European Journal of Medicinal Chemistry 55 (2012) 94–107

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Discovery of a potent, orally bioavailable and highly selective human neuronal nitric oxide synthase (nNOS) inhibitor, *N*-(1-(piperidin-4-yl)indolin-5-yl) thiophene-2-carboximidamide as a pre-clinical development candidate for the treatment of migraine

Subhash C. Annedi [a,*], Shawn P. Maddaford [a], Jailall Ramnauth [a], Paul Renton [a], Taras Rybak [a], Sarah Silverman [a], Suman Rakhit [a], Gabriela Mladenova [a], Peter Dove [a], John S. Andrews [a], Dongqin Zhang [b], Frank Porreca [b]

[a] NeurAxon Inc., 2395 Speakman Drive, Suite #1001, Mississauga, ON, Canada L5K 1B3
[b] Department of Pharmacology, University of Arizona, 1501 North Campbell Avenue, Tucson, AZ 85724, USA

ARTICLE INFO

Article history:
Received 28 February 2012
Received in revised form
1 July 2012
Accepted 4 July 2012
Available online 14 July 2012

Keywords:
1,5-Disubstituted indoline derivatives
Nitric oxide
Selective neuronal nitric oxide synthase inhibitors
Migraine

ABSTRACT

We recently reported a series of 1,6-disubstituted indoline-based thiophene amidine compounds (**5**) as selective neuronal nitric oxide synthase (nNOS) inhibitors to mitigate the cardiovascular liabilities associated with hERG K^+ channel inhibition (IC_{50} = 4.7 μM) with previously reported tetrahydroquinoline-based selective nNOS inhibitors (**4**). The extended structure–activity relationship studies within the indoline core led to the identification of **43** as a selection candidate for further evaluations. The *in vivo* activity in two different pain (spinal nerve ligation and migraine pain) models, the excellent physicochemical and pharmacokinetic properties, oral bioavailability (F_{po} = 91%), and the *in vitro* safety profile disclosed in this report make **43** an ideal candidate for further evaluation in clinical applications related to migraine pain.

1. Introduction

Migraine is a complex central nervous system (CNS) related disorder that involves multiple macromolecules such as $5HT_{1D/1B}$ receptors, calcitonin gene related peptide (CGRP) receptors, and/or nitric oxide synthase (NOS) and regulation of any of these macromolecules activity would offer a therapeutic benefit. For example, the development of the first triptan (**1**, Fig. 1, selective $5HT_{1D/1B}$ agonist) in 1988 was considered revolutionary and became a benchmark for the treatment of acute migraine [1]. However, the success of triptan treatment was limited by the appearance of cutaneous allodynia (pain resulting from a non-noxious stimulus to the skin) and the development of central sensitization (abnormal neuronal activity) during treatment [2]. Although rare, triptans were also associated with coronary vasospasm, an event that can be life threatening in patients with coronary artery disease [3]. Recent clinical trials of acute migraine with orally acting CGRP antagonist (**2**, Fig. 1) showed improvement in pain relief relative to placebo with modest consistency in pain freedom [4]. Unfortunately, the development of **2** was suspended due to liver toxicity issues observed in some patients [5]. Given the cardiovascular liabilities and cutaneous allodynia associated with triptan treatment and potential liver toxicity issues with CGRP antagonist, there is a continued interest in new and effective treatment for migraine pain.

NO is synthesized by three closely related, but separate nitric oxide synthase (NOS) isoforms. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed in the brain and spinal cord and vascular tissue, respectively, while inducible NOS (iNOS) is expressed in glial cells and immune cells during

Abbreviations: CNS, central nervous system; NO, nitric oxide; CGRP, calcitonin gene related peptide; 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-NMMA, *N*-monomethyl L-arginine; SAR, structure–activity relationship studies; SNL, spinal nerve ligation; IM, inflammatory mediator; hERG, human ether-a-go-go-related gene; PK, Pharmacokinetic; CNS MPO, central nervous system multiparameter optimization.

* Corresponding author. Current address: Vibrant Pharma Inc., 45 Dalkeith Drive, Unit #14, Brantford, ON, Canada N3P 1M1. Tel./fax: +1 519 756 9550.
E-mail address: sannedi@vibrantpharma.com (S.C. Annedi).

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2012.07.006

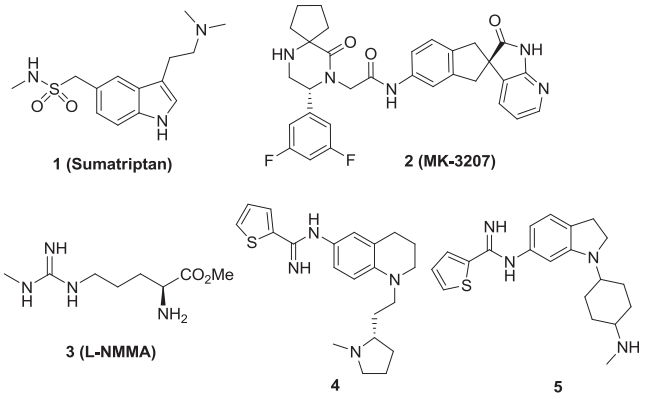


Fig. 1. Compound **1** is the first triptan currently on the market for the treatment of migraine. Compound **2** is a potent CGRP receptor antagonist developed for the treatment of migraine. Compound **3** is a non-selective NOS inhibitor proven to be effective in clinical trials of migraine. Compounds **4** and **5** are recently reported selective nNOS inhibitors.

inflammation [6,7]. NO has been shown to be involved in the development and maintenance of central sensitization and inhibitors of NOS have been shown to be effective in clinical trials of acute migraine and chronic tension type headache (CTTH) [8–11]. For example, **3** (Fig. 1), a non-selective NOS inhibitor showed 67% antimigraine effect compared to placebo in a randomized double-blind clinical study with 15 migraine patients [11]. A similar randomized double-blind, crossover clinical study was conducted on 16 patients with CTTH using **3** (6 mg/kg) and significantly reduced the headache pain intensity on the visual analog scale when compared to placebo [11]. However, a significant increase in blood pressure, most likely due to the inhibition of eNOS, was observed in these patients. Therefore, the selective inhibition of nNOS over the eNOS isoform is necessary in order to avoid the cardiovascular liabilities associated with eNOS inhibition [12,13]. Numerous attempts have been made towards the design and synthesis of selective NOS inhibitors and early NOS inhibitor design, which was based on L-arginine (substrate), produced only non-selective NOS inhibitors. However, subsequent designed inhibitors based on the available crystal structures of NOS enzymes, which targeted the arginine binding site or the (6*R*)-2-amino-6-[(1*R*,2*S*)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1*H*)-one (BH4) co-factor site as well as dimerization inhibitors have been shown to be more potent and selective among the NOS isoforms [14–17].

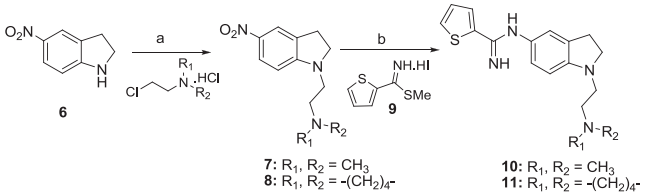
The simplified pharmacophore model adapted by our group for competitively inhibiting NOS at the substrate binding site contains a guanidine isosteric group and a basic amine group both attached through a central aryl scaffold as described earlier [18]. We recently reported a series of 1,6-disubstituted indoline-based selective nNOS inhibitors (**5**) without any cardiovascular liabilities associated with **4** due to hERG K^+ channel inhibition (IC_{50} = 4.7 μM) [19]. To extend the structure–activity relationship studies (SAR) of **5** as part of the lead optimization process, a small focused library containing various alkylamino groups on the 1-position of the indoline ring with thiophene amidine attached at 5-position was synthesized. From these studies, **43** was identified as a potent and selective nNOS inhibitor with exceptional oral bioavailability (91%) without any cardiovascular liabilities (hERG K^+ channel inhibition, IC_{50} >100 μM). As part of our ongoing efforts to find small molecule selective nNOS inhibitors [18–23], herein we report the extended SAR studies on indoline-based thiophene amidine compounds [19] that led to the identification of **43** as a pre-clinical development candidate for the treatment of migraine.

2. Results and discussion

2.1. Chemistry

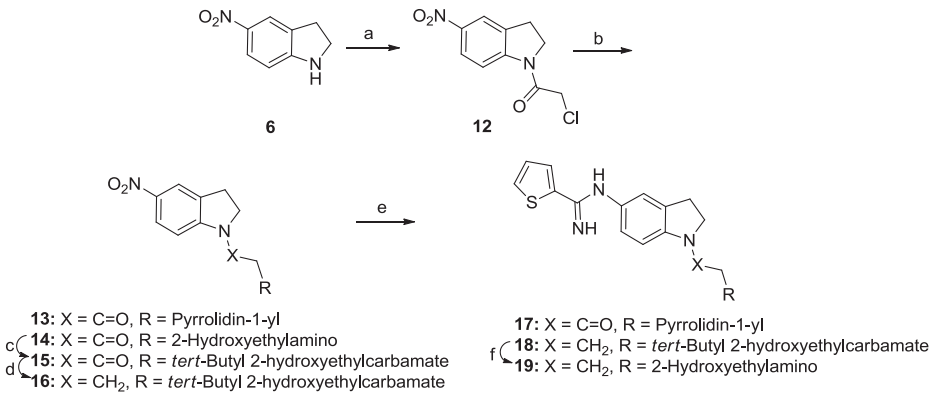
The syntheses of all target compounds were carried out according to the procedures described in Schemes 1–4. The alkylation at the 1-position of the indoline ring was carried out under strong basic conditions to obtain **7** and **8**, respectively (Scheme 1). Reduction of the nitro group under hydrogenation conditions, followed by coupling to the thiophene-2-carbimidothioate **9** [24] provided the target compounds **10** and **11**, respectively. *N*-Acylation of **6** with 2-chloroacetyl chloride [25] provided chloro intermediate **12**, which was substituted with two different amines under basic conditions to obtain **13** and **14**, respectively (Scheme 2). The secondary amine in **14** was protected as the *tert*-butyl carbamate to obtain **15**. The amide group in **15** was reduced into the corresponding amine using borane in THF to obtain **16**. Reduction of the nitro group in **13** and **16** into the corresponding amine followed by the coupling to thiophene-2-carbimidothioate **9** gave **17** and **18**, respectively. Removal of the Boc-protecting group in **18** was carried out under acidic conditions to obtain the target compound **19**.

The reductive amination of **20** with ketones **22**–**24** gave compounds **27**, **31**, and **35**, and analog reaction of indolines **21** and **6** with ketones **25** and **26** gave **37** and **38**, respectively (Scheme 3) [26]. Bromination of **27**, **31** and **35** under neutral conditions with *N*-bromosuccinamide gave 5-bromoindoline derivatives **28**, **32** and **36**, respectively. Removal of the Boc-protecting group in **28** was carried out under strong acidic conditions to obtain **29**; subsequent reductive amination of the obtained amine with formaldehyde in presence of sodium triacetoxyborohydride gave **30**. Similarly, Boc-deprotection in **32**, followed by the N-alkylation with 2-bromoethanol provided **34**. The bromo in **28**, **30**, **32**, **34**, **36** and **37** was converted into the corresponding amine by standard Buchwald–Hartwig amination conditions using LiHMDS as an ammonia surrogate [27], followed by the coupling to thiophene-2-carbimidothioate **9** gave **39**, **41**, **42**, **44**, **45** and **46**, respectively. Reduction of the nitro group in **38** to the corresponding amine was carried out under transfer hydrogenation conditions followed by coupling to thiophene-2-carbimidothioate **9** to give **48**. The Boc-protecting group in **39**, **42**, **46** and **48** was removed under strong acidic conditions to obtain the target compounds **40**, **43**, *trans*-**47**, *cis*-**47** and **49**, respectively. The enantiomeric mixture of *cis*-**47** was separated into pure enantiomers (−)-*cis*-**47** and (+)-*cis*-**47** using chiral HPLC techniques. The stereochemistry of *cis*-**47** and *trans*-**47** was conformed by various 2D NMR studies such as COSY, HSQC, NOESY, HMBC experiments (Supporting information). The NOESY between the 1-3 diaxial protons on C6 and C4 carbons for *cis*-**47** and 1-4 diaxial protons on C7 and C4 for *trans*-**47** is consistent with the proposed structures (Supporting information). No attempts were made to separate the enantiomeric mixtures of **40** and **41** at this time and they were tested as racemic mixtures. At the same time, no attempts were made to determine the stereochemistry of (−)-*cis*-**47** and (+)-*cis*-**47** at this time.



Reagents and conditions: a) NaH, DMF, 90 °C; b) i. Pd-C/H_2, EtOH, rt; ii) **9**, EtOH, rt.

Scheme 1.

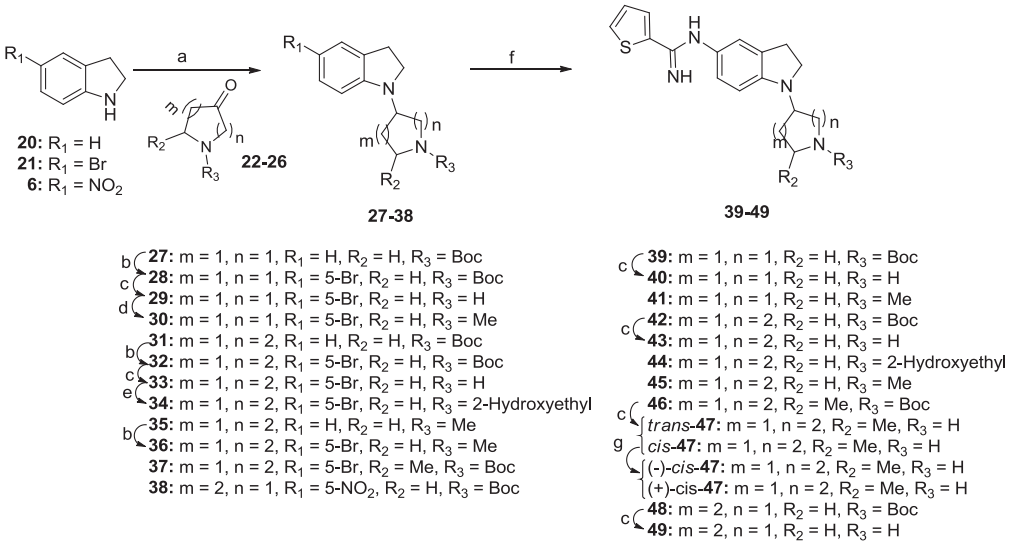


Scheme 2.

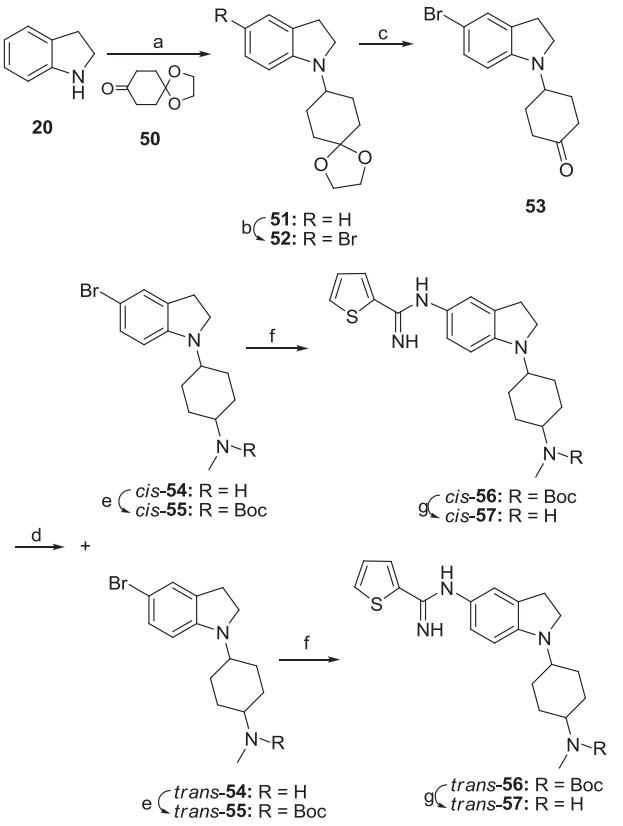
The reductive amination of indoline (**20**) with 1,4-dioxaspiro [4.5]decan-8-one (**50**), followed by bromination under neutral conditions as described above provided **52** (Scheme 4). The deprotection of the acetal group was carried out under acidic conditions to obtain **53**, followed by reductive amination with methylamine hydrochloride provided two diastereomers *cis*-**54** and *trans*-**54** in 1:1.2 ratio, which were separated by regular silica gel column chromatography (the stereochemistry was conformed with the final compounds after the sequence was completed). The protection of secondary amine in *cis*-**54** and *trans*-**54** was achieved under basic conditions to obtain *cis*-**55** and *trans*-**55**, respectively. The aryl bromides in *cis*-**55** and *trans*-**55** were converted into the corresponding amines by the Buchwald–Hartwig amination procedure as described above. The intermediate anilines were then coupled with thiophene-2-carbimidothioate **9** to provide *cis*-**56** and *trans*-**56**, respectively. Finally the Boc-deprotection was carried out under acidic conditions to obtain the final compounds *cis*-**57** and *trans*-**57**, respectively. Both *cis*-**57** and *trans*-**57** 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 certain degree of inter-conversion between boat and chair conformations (Supporting information). However, it was expected that the *trans*-conformation would permit the 1, 4 substituents to reside in a more stable, equatorial position to stabilize the chair conformation. In this conformation the protons in the 2, 3 (and 5, 6) positions (Supporting information) would be predominantly in equatorial and axial positions and experiencing the maximum effect of chemical shift anisotropy from the carbon–carbon bonds [28]. Therefore the *trans*-conformation would have the largest chemical shift range (0.93 ppm) for protons in the 2, 3 (and 5, 6) positions as opposed to the *cis*-compound (0.43 ppm).

2.2. Structure–activity relationship studies (SAR)

All compounds were converted into their corresponding dihydrochloride salts and their inhibitory activities were measured against all three human NOS isoforms (Table 1). Recombinant human nNOS, eNOS and iNOS were produced in Baculovirus-260



Scheme 3.



Reagents and conditions: a) **50**, $NaBH(OAc)_3$, AcOH, DCE, rt; b) NBS, DMF, rt; c) HCl, Acetone, rt; d) $CH_3NH_2.HCl$, $NaBH(OAc)_3$, AcOH, DCE, rt; e) Boc_2O, Et_3N, Dioxane, rt; f) i. $Pd_2(dba)_3$, $^tBu_3P$, LiHMDS, THF, 100 °C; ii. **9**, EtOH, rt; g) 1 N HCl, MeOH, reflux.

Scheme 4.

infected Sf9 cells. In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline. In our present design strategy, we extended the SAR around substituted indoline-based thiophene amidine compounds [19], where the indoline core acts as an aromatic linker with the indoline nitrogen serving as an attachment point for various basic amine side chains, while keeping the thiophene amidine group fixed at 5-position. All compounds showed sub-micromolar potency for the nNOS isoform, good to excellent selectivity over the eNOS isoform, and are comparable to previously reported 1,6-disubstituted indoline derivatives [19]. Compound **10** with *N*,*N*-dimethyl ethyl side chain was most potent ($IC_{50} = 0.04$ μM) for nNOS isoform among the current series with excellent selectivity (287 fold) over eNOS isoform. Compound **11** with a pyrrolidin-1-yl side chain was also potent and selective for the nNOS isoform (nNOS $IC_{50} = 0.12$ μM, eNOS/nNOS = 311). Compound **17** with an amide functionality was 5 times less potent at the nNOS isoform with moderate selectivity (17 fold) over eNOS isoform. This may be due to the restricted flexibility with an amide functionality, which may be preventing the favorable binding orientation as in case of **11** with an amine function and is consistent with our previous observation with 3,4-dihydroquinolin-2(1*H*)-one based nNOS inhibitors [20].

Both (±)-**40** and (±)-**41** with pyrrolidine side chain showed very good potency for nNOS and selectivity over eNOS isoform. Compound (±)-**41** with a tertiary amine with *N*-methyl substitution showed 5 times weaker potency for the nNOS isoform, when compared to (±)-**40** with a secondary amine while maintaining the selectivity over the eNOS isoform. Compound **43** with a piperidine side chain showed excellent potency for nNOS ($IC_{50} = 0.11$ μM) and the best selectivity over eNOS (368 fold) and iNOS (3772 fold) isoforms among the current series. Hydroxyethyl (**44**) and methyl (**45**) substitutions of the secondary amine in **43** resulted up to 2 fold weaker potency for the nNOS isoform and completely lost the selectivity over iNOS (3772 fold for **43** compared to 10 fold for **44** and 23 fold for **45**). Similarly, ortho-substitution to the basic amine (*trans*-**47**, *cis*-**47**, (−)-*cis*-**47** and (+)-*cis*-**47**) resulted up to 9 fold weaker potency for nNOS isoform and became non-selective over the iNOS isoform (2–13 fold) when compared to **43**. Moving the basic amine to 3-position of the piperidine ring ((±)-**49**) also resulted in weaker potency for nNOS isoform and decreased selectivity over iNOS isoform (iNOS/nNOS = 158) when compared to **43**. Moving the basic amine out of the ring by extending one carbon (*cis*-**57** and *trans*-**57**) did not yield any significant difference in nNOS potency, but the selectivity over iNOS was dramatically decreased (20–50 fold compared to 3772 fold for **43**). Overall the functionalization or modification of the piperidine ring in **43** resulted in weaker potency for nNOS isoform and reduced selectivity over other isoforms particularly for iNOS isoform. The two sets of diastereomers *cis*-**47** and *trans*-**47**, *cis*-**57** and *trans*-**57** did not show any significant difference in nNOS potency or selectivity over eNOS or iNOS isoforms. At the same time the separated enantiomers (−)-*cis*-**47** and (+)-*cis*-**47** did not show any significant difference in potency or selectivity among the NOS isoforms, when compared to the racemate *cis*-**47**.

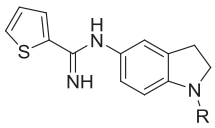
2.3. Cytochrome P450 enzyme inhibition studies

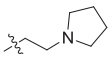
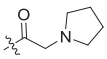
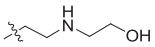
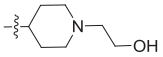
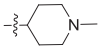
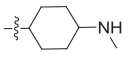
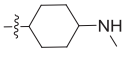
Cytochrome P450 enzyme inhibition studies were performed to assess the potential for metabolism-based drug–drug interactions, which is an inevitable hurdle during the drug development process. This will also rule out the inhibitory activity with cytochrome P450 enzymes that are closely related to NOS [29]. Hence, **43** was tested against the five major human cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4). Compound **43** was inactive up to a maximum concentration of 100 μM at four of the five major human cytochrome P450 enzymes, while an IC_{50} value of 0.75 μM was determined for CYP2D6.

2.4. Spinal nerve ligation (SNL) model and migraine model studies

Evaluated as the most selective compound among the series, **43** was selected for further profiling in two different *in vivo* pain models (Chung and migraine). The Chung or 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 paw [30]. Withdrawal latency to application of radiant heat to the paw was tested. Intraperitoneal administration of **43** at 30 mg/kg resulted in reversal of thermal hyperalgesia with a maximum reversal effect of 71% observed at 60 min (Fig. 2). After the positive results with **43** in the Chung model, the compound was investigated in a rat model of migraine pain. This model involves the application of an inflammatory mediator (IM) onto the dura via a cannula with subsequent measurements in the development of cutaneous allodynia in the hind paws [31]. After administration of the IM, animals develop signs of allodynia which peaks approximately 3 h after cannulation. The appearance of cutaneous allodynia occurring in face and paws after the administration of IM parallels the delayed extra-cranial symptoms seen in patients after the onset of a migraine attack. The oral administration of **43** (30 mg/kg) 15 min prior to IM attenuated the development of allodynia between 1 and 5 h of post-dose, peaking at the 3 h time point (Fig. 3).

Table 1
Inhibition of human NOS enzymes by 1,5-disubstituted indoline derivatives.



Compound	R	Human NOS IC_{50} (μM)[a]			Selectivity	
		nNOS	eNOS	iNOS	eNOS/nNOS	iNOS/nNOS
10		0.04 (0.02–0.05)	11.4 (8.4–15.6)	4.07 (1.93–8.61)	287	102
11		0.12 (0.09–0.17)	37.3 (15.1–92.2)	0.41 (0.27–0.62)	311	3
17		0.62 (0.35–1.08)	10.5 (5.9–18.7)	0.77 (0.50–1.20)	17	1
19		0.35 (0.27–0.45)	10.1 (3.43–29.4)	>100[b]	29	>286
(±)-**40**		0.08 (0.03–0.21)	5.45 (2.62–11.3)	NT[c]	68	NC[d]
(±)-**41**		0.43 (0.32–0.57)	21.2 (14.2–31.4)	NT[c]	49	NC[d]
43		0.11 (0.05–0.25)	40.5 (17.0–96.3)	≈415	368	3772
44		0.16 (0.03–0.85)	39.3 (11.1–138.6)	1.58 (1.16–2.15)	246	10
45		0.2 (0.12–0.34)	58.2 (30.0–112.6)	4.67 (1.60–13.6)	291	23
trans-**47**		0.37 (0.25–0.53)	36.7 (21.4–62.8)	4.75 (2.68–8.42)	99	13
cis-**47**		0.69 (0.50–0.96)	124 (20.6–744)	1.17 (0.91–1.50)	180	2
(−)-*cis*-**47**		0.71 (0.26–1.92)	53.7 (29.4–97.8)	1.09 (0.80–1.49)	76	2
(+)-*cis*-**47**		0.91 (0.31–2.68)	51.7 (28.0–95.0)	1.80 (1.36–2.37)	57	2
(±)-**49**		0.33 (0.19–0.56)	94 (45.6–193.8)	52.2 (37.3–72.9)	285	158
cis-**57**		0.20 (0.11–0.36)	17.9 (11.2–28.3)	4.08 (2.37–7.04)	90	20
trans-**57**		0.10 (0.02–0.56)	34.1 (12.2–94.7)	4.87 (2.56–9.29)	341	50
5[e]		0.37 (0.07–1.93)	195 (146–259)	83 (32.3–212)	527	224

Values reported in parentheses are 95% confidence intervals.
[a] In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline.
[b] >100 = Not active at maximum test concentration of 100 μM.
[c] NT = Not tested.
[d] NC = Not calculable.
[e] Compound **5** is a recently reported selective nNOS inhibitor, reported for comparison.

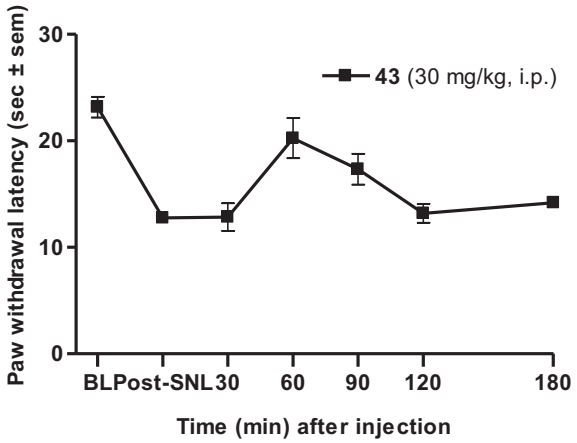


Fig. 2. Compound **43** attenuates thermal hyperalgesia in the L_5/L_6 SNL (Chung) model of neuropathic pain. The reported mean values are from 5 animals.

2.5. hERG K^+ channel inhibition studies

Antiarrhythmic drugs that induce QT prolongation by inhibiting the human ether-a-go-go-related gene (hERG) K^+ channel has been linked to drug induced sudden cardiac death and is now the second leading cause for withdrawing approved drugs from the market [32]. The hERG binding as well as the functional activity (conventional patch-clamp assay) of **43** was tested [33,34] in human recombinant HEK-293 cell lines for its ability to inhibit the hERG K^+ channel considering the moderate hERG activity with **5** (functional activity, $IC_{50} = 13$ μM). Compound **43** was inactive in both binding (IC_{50} of 160 μM) as well as functional activity (not active at the maximum test concentration of 100 μM) against hERG K^+ channel, when compared to reference compound **5**. The inactivity of **43** at hERG channel can be correlated to its lower lipophilicity (clog*P* = 2.57) compared to **5** (clog*P* = 3.15). The data suggests that **43** would be devoid of any cardiovascular liabilities associated with hERG K^+ channel binding that are physiologically relevant based on its 1455 fold weaker potency, when compared to its nNOS potency ($IC_{50} = 0.11$ μM).

2.6. High throughput profile of *43*

To identify off target activity, **43** was tested in 78 validated *in vitro* pharmacological assays that cover a broad range of 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 compound such as **43** for further evaluation in various pre-clinical toxicology studies during the selection process. Accordingly, **43** was tested at a single concentration of 10 μM in 78 different pharmacological assays to identify off target activities (Table 1 from Supporting information). Compound **43** was found to be not active (very weak inhibitor) for most of the targets tested (<50% inhibition at 71 out of 78 targets) and showed significant inhibition (>50%) at the following targets: alpha adrenergic receptor (α_1: 56%, α_2: 74%), human muscarinic receptor (M_1: 68%, M_2: 76%, M_3: 86%, M_4: 91%) and $5HT_{1B}$ transporter (81%). As a follow-up to the high throughput profile, the IC_{50} values were determined for target assays that were showed significant inhibition and the values are: α_{1A} (1.9 μM), α_{1B} (1.8 μM), α_{1D} (2.2 μM), α_{2A} (24 μM), α_{2B} (32 μM), α_{2C} (11 μM), M_1 (8.6 μM), M_2 (3.6 μM), M_3 (4.5 μM) and $5HT_{1B}$ (2.2 μM). Overall **43** possess an excellent *in vitro* safety profile.

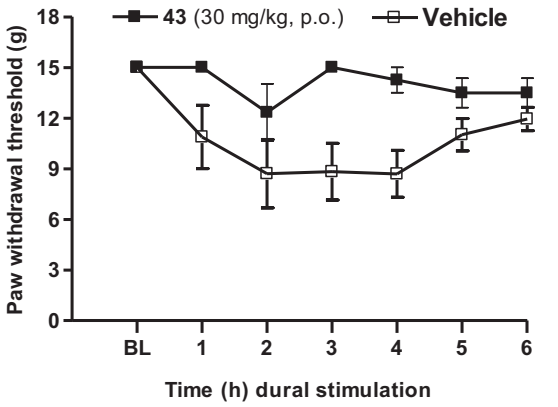


Fig. 3. Oral administration of **43** (30 mg/kg) attenuates tactile hyperesthesia of the hind paw in rats with inflammation of the Dura. The reported mean values are from 6 animals for vehicle and 4 animals for **43**.

Table 2
Physicochemical properties of **43**.

clog*P*	PSA	LogD (pH 7.4)	pK_a	Hb-A	Hb-D	FRB	MW	CNS MPO
3.58	79.39	−1.78	10.44 8.62	4	3	3	326.46	4

2.7. Physicochemical and pharmacokinetic (PK) properties

The nNOS enzyme active site is polar and acidic and the substrate (L-arginine) based nNOS inhibitors are polar and contains basic functional groups. Being polar with a basic amine is not an ideal combination for selective nNOS inhibitors for treating CNS related targets due to the blood–brain barrier and cell membrane permeability issues. Various reports have already appeared in the literature describing the important role of physicochemical properties on the *in vivo* behavior of marketed drugs and drug candidates [36]. Recently, a central nervous system multiparameter optimization (CNS MPO) algorithm was developed using six important physicochemical properties to assess the druglikeness characteristics [37]. Using this tool, a large number of marketed drugs and drug candidates for CNS related targets were evaluated and it was shown that most drugs/drug candidates have a CNS MPO score of ≥4 on a scale of 0–6. To assess the druglike characteristics of **43**, the physicochemical properties and CNS MPO score were determined as shown in Table 2. Compound **43** does not violate any of the Lipinski's rule of five (log *P* < 5, hydrogen bond donors <5, hydrogen bond acceptors <10, molecular weight <500) and has an ideal CNS MPO score of 4. The pKa of the secondary amine and the amidine group were determined as 10.44 and 8.62, respectively for **43** in order to assess the basicity. At physiological pH of 7.4, this compound exists as doubly protonated species to an appreciable amount, which is correlated to its negative log *D* value of −1.78.

Based on its excellent nNOS inhibitory activity and selectivity, its chemical stability (weak P450 inhibition) and favorable physicochemical properties (Table 2) for membrane permeability, **43** was tested further to assess the pharmacokinetic (PK) properties. The PK parameters estimated from non-compartmental analysis of the plasma concentration *vs* time curves for **43** following intravenous (3 mg/kg, iv) and oral (10 mg/kg, po) administration to male Sprague-Dawley rats are summarized in Table 3. Maximum plasma concentration (C_{max} = 101 ng/mL) for **43** was observed at 2 h following the oral dosing and declined with a half-life of 12 h. Compound **43** exhibited a large systemic clearance (CL = 420 mL/

Table 3
Rat pharmacokinetic properties of **43**.[a]

C_{max} (ng/mL)[b]	T_{max} (h)[b]	$t_{1/2}$ (h)[b]	AUC (ng h/mL)	V_{ss} (L/kg)	CL (mL/min/kg)	F_{po} (%)
101	2	12	95.2	330	420	91

[a] 3 mg/kg iv.
[b] 10 mg/kg po.

min/kg) and volume of distribution (V_{ss} = 330 L/kg) with exceptional oral bioavailability (F_{po} = 91%).

PSA, polar surface area; Hb-A, sum of hydrogen bond acceptors; Hb-B, sum of hydrogen bond donors; FRB, number of freely rotatable bonds; MW, molecular weight; CNS MPO, central nervous system multiple parameter optimization score.

3. Conclusions

In conclusion, a series of 1,5-disubstitued indoline-based thiophene amidine compounds were designed, synthesized and shown to be selective inhibitors of human nNOS over the eNOS and iNOS isoforms. By varying the basic amine at 1-position of the indoline ring several potent and highly selective inhibitors of nNOS over eNOS were identified. Compound **43**, a potent and highly selective inhibitor of nNOS having favorable physicochemical properties with exceptional oral bioavailability (91%) was shown to be effective in two different *in vivo* pain models. At the same time **43** is devoid of any cardiovascular liabilities associated with QT prolongation with hERG K^+ channel binding. Compound **43** does not have any off target activities (78 receptors/transporters/ion channels) associated with the side effects, making it an ideal candidate to carry forward through the pre-clinical development process to investigate the role that excess NO produced by nNOS enzyme plays in CNS related disorders such as migraine pain. Finally this report addresses some of the formidable issues in developing selective nNOS inhibitors for therapeutic use.

4. Experimental section

4.1. 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 (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 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. Chemical purity was determined using an Agilent 1100 HPLC system using Zorbax, SB-C18 and Waters XTerra RP8.5 μm reverse phase columns and the purity was determined to be ≥95% until unless specified. Chiral purity was determined using Chiracel OJ-H column and the chiral purity was determined to be >99%. No attempts were made to optimize the yields.

4.1.1. *N,N-Dimethyl-2-(5-nitroindolin-1-yl)ethanamine (**7**)*

A solution of 5-nitroindoline (**6**) (0.5 g, 3.05 mmol) in DMF (10 mL) was treated with NaH (0.39 g, 9.75 mmol, 60% wt in mineral oil) at 0 °C resulting in an orange mixture. The reaction mixture was then treated with 2-chloro-*N,N*-dimethylethanamine hydrochloride (0.87 g, 6.09 mmol) resulting in a dark red mixture. The reaction was heated to 90 °C and stirred for 1.5 h. After allowing the reaction to cool to room temperature, water was added and the product was extracted into EtOAc. The combined ethyl acetate layer was washed with water, brine and dried (Na_2SO_4). Solvent was evaporated and the crude was purified by flash column chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 2.5:97.5) to obtain the title compound (0.4 g, 56%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.96 (dd, 1H, J = 2.1, 8.7 Hz), 7.79 (d, 1H, J = 2.1 Hz), 6.49 (d, 1H, J = 9.0 Hz), 3.72 (t, 2H, J = 8.7 Hz), 3.39 (t, 2H, J = 6.6 Hz), 3.04 (t, 2H, J = 8.7 Hz), 2.44 (t, 2H, J = 6.3 Hz), 2.18 (s, 6H).

4.1.2. *5-Nitro-1-(2-(pyrrolidin-1-yl)ethyl)indoline (**8**)*

Prepared from 5-nitroindoline (**6**) (0.5 g, 3.05 mmol) as described for **7** to obtain the title compound (0.4 g, 50%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.96 (dd, 1H, J = 2.4, 9.0 Hz), 7.80 (d, 1H, J = 2.1 Hz), 6.47 (d, 1H, J = 9.0 Hz), 3.73 (t, 2H, J = 9.0 Hz), 3.41 (t, 2H, J = 6.9 Hz), 3.04 (t, 2H, J = 8.1 Hz), 2.62 (t, 2H, J = 6.9 Hz), 2.58–2.48 (m, 4H), 1.70–1.64 (m, 4H); ESI-MS (*m*/*z*, %): 262 (MH^+, 100).

4.1.3. *N-(1-(2-(Dimethylamino)ethyl)indolin-5-yl)thiophene-2-carboximidamide (**10**)*

A suspension of *N,N*-dimethyl-2-(5-nitroindolin-1-yl)ethanamine (**7**) (0.18 g, 0.77 mmol) and Pd–C (0.08 g, 0.07 mmol, 10% wt) in dry EtOH (5 mL) was purged with hydrogen gas. The reaction was stirred at room temperature overnight under hydrogen atmosphere (balloon pressure). The reaction was filtered through a pad of celite and washed with EtOH. The filtrate was treated with methyl thiophene-2-carbimidothioate hydroiodide (**9**) (0.44 g, 1.55 mmol) and stirred overnight at room temperature. The reaction was diluted with saturated $NaHCO_3$ solution and the product was extracted into CH_2Cl_2. The combined organic layer was dried (Na_2SO_4) and concentrated. The crude was purified by flash column chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 2.5:97.5 to 5:95) to obtain the title compound (0.09 g, 40%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.68 (d, 1H, J = 2.7 Hz), 7.56 (dd, 1H, J = 5.1, 1.2 Hz), 7.07 (dd, 1H, J = 3.6, 5.1 Hz), 6.62 (s, 1H), 6.55–6.46 (m, 2H), 6.28 (s, 2H), 3.32–3.27 (m, 2H), 3.09 (t, 2H, J = 6.6 Hz), 2.84 (t, 2H, J = 8.1 Hz), 2.45 (t, 2H, J = 6.9 Hz), 2.20 (s, 6H); ESI-MS (*m*/*z*, %): 315 (MH^+, 100), 244 (29), 127 (38); ESI-HRMS calculated for $C_{17}H_{23}N_4S$ (MH^+): 315.1637, observed: 315.1645.

4.1.4. *N-(1-(2-(Pyrrolidin-1-yl)ethyl)indolin-5-yl)thiophene-2-carboximidamide (**11**)*

Prepared from 5-nitro-1-(2-(pyrrolidin-1-yl)ethyl)indoline (**8**) (0.40 g, 1.53 mmol) as described for **10** to obtain the title compound (0.23 g, 44%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.68 (dd, 1H, J = 0.9, 3.6 Hz), 7.55 (dd, 1H, J = 0.9, 5.1 Hz), 7.07 (dd, 1H, J = 3.6, 4.8 Hz), 6.62 (s, 1H), 6.50–6.45 (m, 2H), 6.23 (s, 2H), 3.33–3.27 (m, 2H), 3.11 (t, 2H, J = 6.9 Hz), 2.84 (t, 2H, J = 8.1 Hz), 2.62 (t, 2H, J = 7.5 Hz), 2.55–2.45 (m, 4H), 1.70–1.65 (m, 4H); ESI-MS (*m*/*z*, %): 341 (MH^+, 100), 244 (45), 127 (57); ESI-HRMS calculated for $C_{19}H_{25}N_4S$ (MH^+): 341.1794, observed: 341.1788.

4.1.5. *2-Chloro-1-(5-nitroindolin-1-yl)ethanone (**12**)*

A suspension of 5-nitroindoline (**6**) (1.0 g, 6.09 mmol) in toluene (20 mL) was treated with 2-chloroacetyl chloride (0.97 mL, 12.18 mmol) and heated at 110 °C for 15 min. The reaction was cooled to room temperature, treated with sat. $NaHCO_3$ solution and extracted into ethyl acetate. The combined organic layer was dried (Na_2SO_4) and concentrated. The crude was purified by flash column chromatography (EtOAc:hexanes, 1:9 to 1:1 then 2 M NH_3 MeOH:CH_2Cl_2, 1:9) to obtain the title compound (1.08 g, 74%). ^{1}H NMR ($CDCl_3$) δ 8.30–8.27 (m, 1H), 8.14 (dd, 1H, J = 2.1, 9.0 Hz), 8.06 (s, 1H), 4.30 (t, 2H, J = 8.7 Hz), 4.18 (s, 2H), 3.33 (t, 2H, J = 8.4 Hz); ESI-MS (*m*/*z*, %): 241, 243 (MH^+).

*4.1.6. 1-(5-Nitroindolin-1-yl)-2-(pyrrolidin-1-yl)ethanone (**13**)*

A pressure vessel containing 2-chloro-1-(5-nitroindolin-1-yl) ethanone (**12**) (1.05 g, 4.38 mmol) in acetonitrile (30 mL) and water (2 mL) was treated with potassium carbonate (3.03 g, 21.90 mmol) followed by pyrrolidine (1.81 mL, 21.90 mmol) and the resulting mixture was heated at 60 °C for 1 h. The reaction was brought to room temperature and product was extracted into CH_2Cl_2. The CH_2Cl_2 layer was washed with water and sat. sodium carbonate solution. The organic layer was separated and the aqueous layer (pH >9) was further extracted with CH_2Cl_2. The combined organic layer was dried (Na_2SO_4) and concentrated. The crude product was purified by flash column chromatography (2 M NH_3 MeOH:CH_2Cl_2, 5:95) to obtain the title compound (1.2 g, quantitative) as a solid. ^{1}H NMR ($CDCl_3$) δ 8.31 (d, 1H, J = 9.0 Hz), 8.16 (dd, 1H, J = 2.1, 9.0 Hz), 8.08 (s, 1H), 4.31 (t, 2H, J = 8.7 Hz), 4.20 (s, 2H), 3.34 (t, 2H, J = 8.4 Hz); ESI-MS (*m*/*z*, %): 276 (MH^+, 100).

*4.1.7. 2-(2-Hydroxyethylamino)-1-(5-nitroindolin-1-yl)ethanone (**14**)*

Prepared from 2-chloro-1-(5-nitroindolin-1-yl)ethanone (**12**) (6.06 g, 25.2 mmol) as described for **13** to obtain the title compound (3.86 g, 57%) as an oil. ^{1}H NMR ($CDCl_3$) δ 8.32 (d, 1H, J = 9.3 Hz), 8.15 (dd, 1H, J = 2.1, 9.0 Hz), 8.07 (s, 1H), 4.15 (t, 2H, J = 8.4 Hz), 3.67 (t, 2H, J = 4.8 Hz), 3.60 (s, 2H), 3.31 (t, 2H, J = 8.7 Hz), 2.88 (t, 2H, J = 5.1 Hz), 2.23–1.96 (m, 2H); ESI-MS (*m*/*z*, %): 288 (36, M + Na), 266 (MH^+, 100).

*4.1.8. tert-Butyl 2-hydroxyethyl(2-(5-nitroindolin-1-yl)-2-oxoethyl) carbamate (**15**)*

A solution of 2-(2-hydroxyethylamino)-1-(5-nitroindolin-1-yl) ethanone (**14**) (3.81 g, 14.36 mmol) and triethylamine (4.04 mL, 28.7 mmol) in dioxane (55 mL) was treated with di-*tert*-butyl dicarbonate (3.29 g, 15.08 mmol) and stirred for 1 h at room temperature. The reaction was diluted with water and extracted into CH_2Cl_2. The combined organic layer was concentrated and purified by flash column chromatography (2 M NH_3 MeOH:CH_2Cl_2, 5:95) to obtain the title compound (4.54 g, 87%) as a viscous liquid. ^{1}H NMR ($CDCl_3$) δ 8.39–8.31 (m, 1H), 8.23–8.10 (m, 1H), 8.04–7.96 (m, 1H), 4.31–4.13 (m, 4H), 3.85–3.73 (m, 2H), 3.57–3.49 (m, 2H), 3.39–3.29 (m, 2H), 3.18–3.09 (m, 1H), 1.50 (s, 9H); ESI-MS (*m*/*z*, %): 388 (43, MNa^+), 366 (MH^+, 9), 288 (27), 266 (100).

*4.1.9. tert-Butyl 2-hydroxyethyl(2-(5-nitroindolin-1-yl)ethyl) carbamate (**16**)*

A solution of *tert*-butyl 2-hydroxyethyl(2-(5-nitroindolin-1-yl)-2-oxoethyl)carbamate (**15**) (4.42 g, 12.10 mmol) in anhydrous THF (50 mL) was stirred at 0 °C and 1 M borane in THF (36.3 mL, 36.3 mmol) was added drop-wise over a period of 15 min. The reaction was brought to rt and stirred for 1 h. The reaction was quenched with methanol (100 mL) and was stirred for 30 min. The mixture was concentrated and was quenched once more with methanol (100 mL). The crude product was concentrated and filtered through a pad of silica gel (EtOAc:hexanes, 1:1, then 2 M NH_3 in MeOH:CH_2Cl_2, 2.5:97.5 to 5:95) to obtain the title compound (4.01 g, 95%) as an orange-red solid. ESI-MS (*m*/*z*, %): 390 (18, M + K), 374 (71, MNa^+), 351 (MH^+, 31), 296 (100), 252 (86).

*4.1.10. N-(1-(2-(Pyrrolidin-1-yl)acetyl)indolin-5-yl)thiophene-2-carboximidamide (**17**)*

Prepared from 1-(5-nitroindolin-1-yl)-2-(pyrrolidin-1-yl)ethanone (**13**) (1.17 g, 4.26 mmol) as described for **10** to obtain the title compound (0.19 g, 13%) as a foam. ^{1}H NMR (DMSO-d_6) δ 7.99 (d, 1H, J = 8.4 Hz), 7.71 (d, 1H, J = 3.3 Hz), 7.59 (d, 1H, J = 5.1 Hz), 7.08 (dd, 1H, J = 3.9, 4.9 Hz), 6.73 (s, 1H), 6.62 (d, 1H, J = 8.4 Hz), 6.37 (brs, 2H), 4.15 (t, 2H, J = 8.1 Hz), 3.37 (s, 2H), 3.10 (t, 2H, J = 8.1 Hz), 2.57 (brs, 4H), 1.71 (brs, 4H); EI-MS (*m*/*z*, %): 354 (M^+, 14), 245 (43), 176 (100), 133 (56); EI-HRMS calculated for $C_{19}H_{22}N_4OS$ (M^+): 354.1514, observed: 354.1504.

*4.1.11. tert-Butyl 2-hydroxyethyl (2-(5-(thiophene-2-carboximidamido) indolin-1-yl) ethyl)carbamate (**18**)*

Prepared from *tert*-butyl 2-hydroxyethyl(2-(5-nitroindolin-1-yl)ethyl)carbamate (**16**) (3.98 g, 11.35 mmol) as described for **10** to obtain the title compound (4.56 g, 93%) as a foam. ESI-MS (*m*/*z*, %): 431 (MH^+, 100), 429 (46), 242 (16).

*4.1.12. N-(1-(2-(2-Hydroxyethylamino)ethyl)indolin-5-yl) thiophene-2-carboximidamide (**19**)*

A solution of *tert*-butyl 2-hydroxyethyl (2-(5-(thiophene-2-carboximidamido) indolin-1-yl) ethyl)carbamate (**18**) (4.56 g, 10.59 mmol) in methanol (50 mL) was treated with 1 N HCl solution (50 mL) at rt. The resulting mixture was refluxed for 30 min and brought to rt. The reaction was basified with sat. $NaHCO_3$ solution and extracted into CH_2Cl_2. The solvent was evaporated and crude was purified by flash column chromatography (2 M NH_3 MeOH:CH_2Cl_2, 1:99 to 1:9) to obtain the title compound (0.435 g, 12%) as a foam. ^{1}H NMR (DMSO-d_6) δ 7.68 (d, 1H, J = 3.3 Hz), 7.55 (d, 1H, J = 5.1 Hz), 7.07 (t, 1H, J = 4.8 Hz), 6.62 (s, 1H), 6.55–6.47 (m, 2H), 6.36–6.04 (m, 2H), 4.59 (brs, 1H), 3.49–3.48 (m, 2H), 3.30–3.24 (m, 2H), 3.10 (t, 2H, J = 6.6 Hz), 2.88–2.77 (m, 4H), 2.68 (t, 2H, J = 5.7 Hz); ESI-MS (*m*/*z*, %): 331 (MH^+, 100), 285 (26), 127 (22); ESI-HRMS calculated for $C_{17}H_{23}N_4OS$ (MH^+): 331.1585, observed: 331.1587; HPLC purity: 92.45% by area.

*4.1.13. tert-Butyl 3-(indolin-1-yl)pyrrolidine-1-carboxylate (**27**)*

A solution of indoline (**20**) (1.88 mL, 16.78 mmol), *tert*-butyl 3-oxopyrrolidine-1-carboxylate (**22**) (3.73 g, 20.13 mmol) in dry methanol (20 mL) was treated with AcOH (2.37 mL, 41.95 mmol) followed by $NaCNBH_3$ (1.26 g, 20.13 mmol) at 0 °C. The reaction was brought to room temperature and 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 dried (Na_2SO_4) and solvent was evaporated to obtain crude product. The crude was purified by column chromatography (EtOAc:hexanes, 1:9) to obtain the title compound (4.2 g, 87%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.08–7.02 (m, 2H), 6.66 (t, 1H, J = 7.5 Hz), 6.49 (d, 1H, J = 7.8 Hz), 4.17–4.05 (m, 1H), 3.70–3.51 (m, 2H), 3.47–3.37 (m, 4H), 2.95 (t, 2H, J = 8.4 Hz), 2.18–1.99 (m, 2H), 1.46 (s, 9H); ESI-MS (*m*/*z*, %): 289 (MH^+, 16), 233 (100).

*4.1.14. tert-Butyl 3-(5-bromoindolin-1-yl)pyrrolidine-1-carboxylate (**28**)*

A solution of *tert*-butyl 3-(indolin-1-yl)pyrrolidine-1-carboxylate (**27**) (4.15 g, 14.390 mmol) in dry DMF (30 mL) was treated with *N*-bromosuccinimide (2.56 g, 14.390 mmol) in dry DMF (20 mL) at 0 °C and resulting solution was stirred at same temperature for 3 h. The reaction was diluted with water and product was extracted into ethyl acetate. The combined ethyl acetate layer was washed with water, brine and dried (Na_2SO_4). Solvent was evaporated and crude was purified by column chromatography (EtOAc:hexanes, 1:4) to obtain the title compound (5.12 g, 97%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.18–7.07 (m, 2H), 6.33 (d, 1H, J = 8.7 Hz), 4.13–3.99 (m, 1H), 3.68–3.26 (m, 6H), 2.94 (t, 2H, J = 8.1 Hz), 2.17–1.99 (m, 2H), 1.46 (s, 9H); ESI-MS (*m*/*z*, %): 368 (MH^+, 3), 313, 311 (100).

*4.1.15. 5-Bromo-1-(pyrrolidin-3-yl)indoline (**29**)*

Prepared from *tert*-butyl 3-(5-bromoindolin-1-yl)pyrrolidine-1-carboxylate (**28**) (3.0 g, 8.16 mmol) as described for **19** to obtain the title compound (1.75 g, 80%) as a syrup. ^{1}H NMR (DMSO-d_6) δ 7.12–7.06 (m, 2H), 6.45 (d, 1H, J = 8.1 Hz), 4.00–3.92 (m, 1H), 3.34 (t, 2H, J = 8.4 Hz), 2.96–2.66 (m, 7H), 1.92–1.80 (m, 1H), 1.69–1.60 (m, 1H); ESI-MS (*m*/*z*, %): 267, 269 (MH^+, 100).

*4.1.16. 5-Bromo-1-(1-methylpyrrolidin-3-yl)indoline (**30**)*

Prepared from 5-bromo-1-(pyrrolidin-3-yl)indoline (**29**) (1.0 g, 3.74 mmol) and formaldehyde (0.36 g, 4.49 mmol, 37% in water) as described for **27** to obtain the title compound (0.7 g, 67%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.12–7.08 (m, 2H), 6.35 (d, 1H, J = 9.0 Hz), 4.19–4.10 (m, 1H), 3.45–3.39 (m, 2H), 2.91 (t, 2H, J = 8.1 Hz), 2.70–2.63 (m, 3H), 2.51–2.43 (m, 1H), 2.34 (s, 3H), 2.19–2.07 (m, 1H), 1.93–1.81 (m, 1H); ESI-MS (*m*/*z*, %): 281, 283 (MH^+, 100).

*4.1.17. tert-Butyl 4-(indolin-1-yl)piperidine-1-carboxylate (**31**)*

Prepared from indoline (**20**) (2.0 g, 16.78 mmol) and *tert*-butyl 4-oxopiperidine-1-carboxylate (**23**) (3.68 g, 18.46 mmol) as described for **27** to obtain the title compound (5.0 g, 99%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.03 (t, 2H, J = 7.8 Hz), 6.59 (t, 1H, J = 7.2 Hz), 6.40 (d, 1H, J = 7.5 Hz), 4.25–4.20 (m, 2H), 3.55–3.43 (m, 1H), 3.33 (t, 2H, J = 8.4 Hz), 2.93 (t, 2H, J = 8.4 Hz), 2.76 (t, 2H, J = 12.3 Hz), 1.80–1.74 (m, 2H), 1.63–1.51 (m, 2H), 1.45 (s, 9H); ESI-MS (*m*/*z*, %) 303 (MH^+, 5), 247 (100).

*4.1.18. tert-Butyl 4-(5-bromoindolin-1-yl)piperidine-1-carboxylate (**32**)*

Prepared from *tert*-butyl 4-(indolin-1-yl)piperidine-1-carboxylate (**31**) (2.0 g, 6.61 mmol) and *N*-bromosuccinimide (1.17 g, 6.61 mmol) as described for **28** to obtain the title compound (2.5 g, 99%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.13–7.10 (m, 2H), 6.27 (d, 1H, J = 9.0 Hz), 4.28–4.20 (m, 2H), 3.50–3.40 (m, 1H), 3.35 (t, 2H, J = 8.4 Hz), 2.93 (t, 2H, J = 8.4 Hz), 2.75 (t, 2H, J = 12.9 Hz), 1.78–1.72 (m, 2H), 1.60–1.49 (m, 2H), 1.46 (s, 9H); ESI-MS (*m*/*z*, %) 381, 383 (MH^+, 3), 325, 327 (100).

*4.1.19. 5-Bromo-1-(piperidin-4-yl)indoline (**33**)*

Prepared from *tert*-butyl 4-(5-bromoindolin-1-yl)piperidine-1-carboxylate (**32**) (1.8 g, 4.72 mmol) as described for **19** to obtain the title compound (1.3 g, 98%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.12–7.09 (m, 2H), 6.26 (d, 1H, H, J = 8.7 Hz), 3.47–3.34 (m, 3H), 3.22–3.20 (m, 2H), 2.92 (t, 2H, J = 8.4 Hz), 2.73–2.65 (m, 2H), 2.13 (s, 1H), 1.80–1.76 (m, 2H), 1.65–1.52 (m, 2H); ESI-MS (*m*/*z*, %): 283, 281 (MH^+, 100).

*4.1.20. 2-(4-(5-Bromoindolin-1-yl)piperidin-1-yl)ethanol (**34**)*

A solution of 5-bromo-1-(piperidin-4-yl)indoline (**33**) (1.3 g, 4.62 mmol) in dry dioxane (20 mL) was treated with triethylamine (3.25 mL, 23.12 mmol) followed by 2-bromoethanol (0.65 mL, 9.25 mmol) at room temperature and the resulting mixture was refluxed for 4 h. The reaction was brought to room temperature, diluted 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 (2 M NH_3 in MeOH:CH_2Cl_2, 5:95) to obtain the title compound (1.42 g, 94%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.10–7.06 (m, 2H), 6.37 (d, 1H, J = 8.4 Hz), 4.37 (t, 1H, J = 5.1 Hz), 3.50–3.44 (m, 2H), 3.35–3.30 (m, 3H, merged with water peak), 2.95–2.83 (m, 4H), 2.38 (t, 2H, J = 6.3 Hz), 2.08–2.00 (m, 2H), 1.61–1.54 (m, 4H); ESI-MS (*m*/*z*, %): 325, 327 (MH^+, 100).

*4.1.21. 1-(1-Methylpiperidin-4-yl)indoline (**35**)*

Prepared from indoline (**20**) (1.0 g, 8.39 mmol) and *N*-methyl-4-piperidone (**24**) (1.23 mL, 10.06 mmol) as described for **27** to obtain the title compound (1.15 g, 63.5%) as a syrup. ^{1}H NMR (DMSO-d_6) δ 7.06–7.01 (m, 2H), 6.59 (t, 1H, J = 6.6 Hz), 6.40 (d, 1H, J = 7.8 Hz), 3.40–3.34 (m, 3H), 2.97–2.91 (m, 4H), 2.30 (s, 3H), 2.09–2.00 (m, 2H), 1.80–1.72 (m, 4H); ESI-MS (*m*/*z*, %): 217 (MH^+, 42). 98 (100).

*4.1.22. 5-Bromo-1-(1-methylpiperidin-4-yl)indoline (**36**)*

Prepared from 1-(1-methylpiperidin-4-yl)indoline (**35**) (1.12 g, 5.17 mmol) and *N*-bromosuccinimide (0.92 g, 5.176 mmol) as described for **28** to obtain the title compound (1.15 g, 76%) as a syrup. ^{1}H NMR (DMSO-d_6) δ 7.14–7.06 (m, 2H), 6.37 (d, 1H, J = 8.4 Hz), 3.35–3.24 (m, 3H, merged with water peak), 2.93–2.80 (m, 4H), 2.16 (s, 3H), 2.00–1.88 (m, 2H), 1.64–1.55 (m, 4H); EI-MS (*m*/*z*, %): 293, 295 (M^+, 47), 98 (59), 97 (100), 71 (47).

*4.1.23. tert-Butyl 4-(5-bromoindolin-1-yl)-2-methylpiperidine-1-carboxylate (**37**)*

Prepared from 5-bromoindoline (**21**) (0.464 g, 2.34 mmol) and *tert*-butyl 2-methyl-4-oxopiperidine-1-carboxylate (**25**) (0.50 g, 2.34 mmol) as described for **27** to obtain the title compound (0.5 g, 54%) as a 7:3 mixture (*cis*: *trans*) of diastereomers. ^{1}H NMR ($CDCl_3$) δ 7.16–7.08 (m, 2H), 6.29 (d, 0.7 H, J = 8.7 Hz), 6.25 (d, 0.3H, J = 8.8 Hz), 4.71–4.45 (brs, 0.3H), 3.96–3.86 (m, 0.7H), 3.82–3.74 (m, 1H), 3.70–3.53 (m, 1H), 3.38–3.30 (m, 2H), 3.24–3.14 (m, 0.7H) 2.92 (m + t, 2.3H, J = 8.3 Hz), 2.10–1.98 (m, 1H), 1.87–1.54 (m, 3H), 1.48–1.47 (2s, 9H), 1.23 (d, 3H, J = 6.4 Hz); ESI-MS (*m*/*z*, %): 395/393 (MH^+, 7), 339/341 (100).

*4.1.24. tert-Butyl 3-(5-nitroindolin-1-yl)piperidine-1-carboxylate (**38**)*

Prepared from 5-nitroindoline (**6**) (0.5 g, 3.05 mmol) and (**26**) as described for **27** to obtain the title compound (0.585 g, 55%) as a solid. ^{1}H NMR ($CDCl_3$) δ 8.05 (dd, 1H, J = 2.1, 8.8 Hz), 7.88 (s, 1H), 6.35 (d, 1H, J = 8.7 Hz), 4.15–4.07 (m, 2H), 3.76–3.66 (m, 2H), 3.52–3.44 (m, 1H), 3.06 (t, 2H, J = 8.7 Hz), 2.77–2.65 (m, 2H), 2.04–1.45 (m, 13H); EIS-MS (*m*/*z*, %): 370 (MNa^+, 100), 292 (100).

*4.1.25. tert-Butyl 3-(5-(thiophene-2-carboximidamido)indolin-1-yl)pyrrolidine-1-carboxylate (**39**)*

A suspension of $Pd_2(dba)_3$ (0.124 g, 0.13 mmol) and P^tBu_3 (1.65 mL, 0.54 mmol, 10% wt in hexane) in anhydrous THF (5 mL) was treated with a solution of *tert*-butyl 3-(5-bromoindolin-1-yl) pyrrolidine-1-carboxylate (**28**) (1.0 g, 2.72 mmol) in THF (15 mL) followed by LiHMDS (5.44 mL, 5.44 mmol, 1 M solution in THF) at room temperature. The resulting dark brown mixture was heated to 100 °C and stirred for 3 h in a sealed tube. The reaction was cooled to room temperature and treated with TBAF (5 mL, 1 M solution in THF) and stirred for 20 min. The reaction was diluted with water and product was extracted into ether. The combined organic layer was dried (Na_2SO_4) and concentrated to give a dark brown residue. The crude product was purified by column chromatography (2 M NH_3 in MeOH:CH_2Cl_2, 3:97) to obtain the intermediate amine, *tert*-butyl 3-(5-aminoindolin-1-yl)pyrrolidine-1-carboxylate (0.68 g, 82%) as a foam. ESI-MS (*m*/*z*, %): 304 (MH^+, 16), 248 (100).

Prepared from the above amine intermediate *tert*-butyl 3-(5-aminoindolin-1-yl)pyrrolidine-1-carboxylate (0.65 g, 2.142 mmol) and methyl thiophene-2-carbimidothioate hydroiodide (**9**) (1.22 g, 4.28 mmol) as described for **10** to obtain the title compound (0.68 g, 77%) as a foam. ^{1}H NMR (DMSO-d_6) δ 7.42–7.36 (m, 2H), 7.06 (dd, 1H, J = 3.6, 4.9 Hz), 6.79 (brs, 1H), 6.71 (t, 1H, J = 5.1 Hz), 6.48 (d, 1H, J = 8.1 Hz), 4.89 (brs, 2H), 4.12–4.02 (m, 1H), 3.68–3.35 (m, 6H), 2.93 (t, 2H, J = 8.1 Hz), 2.14–2.04 (m, 2H), 1.46 (s, 9H); ESI-MS (*m*/*z*, %): 413 (MH^+, 100).

*4.1.26. N-(1-(Pyrrolidin-3-yl)indolin-5-yl)thiophene-2-carboximidamide (**40**)*

Prepared from *tert*-butyl 3-(5-(thiophene-2-carboximidamido) indolin-1-yl)pyrrolidine-1-carboxylate (**39**) (0.35 g, 0.84 mmol) as described for **19** to obtain the title compound (0.3 g, 92%) as a solid. ^{1}H NMR (DMSO-d_6) δ 11.25 (s, 1H), 9.82–9.60 (m, 3H), 8.63 (s, 1H), 8.15–8.13 (m, 2H), 7.36 (t, 1H, J = 4.5 Hz), 7.12–7.06 (m, 2H), 6.68 (d, 1H, J = 8.4 Hz), 4.39–4.35 (m, 1H), 3.56–3.06 (m, 6H), 2.96 (t, 2H, J = 8.1 Hz), 2.18–2.00 (m, 2H); ESI-MS (*m*/*z*, %): 313 (MH^+, 100), 244 (61); ESI-HRMS calculated for $C_{17}H_{21}N_4S$

(MH^+): 313.1481; observed: 313.1473; HPLC purity: 94.05% by area.

4.1.27. N-(1-(1-Methylpyrrolidin-3-yl)indolin-5-yl)thiophene-2-carboximidamide (**41**)

Prepared from 5-bromo-1-(1-methylpyrrolidin-3-yl)indoline (**30**) (0.64 g, 2.276 mmol) as described for **39** and **10** to obtain the title compound (0.43 g, 72%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.68 (d, 1H, J = 3.0 Hz), 7.58 (dd, 1H, J = 1.2, 5.1 Hz), 7.07 (dd, 1H, J = 3.6, 4.9 Hz), 6.62 (s, 1H), 6.52–6.48 (m, 2H), 6.32 (brs, 2H), 4.16–4.08 (m, 1H), 3.32–3.24 (m, 2H), 2.81 (t, 2H, J = 8.1 Hz), 2.63–2.55 (m, 3H), 2.38–2.31 (m, 1H), 2.24 (s, 3H), 2.09–1.97 (m, 1H), 1.82–1.71 (m, 1H); ESI-MS (m/z, %): 327 (MH^+, 100), 244 (83); ESI-HRMS calculated for $C_{18}H_{23}N_4S$ (MH^+): 327.1637; observed: 327.1650.

4.1.28. tert-Butyl 4-(5-(thiophene-2-carboximidamido)indolin-1-yl) piperidine-1-carboxylate (**42**)

Prepared from *tert*-butyl 4-(5-bromoindolin-1-yl)piperidine-1-carboxylate (**32**) (0.67 g, 1.757 mmol) as described for **39** and **10** to obtain the title compound (0.43 g, 85%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.72 (d, 1H, J = 3.0 Hz), 7.62 (d, 1H, J = 4.8 Hz), 7.10 (dd, 1H, J = 3.6, 4.9 Hz), 6.68 (s, 1H), 6.59 (d, 1H, J = 8.1 Hz), 6.47 (d, 1H, J = 8.4 Hz), 4.05 (d, 2H, J = 12.3 Hz), 3.58–3.50 (m, 1H), 3.28–3.24 (m, 2H), 2.90–2.78 (m, 4H), 1.68 (d, 2H, J = 11.1 Hz), 1.50–1.36 (m, 11H); ESI-MS (m/z, %) 427 (MH^+, 100).

4.1.29. N-(1-(Piperidin-4-yl)indolin-5-yl)thiophene-2-carboximidamide (**43**)

A solution of *tert*-butyl 4-(5-(thiophene-2-carboximidamido) indolin-1-yl)piperidine-1-carboxylate (**42**) (0.23 g, 0.53 mmol) in methanol (10 mL) was treated with 1 N HCl solution (10 mL) and the resulting mixture was refluxed for 30 min. The reaction was brought to room temperature and solvent was evaporated. The crude was dissolved into water (10 mL), filtered and washed with water (2 × 5 mL). The combined water layer was evaporated to obtain the dihydrochloride salt of the title compound (0.18 g, 84%) as a solid. ^{1}H NMR (DMSO-d_6) δ 11.21 (s, 1H), 9.64 (s, 1H), 9.20–9.04 (m, 2H), 8.60 (s, 1H), 8.15–8.12 (m, 2H), 7.36 (t, 1H, J = 4.5 Hz), 7.11–7.03 (m, 2H), 6.66 (d, 1H, J = 8.4 Hz), 3.86–3.74 (m, 1H), 3.44–3.32 (m, 4H), 3.06–2.92 (m, 4H), 1.98–1.78 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 156.37, 149.67, 134.37, 131.61, 129.14, 128.47, 125.19, 124.28, 122.26, 107.90, 55.04, 50.22, 46.70, 42.65, 27.62, 24.00; ESI-MS (m/z, %) 327 (MH^+, 100), 244 (81); ESI-HRMS calculated for $C_{18}H_{23}N_4S$ (MH^+): 327.1637; observed: 327.1636.

4.1.30. N-(1-(1-(2-Hydroxyethyl)piperidin-4-yl)indolin-5-yl) thiophene-2-carboximidamide (**44**)

Prepared from 2-(4-(5-bromoindolin-1-yl)piperidin-1-yl) ethanol (**34**) (1.15 g, 3.54 mmol) as described for **39** and **10** to obtain the title compound (0.85 g, 75%) as a foam. ^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.6 Hz), 7.54 (d, 1H, J = 4.8 Hz), 7.06 (dd, 1H, J = 3.9, 4.9 Hz), 6.61 (s, 1H), 6.51 (d, 1H, J = 8.1 Hz), 6.39 (d, 1H, J = 8.1 Hz), 6.22 (s, 2H), 4.37 (t, 1H, J = 5.1 Hz), 3.51–3.45 (m, 2H), 3.33–3.25 (m, 3H, merged with water peak), 2.96–2.92 (m, 2H), 2.83 (t, 2H, J = 8.4 Hz), 2.38 (t, 2H, J = 6.3 Hz), 2.09–2.00 (m, 2H), 1.66–1.56 (m, 4H); ESI-MS (m/z, %): 371 (MH^+, 100); ESI-HRMS calculated for $C_{20}H_{27}N_4OS$ (MH^+): 371.1900, observed: 371.1917.

4.1.31. N-(1-(1-Methylpiperidin-4-yl)indolin-5-yl)thiophene-2-carboximidamide (**45**)

Prepared from 5-bromo-1-(1-methylpiperidin-4-yl)indoline (**36**) (0.5 g, 1.69 mmol) as described for **39** and **10** to obtain the title compound (0.1 g, 57%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.6 Hz), 7.54 (d, 1H, J = 5.1 Hz), 7.06 (t, 1H, J = 3.9 Hz), 6.61 (s, 1H), 6.51 (d, 1H, J = 8.1 Hz), 6.39 (d, 1H, J = 8.1 Hz), 6.24 (brs, 2H), 3.30–3.24 (m, 3H), 2.86–2.80 (m, 4H), 2.16 (s, 3H), 2.00–1.92 (m, 2H), 1.66–1.56 (m, 4H); ESI-MS (m/z, %): 341 (MH^+, 100), 244 (87); ESI-HRMS calculated for $C_{19}H_{25}N_4S$ (MH^+): 341.1794; observed: 4341.1805.

4.1.32. tert-Butyl 2-methyl-4-(5-(thiophene-2-carboximidamido) indolin-1-yl)piperidine-1-carboxylate (**46**)

Prepared from *tert*-butyl 4-(5-bromoindolin-1-yl)-2-methylpiperidine-1-carboxylate (**37**) (0.25 g, 0.63 mmol) as described for **39** and **10** to obtain the title compound (96 mg, 64.5%) (7:3 mixture of diastereomers) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.71 (d, 1H, J = 3.3 Hz), 7.59 (d, 1H, J = 4.9 Hz), 7.11–7.08 (m, 1H), 6.69 (s, 1H), 6.68–6.22 (m, 4H), 4.51–4.34 (m, 0.4H), 3.99–3.87 (m, 0.4H), 3.83–3.70 (m, 1H), 3.62–3.49 (m, 1H), 3.35–3.17 (m, 3H), 2.86–2.81 (m, 2H), 1.94–1.57 (m, 4H), 1.41, 1.40 (2s, 9H), 1.19 (d, 3H, J = 6.3 Hz); EI-MS (m/z, %): 440 (M^+, 20), 340 (100).

4.1.33. N-(1-(2-Methylpiperidin-4-yl)indolin-5-yl)thiophene-2-carboximidamide (cis-**47** and trans-**47**)

Prepared from *tert*-butyl 2-methyl-4-(5-(thiophene-2-carboximidamido)indolin-1-yl)piperidine-1-carboxylate (**46**) (0.140 g, 0.31 mmol) as described for **19** to obtain the title compounds (90 mg, 83%) as a separable mixture of diastereomers (*cis*:*trans*, 7:3). *cis*-**47**: solid; ^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.6 Hz), 7.55 (d, 1H, J = 5.0 Hz), 7.08–7.05 (m, 1H), 6.61 (s, 1H), 6.53–6.50 (m, 1H), 6.41–6.38 (m, 1H), 6.20 (brs, 2H), 3.46–3.32 (m, 1H), 3.31–3.21 (m, 2H), 3.04–2.95 (m, 1H), 2.83 (t, 2H, J = 8.1 Hz), 2.66–2.56 (m, 2H), 2.20–1.86 (m, 1H), 1.69–1.55 (m, 2H), 1.39 (dq, 1H, J = 11.9, 4.0 Hz), 1.09 (q, 1H, J = 11.6 Hz), 1.00 (d, 3H, J = 6.2 Hz); ESI-MS (m/z, %): 341 (MH^+, 100), 244 (95); ESI-HRMS calculated for $C_{19}H_{25}N_4S$ (MH^+): 341.1794; observed: 341.1802; HPLC purity: 94.65% by area. *trans*-**47**: solid; ^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.5 Hz), 7.55 (d, 1H, J = 5.1 Hz), 7.08–7.05 (m, 1H), 6.62 (s, 1H), 6.53–6.50 (m, 1H), 6.42–6.38 (m, 1H), 6.21 (brs, 2H), 3.65–3.55 (m, 1H), 3.31–3.21 (m, 4H), 2.89–2.75 (m, 4H), 1.77–1.40 (m, 4H), 1.14 (d, 3H, J = 6.8 Hz); ESI-MS (m/z, %): 341 (MH^+, 100), 244 (75); ESI-HRMS calculated for $C_{19}H_{25}N_4S$ (MH^+): 341.1794; observed: 341.1801; HPLC purity: 91.91% by area.

4.1.34. N-(1-(2-Methylpiperidin-4-yl)indolin-5-yl)thiophene-2-carboximidamide [(−)-cis-(**47**) and (+)-cis-(**47**)]

Separation of compounds (−)-*cis*-(**47**) and (+)-*cis*-(**47**) from *cis*-**47** was achieved by preparative HPLC techniques using Chiracel OJ (21 × 250 mm) column eluting with ethanol:hexane (0.1% diethylamine), 18: 82, flow rate: 15 mL/min, wavelength: 254 nm, injection volume: 100 μL of 50 mg/mL (MeOH) solution. (−)-*cis*-(**47**): solid; $[\alpha]_D^{28}$ = −19.6 (c = 0.1, MeOH); ^{1}H NMR (DMSO-d_6) δ 7.67 (dd, 1H, J = 3.6, 0.8 Hz), 7.55 (d, 1H, J = 5.0 Hz), 7.08–7.05 (m, 1H), 6.61 (s, 1H), 6.53–6.50 (m, 1H), 6.41–6.38 (m, 1H), 6.23 (brs, 2H), 3.46–3.32 (m, 1H), 3.31–3.21 (m, 2H), 3.04–2.94 (m, 1H), 2.83 (t, 2H, J = 8.1 Hz), 2.66–2.56 (m, 2H), 2.20–1.86 (m, 1H), 1.69–1.54 (m, 2H), 1.39 (dq, 1H, J = 12.0, 4.0 Hz), 1.09 (q, 1H, J = 11.3 Hz), 1.00 (d, 3H, J = 6.2 Hz); ESI-MS (m/z, %): 341 (MH^+, 100), 244 (95); ESI-HRMS calculated for $C_{19}H_{25}N_4S$ (MH^+): 341.1794; observed: 341.1805. (+)-*cis*-(**47**): solid; $[\alpha]_D^{28}$ = +18.6 (c = 0.1, MeOH); ^{1}H NMR (DMSO-d_6) δ 7.67 (dd, 1H, J = 3.6, 0.9 Hz), 7.55 (d, 1H, J = 5.0 Hz), 7.08–7.05 (m, 1H), 6.61 (s, 1H), 6.53–6.50 (m, 1H), 6.41–6.38 (m, 1H), 6.23 (brs, 2H), 3.46–3.32 (m, 1H), 3.31–3.21 (m, 2H), 3.04–2.95 (m, 1H), 2.83 (t, 2H, J = 8.1 Hz), 2.66–2.56 (m, 2H), 2.20–1.86 (m, 1H), 1.69–1.55 (m, 2H), 1.39 (dq, 1H, J = 11.9, 4.1 Hz), 1.09 (q, 1H, J = 11.3 Hz), 1.00 (d, 3H, J = 6.2 Hz); ESI-MS (m/z, %): 341 (MH^+, 97), 244 (100); ESI-HRMS calculated for $C_{19}H_{25}N_4S$ (MH^+): 341.1794; observed: 341.1811.

*4.1.35. tert-Butyl 3-(5-(thiophene-2-carboximidamido)indolin-1-yl) piperidine-1-carboxylate (**48**)*

Prepared from *tert*-butyl 3-(5-nitroindolin-1-yl)piperidine-1-carboxylate (**38**) (0.55 g, 1.58 mmol) as described for **10** to obtain the title compound (0.51 g, 79%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.68 (d, 1H, J = 3.6 Hz), 7.55 (d, 1H, J = 4.2 Hz), 7.07 (t, 1H, J = 3.6 Hz), 6.64 (s, 1H), 6.53 (d, 1H, J = 8.1 Hz), 6.45 (d, 1H, J = 8.1 Hz), 6.26 (brs, 2H), 4.00–3.86 (m, 2H), 3.78–3.56 (m, 1H), 3.39–3.22 (m, 2H), 2.84 (t, 2H, J = 8.4 Hz), 2.76–2.60 (m, 2H), 1.90–1.23 (m, 13H); ESI-MS (*m*/*z*, %): 427 (MH^+, 100).

*4.1.36. N-(1-(Piperidin-3-yl)indolin-5-yl)thiophene-2-carboximidamide (**49**)*

Prepared from *tert*-butyl 3-(5-(thiophene-2-carboximidamido) indolin-1-yl)piperidine-1-carboxylate (**48**) (0.46 g, 1.078 mmol) as described for **19** to obtain the title compound (0.22 g, 59%) as a solid. ^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.0 Hz), 7.54 (dd, 1H, J = 0.9, 5.1 Hz), 7.06 (dd, 1H, J = 3.9, 5.1 Hz), 6.60 (s, 1H), 6.52 (d, 1H, J = 8.1 Hz), 6.39 (d, 1H, J = 8.1 Hz), 6.20 (brs, 2H), 3.35–3.17 (m, 3H), 2.97–2.79 (m, 4H), 2.53–2.32 (m, 3H), 1.84–1.64 (m, 2H), 1.52–1.44 (m, 2H); ESI-MS (*m*/*z*, %): 327 (MH^+, 80), 244 (100); ESI-HRMS calculated for $C_{18}H_{23}N_4S$ (MH^+): 327.1637, observed: 327.1628; HPLC purity: 94.38% by area.

*4.1.37. 1-(1,4-Dioxaspiro[4.5]decan-8-yl)indoline (**51**)*

Prepared from indoline (**20**) (2.0 g, 16.78 mmol) and 1,4-dioxaspiro[4.5]decan-8-one (**50**) (3.15 g, 20.139 mmol) to obtain the title compound (3.52 g, 81%) as a solid. ^{1}H NMR ($CDCl_3$) δ 7.06–7.02 (m, 2H), 6.62–6.57 (m, 1H), 6.42 (d, 1H, J = 8.1 Hz), 3.46 (s, 4H), 3.46–3.35 (m, 3H), 2.93 (t, 2H, J = 8.2 Hz), 1.87–1.60 (m, 8H); ESI-MS (*m*/*z*, %): 260 (MH^+, 100), 120 (28).

*4.1.38. 5-Bromo-1-(1,4-dioxaspiro[4.5]decan-8-yl)indoline (**52**)*

Prepared from 1-(1,4-dioxaspiro[4.5]decan-8-yl)indoline (**51**) (3.45 g, 13.30 mmol) as described for **27** to obtain the title compound (4.05 g, 90%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.12–7.10 (m, 2H), 6.27–6.24 (m, 1H), 3.95 (s, 4H), 3.38 (t, 3H, J = 8.4 Hz), 2.91 (t, 2H, J = 8.4 Hz), 1.86–1.57 (m, 8H); ESI-MS (*m*/*z*, %): 340, 338 (MH^+, 100), 198 (18).

*4.1.39. 4-(5-Bromoindolin-1-yl)cyclohexanone (**53**)*

A solution of 5-bromo-1-(1,4-dioxaspiro[4.5]decan-8-yl)indoline (**52**) (4.0 g, 11.83 mmol) in acetone (50 mL) was treated with 10% HCl solution (50 mL) and the resulting mixture was stirred for overnight (16 h). Acetone was evaporated, crude was basified with 2 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, 1:4) to obtain the title compound (2.9 g, 83%) as a solid. ^{1}H NMR ($CDCl_3$) δ 7.16–7.14 (m, 2H), 6.32 (d, 1H, J = 8.7 Hz), 3.81 (tt, 1H, J = 3.6, 11.7 Hz), 3.37 (t, 2H, J = 8.4 Hz), 2.95 (t, 2H, J = 8.2 Hz), 2.51–2.41 (m, 4H), 2.18–2.11 (m, 2H), 1.92–1.78 (m, 2H); ESI-MS (*m*/*z*, %): 296, 294 (MH^+, 100), 200 (30).

*4.1.40. 4-(5-Bromoindolin-1-yl)-N-methylcyclohexanamine (cis-**54** and trans-**54**)*

Prepared from 4-(5-bromoindolin-1-yl)cyclohexanone (**53**) (0.5 g, 1.70 mmol) and methylamine hydrochloride (0.11 g, 1.70 mmol) as described for **27** to obtain the title compounds *cis*-**54** and *trans*-**54** (0.48 g, 91%) as partially separable mixture of diastereomers (1:1.2). *Cis*-**54**: ^{1}H NMR (DMSO-d_6) δ 7.09–7.04 (m, 2H), 6.35 (d, 1H, J = 8.1 Hz), 3.36–3.26 (m, 2H), 2.85 (t, 2H, J = 8.4 Hz), 2.59–2.58 (m, 1H), 2.23 (s, 3H), 1.80–1.63 (m, 5H), 1.51–1.33 (m, 5H); ESI-MS (*m*/*z*, %): 311, 309 (MH^+, 100). *trans*-**54**: ^{1}H NMR (DMSO-d_6) δ 7.09–7.05 (m, 2H), 6.36 (d, 1H, J = 8.1 Hz), 3.34–3.29 (m, 3H), 2.85 (t, 2H, J = 8.4 Hz), 2.30–2.16 (m, 5H), 1.95–1.91 (m, 2H), 1.69–1.65 (m, 2H), 1.45–1.32 (m, 2H), 1.32–1.02 (m, 2H); ESI-MS (*m*/*z*, %): 311, 309 (MH^+, 8), 280 (93), 278 (100).

*4.1.41. tert-Butyl 4-(5-bromoindolin-1-yl)cyclohexyl(methyl) carbamate (cis-**55**)*

Prepared from 4-(5-bromoindolin-1-yl)-*N*-methylcyclohexanamine (*cis*-**54**) (0.28 g, 0.90 mmol) as described for **15** to obtain the title compound (0.35 g, 94%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.14–7.10 (m, 2H), 6.32 (d, 1H, J = 8.1 Hz), 4.02–3.98 (m, 1H), 3.70 (s, 2H), 3.56 (t, 2H, J = 8.1 Hz), 3.32–3.30 (m, 1H), 2.94 (t, 2H, J = 8.1 Hz), 2.79–2.74 (m, 3H), 2.15–2.10 (m, 2H), 1.81–1.67 (m, 4H), 1.46 (s, 9H); ESI-MS (*m*/*z*, %): 411 (61), 409 (M^+, 58), 331 (100).

*4.1.42. tert-Butyl 4-(5-bromoindolin-1-yl)cyclohexyl(methyl) carbamate (trans-**55**)*

Prepared from 4-(5-bromoindolin-1-yl)-*N*-methylcyclohexanamine (*trans*-**54**) (0.35 g, 1.14 mmol) as described for **15** to obtain the title compound (0.43 g, 92%) as a syrup. ^{1}H NMR ($CDCl_3$) δ 7.12 (brs, 2H), 6.27 (d, 1H, J = 7.8 Hz), 3.96–3.92 (m, 1H), 3.70 (s, 2H), 3.37 (t, 2H, J = 8.4 Hz), 3.32–3.23 (m, 1H), 2.93 (t, 2H, J = 8.4 Hz), 2.80–2.74 (m, 4H), 1.91–1.73 (m, 5H), 1.52 (s, 9H); ESI-MS (*m*/*z*, %): 411 (MH^+, 14), 331 (100), 156 (85).

*4.1.43. tert-Butylmethyl(4-(5-(thiophene-2-carboximidamido) indolin-1-yl)cyclohexyl)carbamate (cis-**56**)*

Prepared from *tert*-butyl 4-(5-bromoindolin-1-yl)cyclohexyl(methyl)carbamate (*cis*-**55**) (0.36 g, 0.87 mmol) as described for **39** and **10** to obtain the title compound (0.25 g, 67%). ^{1}H NMR (DMSO-d_6) δ 7.68 (d, 1H, J = 3.6 Hz), 7.56 (d, 1H, J = 5.1 Hz), 7.07 (dd, 1H, J = 3.9, 4.8 Hz), 6.63 (s, 1H), 6.51 (d, 1H, J = 7.5 Hz), 6. 42 (d, 1H, J = 8.4 Hz), 6.28–6.27 (m, 2H), 3.88–3.81 (m, 1H), 3.45 (t, 2H, J = 7.9 Hz), 3.26–3.24 (m, 1H), 2.84 (t, 2H, J = 7.8 Hz), 2.72 (s, 3H), 2.10–2.06 (m, 2H), 1.81–1.74 (m, 2H), 1.66–1.57 (m, 2H), 1.51–1.46 (m, 2H), 1.40 (s, 9H); ESI-MS (*m*/*z*, %): 455 (MH^+, 100).

*4.1.44. tert-Butylmethyl(4-(5-(thiophene-2-carboximidamido) indolin-1-yl)cyclohexyl)carbamate (trans-**56**)*

Prepared from *tert*-butyl 4-(5-bromoindolin-1-yl)cyclohexyl (methyl)carbamate (*trans*-**55**) (0.42 g, 1.02 mmol) as described for **39** and **10** to obtain the title compound (0.37 g, 86%). ^{1}H NMR (DMSO-d_6) δ 7.68 (d, 1H, J = 3.0 Hz), 7.56 (d, 1H, J = 5.1 Hz), 7.07 (dd, 1H, J = 3.9, 5.1 Hz), 6.62 (s, 1H), 6.54 (d, 1H, J = 8.4 Hz), 6.41 (d, 1H, J = 8.4 Hz), 6.32–6.28 (m, 2H), 3.81–3.77 (m, 1H), 3.28–3.25 (m, 3H), 2.83 (t, 2H, J = 8.1 Hz), 2.68 (s, 3H), 1.78 (d, 2H, J = 10.8 Hz), 1.64–1.62 (m, 4H), 1.50–1.46 (m, 2H), 1.40 (s, 9H); ESI-MS (*m*/*z*, %): 455 (MH^+, 100).

*4.1.45. N-(1-(4-(Methylamino)cyclohexyl)indolin-5-yl)thiophene-2-carboximidamide (cis-**57**)*

Prepared from *tert*-butylmethyl(4-(5-(thiophene-2-carboximidamido)indolin-1-yl)cyclohexyl)carbamate (*cis*-**56**) (0.23 g, 0.50 - mmol) as described for **19** to obtain the title compound (0.145 g, 81%). ^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.0 Hz), 7.54 (dd, 1H, J = 0.9, 5.1 Hz), 7.06 (dd, 1H, J = 3.9, 4.9 Hz), 6.60 (s, 1H), 6.51 (d, 1H, J = 8.1 Hz), 6.38 (d, 1H, J = 8.1 Hz), 6.21 (brs, 2H), 3.32–3.25 (m, 4H), 2.82 (t, 2H, J = 8.4 Hz), 2.62 (brs, 1H), 2.25 (s, 3H), 1.81–1.66 (m, 4H), 1.53–1.39 (m, 4H); ESI-MS (*m*/*z*, %): 355 (MH^+, 82), 324 (100); ESI-HRMS calculated for $C_{20}H_{27}N_4S$ (MH^+): 355.1949, observed: 355.1950; HPLC purity: 91.42% by area.

*4.1.46. N-(1-(4-(Methylamino)cyclohexyl)indolin-5-yl)thiophene-2-carboximidamide (trans-**57**)*

Prepared from *tert*-butylmethyl(4-(5-(thiophene-2-carboximidamido)indolin-1-yl)cyclohexyl)carbamate (*trans*-**56**) (0.37 g, 0.82 - mmol) as described for **19** to obtain the title compound (0.19 g, 66%).

^{1}H NMR (DMSO-d_6) δ 7.67 (d, 1H, J = 3.3 Hz), 7.54 (dd, 1H, J = 0.6, 5.1 Hz), 7.06 (dd, 1H, J = 3.9, 4.9 Hz), 6.60 (s, 1H), 6.52 (d, 1H, J = 8.1), 6.21 (brs, 2H), 3.31–3.24 (m, 4H), 3.16 (s, 1H), 2.82 (t, 2H, J = 8.2 Hz), 2.31–2.19 (m, 4H), 1.95 (d, 2H, J = 11.4 Hz), 1.73 (d, 2H, J = 11.4 Hz), 1.47–1.35 (m, 2H), 1.16–1.04 (m, 2H); ESI-MS (m/z, %): 355 (MH^+, 100), 324 (81), 133 (35); ESI-HRMS calculated for $C_{20}H_{27}N_4S$ (MH^+): 355.1935, observed: 355.1950; HPLC purity: 93.05% by area.

4.2. General procedure for the conversion of the free base to the dihydrochloride salt

To a solution of the free base (1.0 equiv) in methanol was added 1 M HCl in diethyl ether (3.0 equiv). The solution was stirred at room temperature for 10 min then concentrated to dryness. The residue 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.

4.3. 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 is 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 is 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 pre-incubation time of 15 min at rt. The reaction is initiated by addition of 20 μL 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. The reactions are carried out at 37 °C for 45 min. The reaction is 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 is separated by DOWEX (ion-exchange resin DOWEX 50 W X 8-400, SIGMA) and the DOWEX is removed by spinning at 12,000 g for 10 min in the centrifuge. 70 μL aliquot of the supernatant is added to 100 μL of scintillation fluid and the radio activity is 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 **3**. All assays are performed in duplicate.

4.4. Efficacy in the Chung model of neuropathic pain

Nerve ligation injury was performed according to the literature procedure. Rats were anesthetized with halothane and the L_5 and L_6 spinal nerves were exposed, carefully isolated, and tightly ligated with 4-0 silk suture distal to the DRG. After ensuring homeostatic stability, the wounds were sutured, and the animals allowed 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 non-painful stimuli.

4.5. Migraine model

Male Sprague-Dawley rats were anesthetized using ketamine/xylazine (80 mg/kg, i.p.), the top of the head was shaved using a rodent clipper (Oster Golden A5 w/size 50 blade), and the shaved area was cleaned with betadine and 70% ethanol. Animals were placed into a stereotaxic apparatus (Stoelting model 51600) and the body core temperatures of 37 °C were maintained using a heating pad placed below the animals. Within the shaved and cleaned area on the head, a 2 cm incision was made using a scalpel with a #10 blade and any bleeding was cleaned using sterile cotton swabs. Location of bregma and midline bone sutures were identified as references and a small hole 1 mm in diameter was made using a hand drill without breaking the dura but deep enough to expose the dura. Two additional holes (1 mm in diameter) 4–5 mm from the previous site were made in order to mount stainless steel screws securing the cannula through which an inflammatory soup could be delivered to induce experimental migraine. A modified intracerebroventricular (ICV) cannula was placed into the hole without penetrating into or through the dura. The ICV cannula was modified by cutting it to a length of 1 mm from the bottom of the plastic threads using a Dremel mototool and a file to remove any steel burrs. Once the modified migraine cannula was in place, dental acrylic was placed around the migraine cannula and stainless steel screws in order to assure that the cannula was securely mounted. Once the dental acrylic was dry (i.e., after 10–15 min) the cap of the cannula was secured on top to avoid contaminants entering the cannula and the skin was sutured back using 3-0 silk suture. Animals were given an antibiotic injection (Amikacin C, 5 mg/kg, i.m.) and removed from the stereotaxic frame and allowed to recover from anesthesia on a heated pad. Animals were placed in a clean separate rat cage for a 5 day recovery period. An injection cannula (Plastics One, C313I cut to fit the modified ICV cannulas) connected to a 25 μL Hamilton Syringe (1702SN) by tygon tubing was used to inject 10 μL of the IM solution onto the dura.

Naïve animals prior to the day of migraine surgery are placed in suspended plexiglass chambers (30 cm L × 15 cm W × 20 cm H) with a wire mesh bottom (1 cm^2) and acclimated to the testing chambers for 30 min. The paw withdrawal thresholds to tactile stimuli were determined in response to probing with calibrated von Frey filaments (Stoelting, 58011). The von Frey filaments were applied perpendicularly to the plantar surface of the hind paw of the animal until it buckles slightly, and is held for 3–6 s. A positive response was indicated by a sharp withdrawal of the paw. The 50% paw withdrawal threshold was determined by the non-parametric method of Dixon (9). An initial probe equivalent to 2.00 g was applied and if the response was negative the stimulus was increased one increment, otherwise a positive response resulted in a decrease of one increment. The stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was observed. This "up-down" method was repeated until three changes in behavior were determined. The pattern of positive and negative responses was tabulated. The 50% paw withdrawal threshold is determined as $(10^{[Xf+kM]})/10{,}000$, where Xf = the value of the last von Frey filament employed, k = Dixon value for the positive/negative pattern, and M = the mean (log) difference between stimuli. Only naïve animals with baselines of 11–15 g were used in the experiment. Fifteen grams was used as the maximal cut-off. Five days post migraine surgery animals paw withdrawal thresholds were re-tested using the same habituation and von Frey procedure as stated above. Data were converted to % "antiallodynia" by the formula: % activity = 100 × (post-migraine value − baseline value)/(15 g − baseline value). Only animals that demonstrated no

difference in their tactile hypersensitivity as compared to their pre-migraine surgery values were used in all studies.

After establishing baseline paw withdrawal thresholds, individual animals were removed from the testing chamber, the cap of the migraine cannula was removed and animals received an injection of either a mixture of IM (1 mM Histamine, 1 mM 5-HT [Serotonin], 1 mM Bradykinin, 1 mM PGE_2) or vehicle at 10 μL volume via the migraine cannula over a 5–10 s period. The IM cocktail was made fresh on the day of each experiment. The cap of the migraine cannula was replaced, individual animals were placed back into their corresponding testing chamber and paw withdrawal thresholds were measured at 1 h intervals over a 6 h time course. Data were converted to % "antiallodynia" by the formula: % activity = 100 × (post-IM value − pre-IM baseline value)/(15 g − pre-IM baseline value).

4.6. hERG K^+ channel binding assay

The assay was carried with human recombinant HEK-293 cells using [^{3}H]astemizole as a ligand (2 nM) with incubation at 22 °C for 75 min with reference to astemizole according to the literature procedure. The specific ligand binding to the receptor is defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabelled ligand. The results are expressed as a percent of control specific binding ((measured specific binding/control specific binding) × 100) obtained in the presence of **43**. The IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (*nH*) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting ($Y = D + [(A - D)/(1 + (C/C_{50})^{nH})]$, where Y = specific binding, D = minimum specific binding, A = maximum specific binding, C = compound concentration, $C_{50} = IC_{50}$, and *nH* = slope factor).

4.7. hERG K^+ channel conventional patch-clamp assay

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 ($\sim 2 \times 10^4$ 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, **43** 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 4.

Table 4
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).

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 **43** was constructed from the percentage reductions of current amplitude by sequential concentrations.

4.8. High throughput broad screen

In each experiment, the respective reference compound was tested concurrently with **43**, and the data were compared with historical values determined (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 **43**. 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 unlabelled 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.

Acknowledgments

We are grateful to NoAb BioDiscoveries Inc. (Mississauga, ON, Canada) for performing the human NOS inhibition assays, Cytochrome P450 enzyme inhibition studies and pharmacokinetic studies, Asinex Ltd. (Moscow, Russia) for performing the human iNOS inhibition assays and Cerep (France and USA) for hERG binding assay, hERG patch-clamp assay and broad screen profile. We thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for providing an undergraduate student research award (USRA) for T.R.

Appendix A. Supporting information

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.07.006.

References

[1] J.U. Adelman, E.J. Lewit, Clin. Cornerstone 4 (2002) 53–64.
[2] R. Burstein, B. Collins, M. Jakubowski, Ann. Neurol. 55 (2004) 19–24.
[3] S. Wasson, V.K. Jayam, Clin. Neuropharmacol. 27 (2004) 198–200.
[4] I.M. Bell, S.N. Gallicchio, M.R. Wood, A.G. Quigley, C.A. Stump, C.B. Zartman, J.F. Fay, C.-C. Li, J.J. Lynch, E.L. Moore, S.D. Mosser, T. Prueksaritanont, C.P. Regan, S. Roller, C.A. Salvatore, S.A. Kane, J.P. Vacca, H.G. Selnick, ACS Med. Chem. Lett. 1 (2010) 24–29.
[5] C. Drahl, Chem. Eng. News 87 (2009) 31–33.
[6] G. Wei, V.L. Dawson, J.L. Zweier, Biochim. Biophys. Acta 1455 (1999) 23–34.
[7] D.O. Willenborg, S.A. Fordham, M.A. Staykova, I.A. Ramshawn, W.B. Cowden, J. Immunol. 163 (1999) 5278–5286.
[8] F. Tao, Y.-X. Tao, P. Mao, C. Zhao, D. Li, W.J. Liaw, S.N. Raja, R.A. Johns, Neuroscience 120 (2003) 847–854.
[9] Q. Lin, J. Paleček, V. Palečková, Y.B. Peng, J. Wu, M. Cui, W.D. Willis, J. Neurophysiol. 81 (1999) 1075–1085.
[10] J.H. Hao, X.J. Xu, Pain 66 (1996) 313–319.
[11] J. Olesen, Pharmacol. Ther. 120 (2008) 157–171.
[12] P.L. Huang, Z. Huang, H. Mashimo, K.D. Bloch, M.A. Moskowitz, J.A. Bevan, M.C. Fishman, Nature 377 (1995) 239–242.
[13] H.L. Lassen, H.K. Iverson, J. Olesen, Eur. J. Clin. Pharmacol. 59 (2003) 499–505.
[14] S. Maddaford, S.C. Annedi, J. Ramnauth, S. Rakhit, Annu. Rep. Med. Chem. 44 (2009) 27–50. and references within.
[15] E.P. Erdal, E.A. Litzinger, J. Seo, Y. Zhu, H. Ji, R.B. Silverman, Curr. Top. Med. Chem. 5 (2005) 603–624.
[16] A.C. Tinker, A.V. Wallace, Curr. Top. Med. Chem. 6 (2006) 77–92.
[17] R.B. Silverman, Acc. Chem. Res. 42 (2009) 439–451.

[18] J. Patman, N. Bhardwaj, J. Ramnauth, S.C. Annedi, P. Renton, S.P. Maddaford, S. Rakhit, J.S. Andrews, Bioorg. Med. Chem. Lett. 17 (2007) 2540–2544.
[19] S.C. Annedi, J. Ramnauth, S.P. Maddaford, P. Renton, S. Rakhit, G. Mladenova, P. Dove, S. Silverman, J.S. Andrews, M.D. Felice, F. Porreca, J. Med. Chem. 55 (2012) 943–955.
[20] J. Ramnauth, J. Speed, S.P. Maddaford, P. Dove, S.C. Annedi, P. Renton, S. Rakhit, J. Andrews, S. Silverman, G. Mladenova, S. Zinghini, S. Nair, C. Catalano, D.K.H. Lee, M.D. Felice, F. Porreca, J. Med. Chem. 54 (2011) 5562–5575.
[21] S. Maddaford, P. Renton, J. Speed, S.C. Annedi, J. Ramnauth, S. Rakhit, J. Andrews, G. Mladenova, L. Majuta, F. Porreca, Bioorg. Med. Chem. Lett. 21 (2011) 5234–5238.
[22] P. Renton, J. Speed, S. Maddaford, S.C. Annedi, J. Ramnauth, S. Rakhit, J. Andrews, Bioorg. Med. Chem. Lett. 21 (2011) 5301–5304.
[23] S.C. Annedi, S.P. Maddaford, G. Mladenova, J. Ramnauth, S. Rakhit, J.S. Andrews, D.K.H. Lee, D. Zhang, F. Porreca, D. Bunton, L. Christie, J. Med. Chem. 54 (2011) 7408–7416.
[24] M. Bercot-Vatteroni, Ann. Chim. 7 (1962) 303–337.
[25] V. Hasimbegovic, J. Slätt, J. Bergman, T. Janosik, J. Heterocycl. Chem. 44 (2007) 1213–1217.
[26] A.F. Abdel-Magid, K.G. Carson, B.D. Harris, C.A. Maryanoff, R.D. Shah, J. Org. Chem. 61 (1996) 3849–3862.
[27] X. Huang, S. Buchwald, Org. Lett. 3 (2001) 3417–3419.
[28] R.J. Abraham, J.J. Byrne, L. Griffiths, R. Koniotou, Magn. Reson. Chem. 43 (2005) 611–624.
[29] D.S. Bredt, P.M. Hwang, C.E. Glatt, C. Lowenstein, R.R. Reed, S.H. Snyder, Nature 351 (1991) 714–718.
[30] S.H. Kim, J.M. Chung, Pain 50 (1992) 355–363.
[31] R.M. Edelmayer, T.W. Vanderah, L. Majuta, E.T. Zhang, B. Fioravanti, M.D. Felice, J.G. Chichorro, M.H. Ossipov, T. King, J. Lai, S.H. Kori, A.C. Nelsen, K.E. Cannon, M.M. Heinricher, F. Porreca, Ann. Neurol. 65 (2009) 184–193.
[32] C. Jamieson, E.M. Moir, Z. Rankovic, G. Wilshart, J. Med. Chem. 49 (2006) 5029–5046.
[33] K. Finlayson, A.J. Pennington, J.S. Kelly, Eur. J. Pharmacol. 412 (2001) 203–212.
[34] Z. Zhou, Q. Gong, B. Ye, Z. Fan, J.C. Makielski, G.A. Robertson, C.T. January, Biophys. J. 74 (1998) 230–241.
[35] http://www.cerep.fr/.
[36] M.C. Wenlock, R.P. Austin, P. Barton, A.M. Davis, P.D. Leeson, J. Med. Chem. 46 (2003) 1250–1256. and references therein.
[37] T.T. Wager, X. Hou, P.R. Verhoest, A. Vilalobos, ACS. Chem. Neurosci. 1 (2010) 435–449.