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3,5-Disubstituted indole derivatives as selective human neuronal nitric oxide synthase (nNOS) inhibitors

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ABSTRACT

A series of 3,5-disubstituted indole derivatives was designed, synthesized and evaluated as inhibitors of human nitric oxide synthase (NOS). Various guanidine isosteric groups were explored at the 5-position of the indole ring, while keeping the basic amine side chain such as *N*-methylpiperidine ring, fixed at the 3-position of the indole ring. Compounds having 2-thiophene amidine and 2-furanyl amidine groups (**7**, **8**, **10** and **12**) showed increased activity for human neuronal NOS and good selectivity over endothelial and inducible NOS isoforms. Compound **8** was shown to reverse (10 mg/kg, ip) thermal hyperalgesia in the L_5/L_6 spinal nerve ligation (neuropathic pain) model and was devoid of any significant drug–drug interaction potential due to cytochrome P450 inhibition or cardiovascular liabilities associated with the inhibition of endothelial NOS.

Nitric oxide (NO), synthesized by three distinct but closely related isoforms of nitric oxide synthase (NOS), is a reactive free radical gas involved in a wide range of physiological functions and pathophysiological states.[1] Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, whereas inducible NOS (iNOS) is expressed during bacterial infection and inflammation.[2] Overproduction or excess NO produced by NOS, particularly nNOS, can lead to the development of several disorders such as septic shock, stroke, pain (migraine, CTTH, visceral, and neuropathic), and neurodegenerative disorders (Parkinson's disease, MS, and Alzheimer's disease).[3,4] To gain the therapeutic benefits of nNOS inhibition, it is necessary to achieve selective nNOS inhibition to preserve the important roles of eNOS and iNOS in controlling blood pressure and in immune response, respectively.[5,6]

First generation NOS inhibitors, which were modified analogs of either L-arginine (substrate) or L-citrulline (product), were non-selective among the NOS isoforms.[7] However, later generations of peptidic as well as non-peptidic NOS inhibitors designed based on the combination of X-ray crystallography and enzyme mutation studies were proven to be selective among the NOS isoforms.[8] By exploring the one amino acid difference (Asp597 in nNOS vs Asn368 in eNOS) in the active site of both neuronal and endothelial NOS, up to 100-fold selectivity for nNOS over the eNOS isoform was achieved.[9] The important contributing factor of a guanidine group for NOS inhibition has became one of the most promising strategies for early selective NOS inhibition design.[10] Over the course of more than two decades, various structural classes of selective NOS inhibitors such as guanidines, isothioureas, amidines, aminopyridines, thioureas, imidazoles, indazoles, indoles, quinolones and several others were identified.[11] In particular, amidine and aminopyridine derivatives attracted more attention among the selective nNOS inhibitors due to their druglike nature with better PK/PD properties.[8]

The pharmacophore model adapted by our group that targets the arginine binding site of the NOS enzyme contains a guanidine isosteric group and a basic amine group, attached to a central aryl scaffold as described earlier.[12] The amidine group makes an important bidentate interaction with the conserved glutamic acid residue; whereas the basic amine is shown to improve potency and selectivity among the NOS isoforms (**1** and **2**, Fig. 1).[12–14] On the basis of our previous results with 3,6-disubstituted indole derivatives (**2**, Fig. 1),[15] we selected the *N*-methylpiperidine ring as the basic amine side chain at 3-position of the indole ring and extended our study to explore various guanidine isosteric groups at the 5-position of the indole ring for the optimization process. In our continued search for new small molecule druglike selective nNOS inhibitors,[12–16] herein we report the design, synthesis, and biological activity evaluations of a series of 3,5-disubstituted

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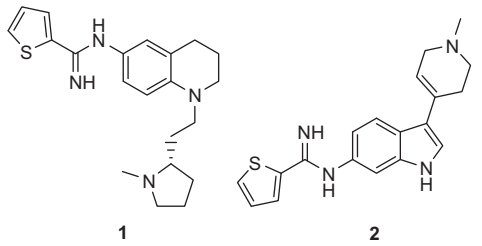
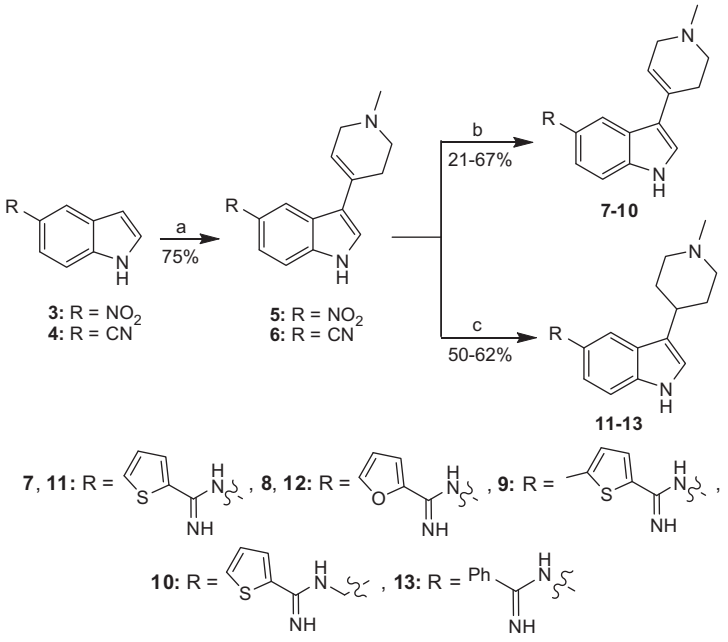


Figure 1. Recently reported selective human nNOS inhibitors.

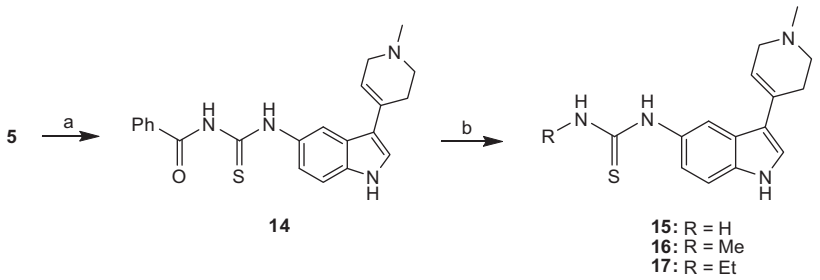
indole derivatives, which led to the identification of **7** and **8** as lead candidates.

Synthesis of 3,5-disubstituted indole derivatives was carried out according to the general procedures described in Schemes 1 and 2. The substitution on 3-position of the indole ring in **3** and **4** was introduced by a key carbon–carbon bond formation reaction with *N*-methyl-4-piperidone under basic conditions to obtain **5** and **6**, respectively, as described earlier (Scheme 1).[17] Reduction of the nitro group in **5** was performed under hydrogenation conditions with hydrazine hydrate to obtain the corresponding amine, which was coupled to methyl thiophene-2-carbimidothioate hydroiodide, benzyl furan-2-carbimidothioate hydrobromide, and 5-methylthiophene-2-carboximidothioic acid methyl ester hydroiodide to obtain the target compounds **7**, **8**, and **9**, respectively.[18,19] To avoid the reduction of the 1-methyl-1,2,3,6-tetrahydropyridine ring, the reduction reaction was carried out in a pre-heated oil bath for a maximum of 5–10 min. or until the yellow color disappears. Reduction of the cyano group in **6** was carried out using $LiAlH_4$, followed by coupling of the free amine to methyl thiophene-2-carbimidothioate hydroiodide provided the target compound **10**. The reduction of the nitro group as well as the 1-methyl-1,2,3,6-tetrahydropyridine ring was carried out under standard hydrogenation conditions using palladium on carbon to obtain the corresponding amine, which was coupled to methyl thiophene-2-carbimidothioate hydroiodide, benzyl furan-2-carbimidothioate hydrobromide, and methyl benzimidate hydrochloride to obtain **11**–**13**, respectively. The reduction of the nitro group in **5** was carried out as described above to obtain the free amine, which was protected with benzoylisothiocyanate to obtain **14** (Scheme 2). The benzoyl group was removed under strong basic conditions to obtain the free amine **15**, which was alkylated either with iodomethane or iodoethane to obtain the *S*-alkyl thiourea derivatives **16** and **17**, respectively.

All compounds were converted into their corresponding dihydrochloride salts,[20] and their inhibitory activities were measured against all three human NOS isoforms (Table 1). In a radiometric method, inhibitory activities were measured by the conversion of [^{3}H]-L-arginine into [^{3}H]-L-citrulline.[21] Compound **7** with 2-thiophene amidine attached directly to the 5-position of the indole ring was the most potent inhibitor for nNOS (0.19 μM) and showed the best selectivity (118- and 105-fold) over eNOS and iNOS isoforms, respectively, among the current series. 2-Furanyl amidine compounds (**8** and **12**) were less potent (twofold) for nNOS isoform when compared to the corresponding 2-thiophene amidine compounds (**7** and **11**). In general, compounds with 2-thiophene amidine and 2-furanyl amidine groups (**7**, **8**, **10**, **11**, and **12**) showed very good potency for human nNOS isoform, when compared to bulky groups such as 5-methyl-2-thiophene amidine, phenyl amidine and carbamothioyl benzamide in **9**, **13**, and **14**, respectively. Reduction of the 1-methyl-1,2,3,6-tetrahydropyridine ring (**7** and **8**) into 1-methylpiperidine ring (**11** and **12**) resulted in 4–5 times weaker potency for nNOS isoform with reduced selectivity over the eNOS and iNOS isoforms. Extension of the 2-thiophene amidine group by one carbon (**10**) resulted in weaker potency (~7-fold) for nNOS isoform with reduced selectivity over the eNOS and iNOS isoforms, when compared to **7**. Substitution of the 2-thiophene amidine in **7** either with 5-methyl-2-thiophene amidine or phenyl amidine in **9** and **13**, resulted in loss of potency (up to 50-fold) for nNOS isoform and selectivity over the eNOS isoform (eNOS/nNOS = 118 for **7** vs 6 and 14 for **9** and **13**, respectively). *S*-Alkyl thiourea derivatives (**16** and **17**) also showed weaker potency (up to 25-fold) for nNOS isoform with reduced selectivity over eNOS

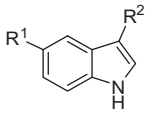


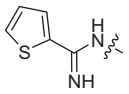
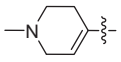
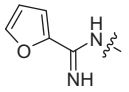
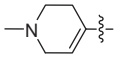
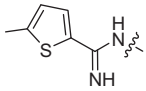
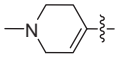
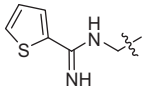
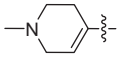
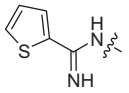
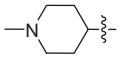
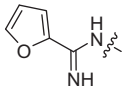
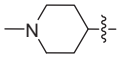
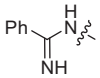
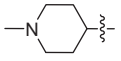
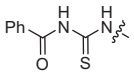
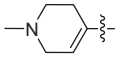
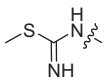
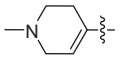
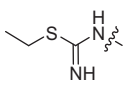
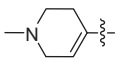
Scheme 1. Reagents and conditions: (a) *N*-Methyl-4-piperidone, pyrrolidine, EtOH, reflux; (b) (i) hydrazine hydrate, Raney-Ni, MeOH, $LiAlH_4$, THF, reflux; (ii) methyl thiophene-2-carbimidothioate hydroiodide or benzyl furan-2-carbimidothioate hydrobromide or 5-methylthiophene-2-carboximidothioic acid methyl ester hydroiodide, EtOH, rt; (c) (i) Pd-C/H_2, EtOH, rt; (ii) methyl thiophene-2-carbimidothioate hydroiodide or benzyl furan-2-carbimidothioate hydrobromide or methyl benzimidate hydrochloride, EtOH, rt.



Scheme 2. Reagents and conditions: (a) (i) Hydrazine hydrate, Raney-Ni, MeOH, reflux; (ii) benzoylisothiocyanate, acetone, rt, 92%. (b) (i) 2 N NaOH, THF, reflux, 79%, (ii) Iodomethane, acetone, reflux 19% or iodoethane , acetone, reflux, 25%.

Table 1
Inhibition of human NOS enzymes by 3,5-disubstituted indole derivatives



Compound	R^1	R^2	nNOS IC_{50} (μM)[a]	eNOS IC_{50} (μM)[a]	iNOS IC_{50} (μM)[a]	eNOS/nNOS	iNOS/nNOS
7			0.19 (0.11–0.31)	22.4 (7.51–66.4)	20.0 (8.63–46.7)	118	105
8			0.55 (0.29–1.05)	36.1 (26.3–49.4)	58.5 (28.3–120.9)	66	106
9			10.4 (5.7–18.6)	65.3 (38.3–111)	NT[b]	6	NC[c]
10			1.29 (0.97–1.71)	64.7 (50.4–82.8)	85.0 (44.8–161)	50	66
11			1.02 (0.68–1.52)	24.2 (16.7–34.9)	58.2 (38.3–88.5)	24	57
12			2.24 (0.66–7.54)	97.4 (68.1–139)	107.9 (55.8–208.4)	43	48
13			18.0 (5.92–54.5)	249 (40.9–1514)	NT[b]	14	NC[c]
14			14.0 (9.8–19.8)	43.0 (32.0–57.6)	NT[b]	3	NC[c]
16			2.25 (1.34–3.78)	36.1 (27.3–47.6)	17.1 (8.5–34.1)	16	8
17			4.83 (2.46–9.4)	105.2 (54.4–203)	NT[b]	22	NC[c]
2[d]			0.80 (0.5–1.4)	26.5 (19.5–35.9)	12.3 (7.3–20.8)	33	15

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] NT = Not tested.
[c] NC = Not calculable.
[d] Compound **2** is a recently reported selective human nNOS inhibitor, included for comparison.

isoform, when compared to **7** and is consistent with our previous observation with 2-aminobenzothiazole compounds.[12] Overall, 2-thiophene amidine and 2-furanyl amidine groups were determined to be ideal guanidine isosteric groups for designing selective nNOS inhibitors using this particular 3,5-disubstituted indole series of compounds. At the same time moving the 2-thiophene amidine group from 6-position of the indole ring (**2**) to 5-position of the indole ring (**7**) was shown to increase the potency for nNOS (fourfold) isoform and selectivity over the eNOS (3.5-fold) and iNOS (sevenfold) isoforms and is consistent with our previous observation with 2-aminobenzothiazole compounds.[12] Compounds **7** and **8** were tested for their inhibitory activity in rat nNOS isoform and showed 12- and 5-fold weaker inhibitory activity (**7**: IC_{50} = 2.3 μM, **8**: IC_{50} = 2.6 μM), respectively.

To investigate the potential therapeutic application of these new nNOS inhibitors, **8** was selected for further profiling in an in vivo neuropathic pain model (Fig. 2). 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 produce thermal and mechanical allodynia of the affected paw.[22] The thermal hyperalgesia was tested by the withdrawal latency to application of radiant heat to the paw. Intraperitoneal administration of **8** at 10 mg/kg resulted in reversal of thermal hyperalgesia between 30 and 120 min with a maximum effect of 94% reversal at 30 min (Fig. 2). After the positive in vivo results with **8**, it was tested for five major human cytochrome P450 enzyme inhibition studies to assess the potential for metabolism-based drug–drug interactions. This will also rule out any inhibitory activity with cytochrome P450 enzymes that are closely related to NOS.[23] Compound **8** was tested in a range of concentrations (up to 100 μM) against the five major human cytochrome P450 enzymes. Compound **8** showed very little drug–drug interaction potential, as the compound was not active (CYP 2C9, CYP 2C19, and CYP 3A4: IC_{50} > 100 μM) or very weak (CYP 2D6: IC_{50} = 20.5 μM) or weak (CYP 1A2: IC_{50} = 9.52 μM) inhibitor of the five major human cytochrome P450 enzymes.

Compound **8** was also assessed for the contractile response (inhibition of acetylcholine (ACh)-mediated vasorelaxation) on isolated human resistance arteries to assess the undesirable cardiovascular liability associated with the inhibition of eNOS isoform (Fig. 3).[5,24] The arteries were preconstricted with U46619, a thromboxane A2 (TxA2) mimetic agent, and then exposed to ACh, an endothelium and nitric oxide dependent vasodilator as described earlier.[15] The response to ACh provides information on the activity of eNOS in an active human biological tissue and thereby any inhibitory effect of **8** on the eNOS isoform. Compound **8** did not possess any significant vasoconstrictive effect associated with the inhibition of human eNOS at a concentration of 10 μM (Fig. 3). This result suggests that **8** is devoid of any cardiovascular liabilities associated with the inhibition of eNOS based on its eNOS potency (36.1 μM), and is consistent with our previous observation with the reference compound **2**.[15]

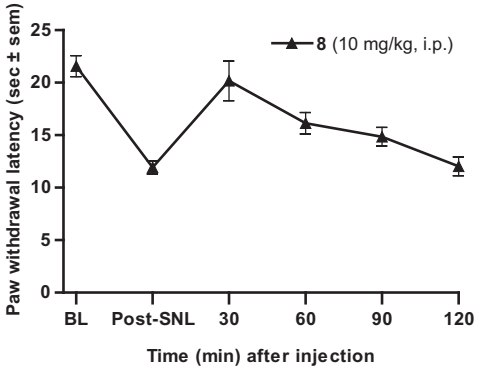


Figure 2. Intraperitoneal administration of **8** (10 mg/kg) attenuates thermal hyperalgesia in the L_5/L_6 SNL model of neuropathic pain. The mean values reported are from six animals.

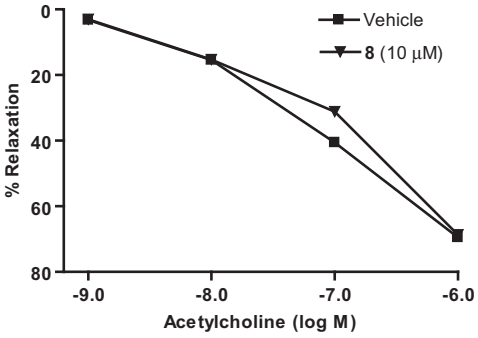


Figure 3. Compound **8** did not show any significant vasoconstrictive effect (inhibition of ACh-mediated vasorelaxation) associated with the inhibition of human eNOS at a concentration of 10 μM.

In conclusion, a series of 3,5-disubstituted indole derivatives was designed, synthesized and shown to be selective inhibitors of human nNOS. In this study, we picked the most promising piperidine basic amine side chain at 3-position of the indole ring and explored various guanidine isosteric groups at the 5-position of the indole ring. Two of the guanidine isosteric groups (2-thiophene amidine and 2-furanyl amidine) were shown to be sub-micromolar potent at human nNOS and very selective over the eNOS and iNOS isoforms. The potential therapeutic application of these compounds was demonstrated by the reversal of thermal hyperalgesia in an in vivo model of neuropathic pain with **8**. The weak inhibitory activity at the cytochrome P450 enzymes and the lack of eNOS mediated vasoconstrictive effect associated with eNOS inhibition with **8** mitigates drug–drug interaction potential as well as the cardiovascular liabilities associated with these selective nNOS inhibitors, respectively. Further lead optimization (based on **7** and **8**) and various in vivo activity evaluations are underway and will be communicated in due course.

Acknowledgments

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