mmol) as described for **10** to obtain the title compound (7.91 g, 85%) as a syrup. ^{1}H NMR (DMSO-d_6) δ 6.93 (d, 1H, J = 7.5 Hz), 6.69–6.65 (m, 2H), 4.28–4.16 (m, 1H), 3.49–3.16 (m, 6H), 2.83 (t, 2H, J = 8.4 Hz), 2.07–1.09 (m, 2H), 1.40 (s, 9H). ESI-MS (m/z, %): 391 and 389 (MNa^+, 65%), 369 and 367 (MH^+, 2%), 313 and 311 (100%).

***tert*-Butyl 4-(6-Nitroindolin-1-yl)piperidine-1-carboxylate (31).** Prepared from 6-nitroindoline (**6**) (5.0 g, 30.5 mmol) and *tert*-butyl 4-oxopiperidine-1-carboxylate (**29**) (12.14 g, 60.9 mmol) as described for **10** to obtain the title compound (8.88 g, 84%). ^{1}H NMR (DMSO-d_6) δ 7.40 (dd, 1H, J = 8.1, 2.1 Hz), 7.19 (d, 1H, J = 8.1 Hz), 7.17 (d, 1H, J = 2.1 Hz), 4.04 (brd, 2H, J = 11.7 Hz), 3.81–3.71 (m, 1H), 3.47 (t, 2H, J = 8.6 Hz), 3.00 (t, 2H, J = 8.6 Hz), 2.95–2.75 (m, 2H), 1.64 (brd, 2H, J = 11.1 Hz), 1.52–1.42 (m, 2H), 1.40 (s, 9H). EI-MS (m/z, %): 347 (M^+, 100), 274 (78).

***tert*-Butyl 4-(5-Fluoro-6-nitroindolin-1-yl)piperidine-1-carboxylate (32).** Prepared from 5-fluoro-6-nitroindoline (**27**) (0.115 g, 0.63 mmol) and *tert*-butyl 4-oxo-1-piperidinecarboxylate (**29**) (0.25 g, 1.26 mmol) as described for **10** to obtain the title compound (0.206 g, 89%) as an orange oil. ^{1}H NMR (DMSO-d_6) δ 7.23 (d, 1H, J = 11.0

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943–955

Hz), 7.02 (d, 1H, J = 5.9 Hz), 4.03 (app d, 2H, J = 12.0 Hz), 3.77−3.67 (m, 1H), 3.42 (app t, 2H, J = 8.5 Hz), 2.99 (app t, 2H, J = 8.3 Hz), 2.93−2.70 (m, 2H), 1.73−1.58 (m, 2H), 1.51−1.40 (m, 2H), 1.40 (s, 9H).

***tert*-Butyl 3-(6-(Thiophene-2-carboximidamido)indolin-1-yl)pyrrolidine-1-carboxylate (33).** A suspension of tris-(dibenzylideneacetone)dipalladium(0) (0.125 g, 0.13 mmol) in THF (3 mL) was treated with tri*tert*-butylphosphine in hexanes, 10 wt % (1.65 mL, 0.54 mmol) and then stirred for 5 min at room temperature. To this mixture was added lithium hexamethyldisilizane (1 M in THF) (5.45 mL, 5.45 mmol) and *tert*-butyl 3-(6-bromoindolin-1-yl)-pyrrolidine-1-carboxylate (**30**) (1.0 g, 2.72 mmol) in THF (20 mL). The reaction vessel was sealed and the dark mixture was heated at 100 °C for 3 h and then allowed to cool to room temperature, treated with tetrabutylammonium fluoride (1 M in THF) (13.61 mL, 13.61 mmol), and stirred for 30 min. The mixture was concentrated and partitioned between EtOAc and H_2O in a separatory funnel and extracted. The organic layer was separated and rinsed with brine, dried (Na_2SO_4), filtered, and concentrated to give a dark residue. This residue was purified by silica gel column chromatography using the Biotage purification system (column:, Silicycle 80 g; EtOAc:hexanes, 1:3 gradient to EtOAc over 10 column volumes; flow rate, 30 mL/min; collection wavelength, 254 nm) to obtain *tert*-butyl 3-(6-aminoindolin-1-yl)pyrrolidine-1-carboxylate as a light-brown semisolid.

A solution of above *tert*-butyl 3-(6-aminoindolin-1-yl)pyrrolidine-1-carboxylate in dry EtOH (20 mL) was treated with methyl thiophene-2-carbimidothioate hydroiodide (**13**) (1.53 g, 5.44 mmol) and stirred at room temperature for 17 h. The reaction was worked up and purified as described for **14** to obtain the title compound (0.7 g, 77%, over two steps) as a light-yellow solid. ^{1}H NMR (DMSO-d_6) δ 7.72 (d, 1H, J = 8.7 Hz), 7.59 (d, 1H, J = 4.8 Hz), 7.08 (dd, 1H, J = 3.9, 4.8 Hz), 6.94 (d, 1H, J = 7.5 Hz), 6.32 (brs, 2H), 6.09−6.07 (m, 2H), 4.28−4.08 (m, 1H), 3.49−3.14 (m, 6H, overlap with H_2O peak), 2.82 (t, 2H, J = 7.8 Hz), 2.08−1.91 (m, 2H), 1.39 (s, 9H). ESI-MS (m/z, %): 413 (MH^+, 100%).

***tert*-Butyl 4-(6-(Thiophene-2-carboximidamido)indolin-1-yl)piperidine-1-carboxylate (34).** Prepared from *tert*-butyl 4-(6-nitroindolin-1-yl)piperidine-1-carboxylate (**31**) (1.0 g, 2.88 mmol) as described for **14** to obtain the title compound (1.08 g, 90%). ^{1}H NMR (DMSO-d_6) δ 7.74−7.69 (m, 1H), 7.61−7.57 (m, 1H), 7.12−7.06 (m, 1H), 7.93−6.90 (m, 1H), 6.23 (brs, 2H), 6.03−6.00 (m, 2H), 4.12−3.99 (m, 3H), 3.61−3.52 (m, 1H), 3.33 (m, 2H), 2.84−2.78 (m, 3H), 1.72−1.62 (m, 2H), 1.49−1.39 (m, 11H). EI-MS (m/z, %): 426 (M^+, 100).

***tert*-Butyl 4-(5-Fluoro-6-(thiophene-2-carboximidamido)-indolin-1-yl)piperidine-1-carboxylate (35).** Prepared from *tert*-butyl 4-(5-fluoro-6-nitroindolin-1-yl)piperidine-1-carboxylate (**32**) (0.20 g, 0.54 mmol) as described for **14** to obtain the title compound (0.15 g, 56%) as a sticky yellow residue. ^{1}H NMR (DMSO-d_6) δ 7.74 (d, 1H, J = 4.2 Hz), 7.63 (d, 1H, J = 4.9 Hz), 7.16−7.05 (m, 1H), 6.68 (d, 1H, J = 10.0 Hz), 6.53 (brs, 2H), 6.06 (d, 1H, J = 6.8 Hz), 4.10−3.92 (m, 2H), 3.60−3.45 (m, 1H), 3.30−3.19 (m, 2H), 2.92−2.65 (m, 4H), 1.73−1.57 (m, 2H), 1.49−1.30 (m, 11H). ^{19}F NMR (DMSO-d_6) δ −137.856 to −137.924 (m). ESI-MS (m/z, %): 445 (MH^+, 100).

***N*-(1-(Pyrrolidin-3-yl)indolin-6-yl)thiophene-2-carboximidamide (36).** A solution of *tert*-butyl 3-(6-(thiophene-2-carboximidamido)indolin-1-yl)pyrrolidine-1-carboxylate (**33**) (0.65 g, 1.57 mmol) in MeOH (15 mL) was treated with 3 N HCl (5.25 mL, 15.76 mmol) and then heated at 100 °C for 30 min. The mixture was concentrated to remove most of the methanol, diluted with H_2O (20 mL), and washed with CH_2Cl_2. The aqueous layer was basified with 3 N NaOH solution (~15 mL) and extracted into CH_2Cl_2. The organic layer was separated, dried (Na_2SO_4), filtered, and concentrated to obtain the title compound (0.435 g, 88%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 7.69 (d, 1H, J = 3.0 Hz), 7.58 (dd, 1H, J = 0.9, 5.1 Hz), 7.08 (dd, 1H, J = 3.6, 4.8 Hz), 6.92 (d, 1H, J = 7.2 Hz), 6.26 (brs, 2H), 6.06−6.02 (m, 2H), 4.03−3.91 (m, 1H), 3.34 (t, 2H, J = 8.4 Hz, overlap with H_2O peak), 2.97 (dd, 1H, J = 4.5, 8.1 Hz), 2.87−2.69 (m, 5H), 1.94−1.83 (m, 1H), 1.77−1.65 (m, 1H). ESI-MS (m/z, %): 313 (MH^+, 100%). ESI-HRMS calculated for $C_{17}H_{21}N_4S$ (MH^+), 313.1481; observed, 313.1471. HPLC purity: 99.57%.

***N*-(1-(Piperidin-4-yl)indolin-6-yl)thiophene-2-carboximidamide (37).** Prepared from *tert*-butyl 4-(6-(thiophene-2-carboximidamido)indolin-1-yl)piperidine-1-carboxylate (**34**) (1.05 g, 2.46 mmol) as described for **36** to obtain the title compound (0.214 g, 27%). ^{1}H NMR (DMSO-d_6) δ 7.69 (d, 1H, J = 3.0 Hz), 7.58 (d, 1H, J = 5.1 Hz), 7.08 (dd, 1H, J = 5.1, 3.9 Hz), 6.91 (d, 1H, J = 7.5 Hz), 6.20 (brs, 2H), 6.00 (d, 1H, J = 7.5 Hz), 5.94 (s, 1H), 3.45−3.29 (m, 3H), 2.98 (m, 2H), 2.81 (t, 2H, J = 8.2 Hz), 2.57−2.49 (m, 2H), 1.64−1.60 (m, 2H), 1.52−1.38 (m, 2H). EI-MS (m/z, %): 326 (M^+, 100). EI-HRMS calculated for $C_{18}H_{22}N_4S$ (M^+), 326.1564; observed, 326.1565. HPLC purity: 97.99%.

***N*-(5-Fluoro-1-(piperidin-4-yl)indolin-6-yl)thiophene-2-carboximidamide (38).** Prepared from *tert*-butyl 4-(5-fluoro-6-(thiophene-2-carboximidamido)indolin-1-yl)piperidine-1-carboxylate (**35**) (0.125 g, 0.28 mmol) as described for **36** to obtain the title compound (0.067 g, 69%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 7.71 (d, 1H, J = 3.5 Hz), 7.60 (d, 1H, J = 5.0 Hz), 7.12−7.04 (m, 1H), 6.84 (d, 1H, J = 10.1 Hz), 6.43 (brs, 2H), 5.97 (d, 1H, J = 6.8 Hz), 3.44−3.19 (m, 3H, merged with H_2O peak), 2.98 (app d, 2H, J = 11.9 Hz), 2.81 (t, 2H, J = 8.0 Hz), 2.61−2.50 (m, 2H), 1.69−1.52 (m, 2H), 1.46−1.33 (m, 2H). ^{19}F NMR (DMSO-d_6) δ −138.130 to −138.190 (m). ESI-MS (m/z, %): 345 (MH^+, 100). ESI-HRMS calculated for $C_{18}H_{22}N_4FS$ (MH^+), 345.1543; observed, 345.1541. HPLC purity: 98.21%.

6-Nitro-1-(1,4-dioxaspiro[4.5]decan-8-yl)indoline (40). Prepared from 6-nitroindoline (**6**) (3.0 g, 18.27 mmol) and 1,4-dioxaspiro[4.5]decan-8-one (**39**) (4.28 g, 27.4 mmol) as described for **10** to obtain the title compound (5.02 g, 90%) as a dark-orange solid. ^{1}H NMR ($CDCl_3$) δ 7.46 (dd, 1H, J = 2.1, 7.8 Hz), 7.08−7.04 (m, 2H), 3.96 (s, 4H), 3.55−3.43 (m, 3H), 3.01 (t, 2H, J = 8.4 Hz), 1.88−1.67 (m, 8H). ESI-MS (m/z, %): 305 (MH^+, 100).

4-(6-Nitroindolin-1-yl)cyclohexanone (41). To a solution of 6-nitro-1-(1,4-dioxaspiro[4.5]decan-8-yl)indoline (**40**) (4.8 g, 15.77 mmol) in acetone (30 mL) was added hydrogen chloride (20 mL, 20.00 mmol) and the mixture left to stir at room temperature overnight. The solvent was removed and diluted with dichloromethane and 1 N NaOH solution. The organic layer was extracted, washed with brine, dried (Na_2SO_4), and solvent was evaporated to obtain the crude title compound (4.2 g, quantitative). ^{1}H NMR ($CDCl_3$) δ 7.53 (dd, 1H, J = 1.8, 7.8 Hz), 7.17 (d, 1H, J = 1.8 Hz), 7.10 (d, 1H, J = 7.8 Hz), 3.99−3.89 (m, 1H), 3.52 (t, 2H, J = 8.7 Hz), 3.05 (t, 2H, J = 8.7 Hz), 2.57−2.51 (m, 4H), 2.20−2.14 (m, 2H), 1.97−1.83 (m, 2H). ESI-MS (m/z, %): 261 (MH^+, 40).

***N*-Methyl-4-(6-nitroindolin-1-yl)cyclohexanamine (*cis*-42 and *trans*-42).** Prepared from 4-(6-nitroindolin-1-yl)cyclohexanone (**41**) (2.0 g, 7.68 mmol) and methylamine hydrochloride (0.62 g, 9.22 mmol) as described for **10** to obtain two partially separable mixture of diastereomers (1.21 g, 57%) in 1:1 ratio. *cis*-**42**: dark-orange solid; ^{1}H NMR ($CDCl_3$) δ 7.45 (dd, 1H, J = 2.1, 8.1 Hz), 7.06−7.04 (m, 2H), 3.56 (t, 2H, J = 8.7 Hz), 3.47−3.36 (m, 1H), 3.00 (t, 2H, J = 8.7 Hz), 2.83−2.81 (m, 1H), 2.44 (s, 3H), 1.93−1.87 (m, 2H), 1.83−1.74 (m, 2H), 1.68−1.57 (m, 4H); ESI-MS (m/z, %) 276 (MH^+, 100). *trans*-**42**: dark-orange solid; ^{1}H NMR ($CDCl_3$) δ 7.46 (dd, 1H, J = 1.8, 7.8 Hz), 7.07−7.04 (m, 2H), 3.51 (t, 2H, J = 8.7 Hz), 3.47−3.37 (m, 1H), 3.01 (t, 2H, J = 8.7 Hz), 2.45 (s, 3H), 2.41−2.33 (m, 1H),2.15−2.04 (m, 2H), 1.93−1.83 (m, 2H), 1.76−1.55 (m, 4H); ESI-MS (m/z, %): 276 (MH^+, 100), 245 (60).

***tert*-Butyl Methyl(4-(6-nitroindolin-1-yl)cyclohexyl)-carbamate (*cis*-43).** Prepared from *N*-methyl-4-(6-nitroindolin-1-yl)cyclohexanamine (*cis*-**42**) (0.15 g, 0.54 mmol) and di-*tert*-butyl dicarbonate (0.13 g, 0.59 mmol) as described for **20** to obtain the title compound as an orange solid (0.2 g, 98%). ^{1}H NMR ($CDCl_3$) δ 7.51 (dd, 1H, J = 2.1, 7.8 Hz), 7.12 (d, 1H, J = 2.1 Hz), 7.09 (d, 1H, J = 7.8 Hz), 4.03−3.95 (m, 1H), 3.70 (t, 2H, J = 8.1 Hz), 3.49−3.44 (m, 1H), 3.05 (t, 2H, J = 8.1 Hz), 2.78 (s, 3H), 2.17−2.10 (m, 2H), 1.79−1.62 (m, 6H), 1.47 (s, 9H). ESI-MS (m/z, %): 376 (MH^+, 10), 276 (95), 245 (100).

dx.doi.org/10.1021/jm201564u | *J. Med. Chem.* 2012, 55, 943−955

***tert*-Butyl Methyl(4-(6-nitroindolin-1-yl)cyclohexyl)-carbamate (*trans*-43).** Prepared from *N*-methyl-4-(6-nitroindolin-1-yl)cyclohexanamine (*trans*-**42**) (0.195 g, 0.70 mmol) and di-*tert*-butyl dicarbonate (0.17 g, 0.77 mmol) as described for **20** to obtain the title compound (0.26 g, 98%) as an orange solid. ^{1}H NMR ($CDCl_3$) δ 7.46 (dd, 1H, J = 2.1, 8.1 Hz), 7.07−7.05 (m, 2H), 4.07−3.95 (m, 1H), 3.50 (t, 2H, J = 8.7 Hz), 3.42−3.34 (m, 1H), 3.02 (t, 2H, J = 8.7 Hz), 2.76 (s, 3H), 1.92−1.81 (m, 4H), 1.68−1.55 (m, 4H), 1.47 (s, 9H). ESI-MS (m/z, %): 376 (MH^+, 10), 276 (90), 245 (100).

***tert*-Butyl Methyl(4-(6-(thiophene-2-carboximidamido)-indolin-1-yl)cyclohexyl)carbamate (*cis*-44).** Prepared from *tert*-butyl methyl(4-(6-nitroindolin-1-yl)cyclohexyl)carbamate (*cis*-**43**) (0.19 g, 0.50 mmol) as described for **14** to obtain the title compound (0.19 g, 90%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.42−7.38 (m, 2H), 7.11−7.05 (m, 1H), 7.01 (d, 1H, J = 7.5 Hz), 6.24 (dd, 1H, J = 1.8, 7.8 Hz), 6.12 (s, 1H), 5.71 (brs, 2H), 4.02−3.92 (m, 1H), 3.56 (t, 2H, J = 8.1 Hz), 3.38−3.31 (m, 1H), 2.93 (t, 2H, J = 8.1 Hz), 2.77 (s, 3H), 2.17−2.11 (m, 2H), 1.81−1.56 (m, 6H), 1.46 (s, 9H). ESI-MS (m/z, %): 455 (MH^+, 100).

***tert*-Butyl Methyl(4-(6-(thiophene-2-carboximidamido)-indolin-1-yl)cyclohexyl)carbamate (*trans*-44).** Prepared from *tert*-butyl methyl(4-(6-nitroindolin-1-yl)cyclohexyl)carbamate (*trans*-**43**) (0.25 g, 0.66 mmol) as described for **14** to obtain the title compound (0.22 g, 84%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.42−7.37 (m, 2H), 7.11−7.05 (m, 1H), 6.95 (d, 1H, J = 7.8 Hz), 6.21 (d, 1H, J = 7.2 Hz), 6.08 (s, 1H), 4.87 (brs, 2H), 4.02−3.90 (m, 1H), 3.40−3.24 (m, 3H), 2.91 (t, 2H, J = 7.8 Hz), 2.72 (s, 3H), 1.92−1.76 (m, 4H), 1.67−1.55 (m, 4H), 1.46 (s, 9H). ESI-MS (m/z, %): 455 (MH^+, 100).

***N*-(1-(4-(Methylamino)cyclohexyl)indolin-6-yl)thiophene-2-carboximidamide (*cis*-45).** To a solution of *tert*-butyl methyl(4-(6-(thiophene-2-carboximidamido)indolin-1-yl)cyclohexyl)carbamate (*cis*-**44**) (0.18 g, 0.39 mmol) in CH_2Cl_2 (10 mL) was added TFA (2 mL) and the mixture left to stir for 2 h at room temperature. The contents were transferred to a separatory funnel, 3 N NaOH was added, and the organic layer was extracted and evaporated. The crude was purified on a silica gel column chromatography (2 M NH_3 in MeOH:CH_2Cl_2 1:9) to obtain the title compound (0.075 g, 53%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.41−7.39 (m, 2H), 7.06 (dd, 1H, J = 3.9, 4.8 Hz), 6.98 (d, 1H, J = 7.8 Hz), 6.19 (dd, 1H, J = 1.5, 7.5 Hz), 6.07 (s, 1H), 4.86 (brs, 2H), 3.45 (t, 2H, J = 8.4 Hz), 3.38−3.31 (m, 1H), 2.90 (t, 2H, J = 8.4 Hz), 2.81 (brs, 1H), 2.45 (s, 3H), 1.96−1.92 (m, 2H), 1.88−1.72 (m, 2H), 1.63−1.54 (m, 4H). ESI-MS (m/z, %): 355 (MH^+, 100), 324 (50), 178 (60). ESI-HRMS calculated for $C_{20}H_{27}N_4S$ (MH^+), 355.1950; observed, 355.1950. HPLC purity: 99.17%.

***N*-(1-(4-(Methylamino)cyclohexyl)indolin-6-yl)thiophene-2-carboximidamide (*trans*-45).** Prepared from *tert*-butyl methyl(4-(6-(thiophene-2-carboximidamido)indolin-1-yl)cyclohexyl)carbamate (*trans*-**44**) (0.20 g, 0.44 mmol) as described for *cis*-**44** to obtain the title compound (0.13 g, 83%) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.41−7.38 (m, 2H), 7.06 (dd, 1H, J = 3.9, 4.8 Hz), 6.98 (d, 1H, J = 7.5 Hz), 6.20 (dd, 1H, J = 1.5, 7.5 Hz), 6.08 (s, 1H), 4.86 (brs, 2H), 3.41−3.29 (m, 3H), 2.91 (t, 2H, J = 8.1 Hz), 2.45 (s, 3H), 2.40−2.25 (m, 1H), 2.07−1.99 (m, 2H), 1.1.91−1.86 (m, 2H), 1.53−1.36 (m, 2H), 1.28−1.10 (m, 2H). ESI-MS (m/z, %): 355 (MH^+, 100), 324 (50), 178 (60). ESI-HRMS calculated for $C_{20}H_{27}N_4S$ (MH^+), 355.1950; observed, 355.1942. HPLC purity: 98.65%.

***tert*-Butyl 4-(5-Fluoro-6-nitroindolin-1-yl)cyclohexyl-(methyl)carbamate (47).** Prepared from 5-fluoro-6-nitroindoline (**27**) (0.21 g, 1.15 mmol) and *tert*-butyl methyl(4-oxocyclohexyl)-carbamate (**46**) (0.524 g, 2.30 mmol) as described for **10** to obtain the title compound (0.215 g, 47%) as a mixture of diastereomers (*trans*:*cis*, 1.5:1). ^{1}H NMR (DMSO-d_6) δ 7.27−7.20 (m, 1H), 6.96 (d, 0.6H, J = 5.9 Hz), 6.82 (d, 0.4H, J = 5.9 Hz), 3.91−3.78 (m, 1H), 3.68 (app t, 1H, J = 8.3 Hz), 3.55−3.39 (m, 2H), 3.08−2.93 (m, 2H), 2.72 and 2.69 (2s, 3H), 2.06−1.94 (m, 1H), 1.81−1.44 (m, 7H), 1.40 (s, 9H). ^{19}F NMR (DMSO-d_6) δ −132.311 to −132.370 (m), −132.645 to −132.705 (m). ESI-MS (m/z, %): 416 (MNa^+, 48%), 394 (MH^+, 30), 338 (100).

***tert*-Butyl 4-(5-Fluoro-6-(thiophene-2-carboximidamido)-indolin-1-yl)cyclo-hexyl(methyl)carbamate (48).** A solution of *tert*-butyl 4-(5-fluoro-6-nitroindolin-1-yl)cyclohexyl(methyl)carbamate (**47**) (0.106 g, 0.26 mmol) in dry EtOH (7 mL) was treated with 10% palladium on carbon (0.029 g, 0.02 mmol) and stirred for 1 h under hydrogen atm. The mixture was filtered through a pad of Celite and washed with EtOH to obtain crude *tert*-butyl 4-(6-amino-5-fluoroindolin-1-yl)cyclohexyl(methyl)carbamate.

To the above ethanolic solution of *tert*-butyl 4-(6-amino-5-fluoroindolin-1-yl)cyclohexyl(methyl)carbamate was added methyl thiophene-2-carbimidothioate hydroiodide (**13**) (0.154 g, 0.539 mmol) and the resulting mixture stirred at room temperature for 66 h. The reaction was worked up and purified as described for **14** to obtain the title compound (70 mg, 55% over two steps) as a mixture of diastereomers (*trans*:*cis*, 1.5:1). ^{1}H NMR (DMSO-d_6) δ 7.72 (d, 1H, J = 3.8 Hz), 7.60 (d, 1H, J = 5.0 Hz), 7.12−7.05 (m, 1H), 6.90−6.80 (m, 1H), 6.44 (brs, 2H), 5.98 (d, 0.6H, J = 6.9 Hz), 5.93 (d, 0.4H, J = 6.9 Hz), 3.91−3.69 (m, 1H), 3.54−3.45 (m, 1H), 3.30−3.16 (m, 2H), 2.89−2.75 (m, 2H), 2.72 and 2.65 (2s, 3H), 2.09−1.93 (m, 1H), 1.85−1.44 (m, 7H), 1.39 (s, 9H). ^{19}F NMR (DMSO-d_6) δ −137.973 to −138.033 (m), −138.197 to −138.256 (m).

***N*-(5-Fluoro-1-(4-(methylamino)cyclohexyl)indolin-6-yl)-thiophene-2-carbox -imidamide (*cis*-49 and *trans*-49).** Prepared from *tert*-butyl 4-(5-fluoro-6-(thiophene-2-carboximidamido)indolin-1-yl)cyclohexyl(methyl)carbamate (**48**) (0.105 g, 0.22 mmol) as described for (±)-**36** to obtain the title compound as a separable mixture of diastereomers. *cis*-**49**: yellow solid (20 mg, 24%); ^{1}H NMR (DMSO-d_6) δ 7.72 (d, 1H, J = 3.0 Hz), 7.60 (d, 1H, J = 5.0 Hz), 7.09 (dd, 1H, J = 5.0, 3.8 Hz), 6.83 (d, 1H, J = 10.1 Hz), 6.42 (brs, 2H), 5.93 (d, 1H, J = 6.8 Hz), 3.33−3.19 (m, 4H, merged with H_2O peak), 2.80 (t, 2H, J = 8.1 Hz), 2.62−2.53 (m, 1H), 2.23 (s, 3H), 1.83−1.61 (m, 4H), 1.56−1.30 (m, 4H); ^{19}F NMR (DMSO-d_6) δ −138.388 to −138.448 (m); ESI-MS (m/z, %), 373 (MH^+, 69), 342 (100); ESI-HRMS calculated for $C_{20}H_{26}N_4FS$ (MH^+), 373.1856; observed:, 373.1869; HPLC purity:, 97.74%. *trans*-**49**: yellow solid (30 mg, 36%); ^{1}H NMR (DMSO-d_6) δ 7.72 (d, 1H, J = 2.9 Hz), 7.60 (d, 1H, J = 5.0 Hz), 7.09 (dd, 1H, J = 5.0, 3.8 Hz), 6.83 (d, 1H, J = 10.1 Hz), 6.42 (brs, 2H), 5.93 (d, 1H, J = 6.8 Hz), 3.33−3.15 (m, 4H, merged with H_2O peak), 2.80 (t, 2H, J = 8.0 Hz), 2.32−2.15 (m, 1H), 2.27 (s, 3H), 1.99−1.86 (m, 2H), 1.77−1.63 (m, 2H), 1.49−1.28 (m, 2H), 1.20−1.00 (m, 2H); ^{19}F NMR (DMSO-d_6) δ −138.231 to −138.290 (m); ESI-MS (m/z, %), 373 (MH^+, 46), 342 (100); ESI-HRMS calculated for $C_{20}H_{26}N_4FS$ (MH^+), 373.1856; observed, 373.1865; HPLC purity, 97.96%.

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 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 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 are carried out at 37 °C for 45 min. The reaction

dx.doi.org/10.1021/jm201564u | J. Med. Chem. 2012, 55, 943−955

Table 2. Experimental Conditions for hERG K^+ Channel Conventional Patch-Clamp Assay

cells	solutions	incubation	detection
HEK-293 cell line stably expressing hERG	**extracellular solutions:** 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 D(+)-glucose, 10 HEPES (pH 7.4 by NaOH). **intracellular solutions:** 130 KCl, 10 NaCl, 1 $MgCl_2$, 10 EGTA, 5 MgATP, 10 HEPES (pH 7.2 by KOH).	5−10 min for concentration at rt (22−24 °C) cumulatively.	conventional whole-cell patch clamp (by Axopatch 200 B or HEKA EPC9).

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 radio activity 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 (**1**). All assays were performed in duplicate.

Efficacy in the Chung Model of Neuropathic Pain. Nerve ligation injury was performed according to the literature procedure.[30] 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 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 Conventional Patch-Clamp Assay.[32] Cultured cells (1−7 days) were used for patch clamp assay. The cells were cultured in DMEM/GlutaMax-1 + 10% FBS and were planted on collagen-coated dishes at low density (~2×10^4 cells/dish). The cell was held at −80 mV. A 50 ms pulse to −40 mV was delivered to measure the leaking currents, which were subtracted from the tail currents online. Then the cell was depolarized to +20 mV for 2 s, followed by a second pulse to −40 mV for 1 s to reveal the tail currents. This paradigm was delivered once every 5 s to monitor the current amplitude. After the current amplitude stabilized, cis-**45** was delivered to the extracellular medium by a rapid solution changer perfusion system. During superfusion, the cell was repetitively stimulated with the protocol described above, and the current amplitude was continuously monitored. The experimental conditions are described in Table 2.

The degree of inhibition (%) was obtained by measuring the tail current amplitude before and after drug superfusion (the difference current was normalized to control and multiplied by 100 to obtain the percent of inhibition). Concentration (log) response curves were fitted to a logistic equation to generate estimates of IC_{50}. The concentration−response relationship of *cis*-**45** was constructed from the percentage reductions of current amplitude by sequential concentrations.

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 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 (100 μM). *cis*-**45** (±L-arginine) was added at the selected concentration for a period of 50 min. In the presence of *cis*-**45** (±L-arginine), all vessels were then submaximally vasoconstricted with U46619 prior to CCRCs to ACh (1×10^{-10} M to 1×10^{-5} M). Compound **1** was studied in each artery ring (100 μM) in the presence and absence of L-arginine (100 μM) as a positive control and reference compound **5** was studied for comparison.

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.

High Throughput Broad Screen. In each experiment, the respective reference compound was tested concurrently with *cis*-**45**, and the data were compared with historical values (Table 1 from Supporting Information). Results showing an inhibition (or stimulation for assays run in basal conditions) higher than 50% are considered to represent significant effect of *cis*-**45**. 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

Full details on the HPLC methods for all final compounds, 2D spectral data for *cis*-**45** and *trans*-**45** and the broad screen results for *cis*-**45** with 80 receptors. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1-416-673-8457. Fax: 1-905-823-5836. E-mail: sannedi@neuraxon.com.

■ ACKNOWLEDGMENTS

We are grateful to NoAb BioDiscoveries Inc. (Mississauga, ON, Canada) and Asinex Ltd. (Moscow, Russia) for performing the human NOS inhibition assays, Biopta Ltd. (Glasgow, UK) for studying the contractile effect on human resistance arteries, and Cerep (USA and France) for hERG binding assay, hERG patch-clamp assay, and broad screen profile.

■ 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; FAD, flavinadenine dinucleotide; FMN, flavine mononucleotide; BH4, (6*R*)-2-amino-6-[(1*R*,2*S*)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1*H*)-one; L-NMMA, *N*-monomethyl L-arginine; hERG, human ether-a-go-go-related gene; SAR, structure−activity relationship; SNL, spinal nerve ligation; ACh, acetylcholine

■ REFERENCES

(1) Mustafa, A. K.; Gadalla, M. M.; Synder, S. H. Signaling by gasotransmitters. *Sci. Signaling* **2009**, *2*, 1−8.

(2) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric Oxide: Physiology, Pathophysiology, and Pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109−142.

(3) Alderton, W. K.; Cooper, C. E.; Knowles, R. G. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **2001**, *357*, 593−615.

(4) Stuehr, D. J. Structure−function aspects in the nitric oxide synthases. *Annu. Rev. Pharmacol. Toxicol.* **1997**, *37*, 339−359.

(5) Vallance, P.; Leiper, J. Blocking NO synthesis: how, where and why? *Nature Rev. Drug Discovery* **2002**, *1*, 939−950.

(6) Calabrese, V.; Mancuso, C.; Calvani, M.; Rizzarelli, E.; Butterfield, A. D.; Stella, A. M. G. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nature Rev. Neurosci.* **2007**, *8*, 766−775.

(7) Miclescu, A.; Gordh, T. Nitric oxide and pain: "something old, something new". *Acta Anaesthesiol. Scand.* **2009**, *53*, 1107−1120.

(8) Huang, P. L.; Huang, Z.; Mashimo, H.; Bloch, K. D.; Moskowitz, M. A.; Bevan, J. A.; Fishman, M. C. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **1995**, *377*, 239−242.

(9) Lassen, H. L.; Iverson, H. K.; Olesen, J. A dose−response study of nitric oxide synthase inhibition in different vascular beds in man. *Eur. J. Clin. Pharmacol.* **2003**, *59*, 499−505.

(10) Maddaford, S.; Annedi, S. C.; Ramnauth, J.; Rakhit, S. Advancements in the development of nitric oxide synthase inhibitors. *Annu. Rep. Med. Chem.* **2009**, *44*, 27−50 and references within..

(11) Erdal, E. P.; Litzinger, E. A.; Seo, J.; Zhu, Y.; Ji, H.; Silverman, R. B. Selective neuronal nitric oxide synthase inhibitors. *Curr. Top. Med. Chem.* **2005**, *5*, 603−624.

(12) Tinker, A. C.; Wallace, A. V. Selective inhibitors of inducible nitric oxide synthase: potential agents for the treatment of inflammatory diseases? *Curr. Top. Med. Chem.* **2006**, *6*, 77−92.

(13) Silverman, R. B. Design of selective neuronal nitric oxide synthase inhibitors for the prevention and treatment of neurodegenerative diseases. *Acc. Chem. Res.* **2009**, *42*, 439−451.

(14) Patman, J.; Bhardwaj, N.; Ramnauth, J.; Annedi, S. C.; Renton, P.; Maddaford, S. P.; Rakhit, S.; Andrews, J. S. Novel 2-aminobenzothiazoles as selective neuronal nitric oxide synthase inhibitors. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2540−2544.

(15) Maddaford, S.; Renton, P.; Speed, J.; Annedi, S. C.; Ramnauth, J.; Rakhit, S.; Andrews, J.; Mladenova, G.; Majuta, L.; Porreca, F. 1,6-Disubstituted indole derivatives as selective human neuronal nitric oxide synthase inhibitors. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5234−5238.

(16) Renton, P.; Speed, J.; Maddaford, S.; Annedi, S. C.; Ramnauth, J.; Rakhit, S.; Andrews, J. 1,5-Disubstituted indole derivatives as selective human neuronal nitric oxide synthase inhibitors. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5301−5304.

(17) Annedi, S. C.; Maddaford, S. P.; Mladenova, G.; Ramnauth, J.; Rakhit, S.; Andrews, J. S.; Lee, D. K. H.; Zhang, D.; Porreca, F.; Bunton, D.; Christie, L. 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. *J. Med. Chem.* **2011**, *54*, 7408−7416.

(18) Ramnauth, J.; Speed, J.; Maddaford, S. P.; Dove, P.; Annedi, S. C.; Renton, P.; Rakhit, S.; Andrews, J.; Silverman, S.; Mladenova, G.; Zinghini, S.; Nair, S.; Catalano, C.; Lee, D. K. H.; Felice, M. D.; Porreca, F. Design, synthesis and biological evaluation of 3,4-dihydroquinolin-2(1*H*)-one and 1,2,3,4-tetrahydroquiniline-based selective human neuronal nitric oxide synthase (nNOS) inhibitors. *J. Med. Chem.* **2011**, *54*, 5562−5575.

(19) O'Neill, M. J.; Murray, T. K.; McCarty, D. R.; Hicks, C. A.; Dell, C. P.; Patrick, K. E.; Ward, M. A.; Osborne, D. J.; Wiernicki, T. R.; Roman, C. R.; Lodge, D.; Fleisch, J. H.; Singh, J. ARL 17477, a selective nitric oxide synthase inhibitor, with neuroprotective effects in animal models of global and focal cerebral ischaemia. *Brain Res.* **2000**, *871*, 234−244.

(20) Leeson, P. D.; Springthorpe, B. The influence of druglike concepts on decision-making in medicinal chemistry. *Nature Rev. Drug Discovery* **2007**, *6*, 881−890.

(21) Waring, M. J. Lipophilicity in drug discovery. *Expert Opin. Drug Discovery* **2010**, *5*, 235−248.

(22) Abdel-Magid, A. F.; Carson, K. G.; Harris, B. D.; Maryanoff, C. A.; Shah, R. D. Reductive amination of aldehydes and ketones with sodium triacetoxyborohydride. Studies on direct and indirect reductive amination procedures. *J. Org. Chem.* **1996**, *61*, 3849−3862.

(23) Bercot-Vatteroni, M. Thiophene derivatives of possible therapeutic interest. *Ann. Chim.* **1962**, *7*, 303−337.

(24) Hasimbegovic, V.; Slätt, J.; Bergman., J.; Janosik, T. The synthesis of some 3-acylindoles revisited. *J. Heterocycl. Chem.* **2007**, *44*, 1213−1217.

(25) Chamberlain, S.; Deanda, F. J.; Gerding, R.; Hasegawa, M.; Kuntz, K.; Shotwell, J. B.; Wilson, J.; Lei, H. J.; Miyazaki, Y.; Nishigaki, N.; Patnaik, S.; Redman, A.; Stevens, K.; Yang, B. 2-[(2-{Phenylamino}-1*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino]benzamide derivatives as IGF-1r inhibitors for the treatment of cancer. WO 2009020990 A1, 2009.

(26) Huang, X.; Buchwald, S. New ammonia equivalents for the Pd-catalyzed amination of aryl halides. *Org. Lett.* **2001**, *3*, 3417−3419.

(27) Abraham, R. J.; Byrne, J. J.; Griffiths, L.; Koniotou, R. ^{1}H chemical shifts in NMR. Part 22. Prediction of the ^{1}H chemical shifts of alcohols, diols and inositols in solution, a conformational and solvation investigation. *Magn. Reson. Chem.* **2005**, *43*, 611−624.

(28) Leeson, P. D.; St-Gallay, S. A. The influence of the "organizational factor" on compound quality in drug discovery. *Nature Rev. Drug Discovery* **2011**, *10*, 749−765 and references within..

(29) Jaroch, S.; Rehwinkel, H.; Hölscher, P.; Sülzle, D.; Burton, G.; Hillmann, M.; McDonald, F. M.; Miklautz, H. Fluorinated dihydroquinolines as potent *n*-NOS inhibitors. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 743−746.

(30) Kim, S. H.; Chung, J. M. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **1992**, *50*, 355−363.

(31) Fermini, B.; Fossa, A. A. The impact of drug-induced QT interval prolongation on drug discovery and development. *Nature Rev.* **2003**, *2*, 439−447.

(32) Zhou, Z.; Gong, Q.; Ye, B.; Fan, Z.; Makielski, J. C.; Robertson, G. A.; January, C. T. Properties of HERG channels stably expressed in HEK 293 cells studied at physiological temperature. *Biophys. J.* **1998**, *74*, 230−241.

(33) Recanatini, M.; Poluzzi, E.; Masetti, M.; Cavalli, A.; Ponti, F. D. QT Prolongation through hERG K^+ channel blockade: current

knowledge and strategies for the early prediction during drug development. *Med. Res. Rev.* **2005**, *25*, 133−166.
(34) Jamieson, C.; Moir, E. M.; Rankovic, Z.; Wilshart, G. Medicinal chemistry of hERG optimizations: Highlights and hung-ups. *J. Med. Chem.* **2006**, *49*, 5029−5046.
(35) *Cerep*; http://www.cerep.fr/.