Published in final edited form as:
Bioorg Med Chem Lett. 2010 January 15; 20(2): 554. doi:10.1016/j.bmcl.2009.11.086.

Potent and selective neuronal nitric oxide synthase inhibitors with improved cellular permeability

Fengtian Xue[a], **Jianguo Fang**[a], **William W. Lewis**[a], **Pavel Martásek**[b,c], **Linda J. Roman**[b], and **Richard B. Silverman**[a,*]

[a]Department of Chemistry, Department of Biochemistry, Molecular Biology, and Cell Biology, Center for Molecular Innovation and Drug Discovery, Chemistry of Life Processes Institute, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113, USA

[b]Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas

[c]Department of Pediatrics and Center for Applied Genomics, 1st School of Medicine, Charles University, Prague, Czech Republic

Abstract

Recently, a series of potent and selective neuronal nitric oxide synthase inhibitors containing two basic nitrogen atoms was reported (Ji, H.; Stanton, B. Z.; Igarashi, J.;, Li, H.; Martásek, P.; Roman, L. J.; Poulos, T. L.; Silverman, R. B. *J. Am. Chem. Soc.* **2008**, *130(12)*, 3900–3914). In an effort to improve their bioavailability, three compounds (**2a**–**c**) were designed with electron-withdrawing groups near one of the basic nitrogen atoms to lower its pK_a. Inhibition studies with these compounds showed that two of them not only retained most of the potency and selectivity of the best analogue of the earlier series, but also showed improved membrane permeability based on data from a cell-based assay.

Neuronal nitric oxide synthase (nNOS) catalyzes the oxidation of L-arginine to L-citrulline via the intermediate N^G-hydroxy-L-arginine in the central nervous system (CNS), generating the highly reactive free radical neurotransmitter nitric oxide (NO).[1–3] Significant research has shown that overproduction of NO from nNOS is implicated in various neurodegenerative diseases,[4–5] including Parkinson's,[6–8] Alzheimer's,[9] Huntington's[10] diseases, and stroke.[11] Since nNOS plays a critical role in the production of neuronal NO, it is considered to be a promising neurodegenerative therapeutic target.[12–14]

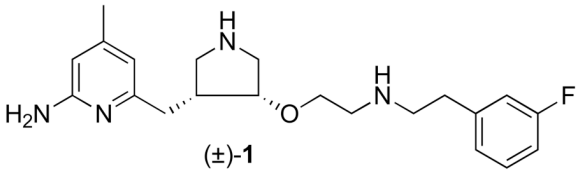
Although intense research efforts have been devoted to the design and development of small molecules to inhibit the activity of nNOS,15 none has been reported to enter clinical trials for neurodegenerative disease. There are two major challenges, in addition to high potency, involved in developing novel nNOS inhibitors. The first is the selectivity of inhibitors for nNOS over its closely related isozymes, endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is a key regulator of blood pressure and vascular tissues. It has been shown that inhibition of eNOS activity leads to high blood pressure and vascular effects.16 On the other hand, iNOS

*Address correspondence to Professor Richard B. Silverman at the Department of Chemistry.

Supporting Information: Detailed synthetic procedures and full characterization (^{1}H NMR, ^{13}C NMR and mass spectrum) of compounds **5**–**6**, **8**–**9**, **3b**–**c**, **12a**–**c**, and **2a**–**c** are available.

is the enzyme responsible for the generation of cytotoxic NO, playing an important role in the immune system.16 Therefore, when blocking the activity of nNOS, designed inhibitors should not interfere with the activity of either eNOS or iNOS.15 The second challenge for drugs that target nNOS, which primarily resides in the CNS, is the ability of the designed compounds to penetrate the blood brain barrier (BBB). The BBB is a unique barrier formed by brain capillary endothelial cells that are linked via tight junctions. Accordingly, drug 5 molecules need to cross the endothelial cell monolayer via the transcellular pathway to exhibit a pharmaceutical effect in the CNS.

Our laboratory has previously reported the structure-based design, synthesis, and biological evaluation of a series of pyrrolidine-based nNOS selective inhibitors.[17–19] Among them, **1** demonstrates great potency (K_i = 15 nM) and very high selectivity for nNOS over eNOS (2100 fold) and iNOS (630 fold). These data indicated that **1** is a promising candidate to be considered as a lead compound for neurodegenerative drugs.[19]



(±)-**1**

Although **1** exhibits high *in vitro* potency and selectivity, its therapeutic utility is limited by poor BBB permeability.[19] We speculated that the two positive charges of **1** at physiological pH, derived from the two secondary amino groups, dramatically impaired the ability of **1** to penetrate the highly lipophilic BBB by passive diffusion.

The low membrane permeability of **1** represents a major challenge for further investigation of this compound. To circumvent this problem, different strategies have been applied in an attempt to improve the bioavailability of **1**.[19–21] In this report, we describe the design, synthesis, and biological evaluation of a new series of analogues of **1** with electron-withdrawing groups (Figure 1) to lower the pK_a of one of the basic nitrogens (**2a**–**c**), and demonstrate the possibility of using them as inhibitors with improved bioavailability.

In the current design, different electron withdrawing groups, including ether (**2a**), monofluoro methylene (**2b**), and difluoro methylene (**2c**), were introduced at a vicinal position to the amino group in the lipophilic tail of **1**. Chemical structures of these new inhibitors are closely related to that of **1**, while the predicted pK_a values of the protonated vicinal nitrogen atoms are significantly lower than the corresponding protonated nitrogen atom in **1** (Table 1). We anticipate that the additional electron withdrawing groups, which partially remove the positive charge from the vicinal nitrogen atom by induction, would decrease the cationic character of inhibitors **2a**–**c** compared to **1**, and therefore, improve membrane permeability of the inhibitors.

As shown in Scheme 1, the synthesis of **3b** began with 3-fluorostyrene (**4**). Bromofluorination of **4** using NBS and $Et_3N{\cdot}3HF$ generated 1-(2-bromo-1-fluoroethyl)-3-fluorobenzene (**5**) in high yields.[22] Next, **5** was converted to azide (**6**) using NaN_3 in DMSO at 65 °C in good yields.[23] Finally, catalytic hydrogenation of **6** in a mixture of EtOH and 1 N HCl (2:1) provided **3b** as a HCl salt in excellent yields.

2,2-Difluoro-2-(3-fluorophenyl)ethanamine (**3c**) was synthesized as shown in Scheme 2. 2-Bromo-3'-fluoroacetophenone (**7**) was treated with diethylaminosulfur trifluoride (DAST) to give 1-(2-bromo-1,1-difluoroethyl)-3-fluorobenzene (**8**) in good yields.[24] Next, **8** was allowed to react with NaN_3 in DMSO at 110 °C to give azide (**9**) in good yields. Finally, **9** was subjected to catalytic hydrogenation under acidic conditions to give **3c** as an HCl salt in excellent yields.

With **3a**–**c** in hand (**3a** is commercially available), we finished the syntheses of inhibitors **2a**–**c** using a three-step procedure (Scheme 3). First, reductive amination between **10**[19] and **3a**–**c** using $NaHB(OAc)_3$ gave **11a**–**c** in modest yields. To simplify the purification process, the resulting secondary amines (**11a**–**c**) were protected with $(Boc)_2O$ to give **12a**–**c**. Finally, the Bn-protecting group and the three Boc-protecting groups were removed at the same time in a mixture of EtOH and 12 N HCl (2:1) under high pressure catalytic hydrogenation conditions to provide **2a**–**c** as HCl salts in good yields.

Inhibitors **2a**–**c** were evaluated for *in vitro* inhibition against three isozymes of NOS: rat nNOS, bovine eNOS, and murine iNOS using known methods.[25] Inhibitor **2a**, with an electronegative oxygen atom inserted into the lipophilic tail of **1**, is almost six-fold less potent for nNOS compared to the lead compound (**1**), while the selectivity of this inhibitor for nNOS over eNOS and iNOS decreases by ten-fold and three-fold, respectively. There are two likely reasons for these results. First, compound **2a** is one atom longer than **1**, which probably makes it too long to fit snugly into the active site of nNOS. Moreover, it has been shown previously that the positively charged amine functionality in the lipophilic tail plays an important role for tight binding of **1** to nNOS;[19] therefore, partial removal of the positive charge from this group may impair the activity of inhibitors.[19] Inhibitor **2b**, with monofluoromethylene vicinal to one of the basic amino groups, shows very good potency for nNOS and excellent selectivity for nNOS over eNOS and iNOS. Given that the pK_a of the vicinal amino group in **2b** is lower than that in **2a**, partial removal of the positive charge on that amino group cannot explain the lower potency of **2a**. Finally, inhibitor **2c**, with a difluoromethylene in the lipophilic tail of **1**, exhibited a 2.2-fold drop in potency relative to **2b**. The introduction of the strongly electron-withdrawing difluoromethylene group essentially completely removes the positive charge on the amino group in the lipophilic tail at neutral pH, which decreases its ability for an electrostatic interaction with the heme carboxylate. However, inhibitor **2c** still showed excellent selectivity for nNOS over eNOS and iNOS.

The two best inhibitors (**2b** and **2c**), together with the lead compound (**1**), were tested for their potency in a cell-based assay.[26] This assay can provide information about their membrane permeability. The results are summarized in Table 3. The IC_{50} values for inhibitors **2b** and **2c** are compared to the lead compound (**1**) in the first ratio column. Compound **2b** is 0.42 times as potent as **1**; compound **2c** is 0.19 times as potent as **1**. The second ratio column compares $IC_{50(cell)}$ values for **2b** and **2c** relative to **1** in the cell-based assay, which should relate to the relative ability of the compounds to penetrate the cell membrane. Compound **2b** is only 0.9 as potent as **1**, which indicates that **2b** crosses the cell membrane 2.1 times (0.9/0.42) better than **1**. Compound **2c** is 0.47 times as potent as **1** in the cell-based assay, indicating that **2c** crosses the cell membrane 2.5 times (0.47/0.19) better than **1**. This suggests that the difluoromethylene electron-withdrawing group of **2c**, which decreases the pK_a of the vicinal amino group compared to the monofluoromethylene group of **2b** and no electron-withdrawing group of **1**, improves cell permeability. On the other hand, the absolute potency of inhibitors may be sacrificed because of the importance of the positively charged amino group in binding to nNOS.

In conclusion, a new series of potent and selective nNOS inhibitors (**2a**–**2c**) were designed and synthesized. Purified enzyme and cell-based evaluation of these new inhibitors led to the discovery of inhibitors **2b** and **2c**, which not only retain most of the activity of the lead compound (**1**), but also have improved membrane permeability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to the National Institutes of Health (GM49725 to R.B.S. and GM52419 to Professor Bettie Sue Siler Masters, with whose laboratory P.M. and L.J.R. are associated) for financial support of this research. B.S.S.M. also is grateful to the Welch Foundation for a Robert A. Welch Foundation Distinguished Professorship in Chemistry (AQ0012). P.M. is supported by grants 0021620806 and 1M0520 from MSMT of the Czech Republic.

References and Notes

1. Hall AV, Antoniou H, Wang Y, Cheung AH, Arbus AM, Olson SL, Lu WC, Kau CL, Marsden PA. J. Biol. Chem 1994;269:33082. [PubMed: 7528745]
2. Wang Y, Newton DC, Marsden PA. Crit. Rev. Neurobiol 1999;13:21. [PubMed: 10223522]
3. Rosen GM, Tsai P, Pou S. Chem. Rev 2002;102:1191. [PubMed: 11942793]
4. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Science 1994;265:1883. [PubMed: 7522345]
5. Duncan AJ, Heales SJ. Mol. Aspects. Med 2005;26:67. [PubMed: 15722115]
6. Przedborski S, Jackson-Lewis V, Yokoyama R, Shibata T, Dawson VL, Dawson TM. Proc. Natl. Acad. Sci. U.S.A 1996;93:4565. [PubMed: 8643444]
7. Hantraye P, Brouillet E, Ferrante R, Palfi S, Dolan R, Matthews RT, Beal MF. Nat. Med 1996;2:1017. [PubMed: 8782460]
8. Zhang L, Dawson VL, Dawson TM. Pharmacol. Ther 2006;109:33. [PubMed: 16005074]
9. Dorheim M-A, Tracey WR, Pollock JS, Grammas P. Biochem. Biophys. Res. Commun 1994;205:659. [PubMed: 7528015]
10. Norris PJ, Waldvogel HJ, Faull RLM, Love DR, Emson PC. Neuronsicence 1996;72:1037.
11. Sims NR, Anderson MF. Neurochem. Int 2002;40:511. [PubMed: 11850108]
12. Southan GJ, Szabo C. Biochem. Pharmacol 1996;51:383. [PubMed: 8619882]
13. Babu BR, Griffith OW. Curr. Opin. Chem. Biol 1998;2:491. [PubMed: 9736922]
14. Hobbs AJ, Higgs A, Moncada S. Annu. Rev. Pharmacol. Toxicol 1999;39:191. [PubMed: 10331082]
15. Ji, H.; Erdal, EP.; Litzinger, EA.; Seo, J.; Zhu, Y.; Xue, F.; Fang, J.; Huang, J.; Silverman, RB. Frontiers in Medicinal Chemistry. Reitz, AB.; Choudhary, MI., editors. Vol. Volume 4. Bentham Science Publishers; 2009. p. 842
16. Alderton WK, Cooper CE, Knowles RG. Biochem. J 2001;357:593. [PubMed: 11463332]
17. Ji H, Tan S, Igarashi J, Li H, Derrick M, Martásek P, Roman LJ, Vásquez-Vivar J, Poulos TL, Silverman RB. Ann. Neurol 2009;65:209. [PubMed: 19235180]
18. Ji H, Li H, Martásek P, Roman L, Poulos TL, Silverman RB. J. Med. Chem 2009;53:779. [PubMed: 19125620]
19. Lawton GR, Ranaivo HR, Wing LK, Ji H, Xue F, Martesek P, Roman LJ, Watterson DM, Silverman RB. Bioorg. Med. Chem 2009;17:2371. [PubMed: 19268602]
20. Xue F, Silverman RB. unpublished results.
21. Xue F, Ji H, Huang J, Fang J, Silverman RB. unpublished results.
22. Rosen TC, Yoshida S, Fröehlich R, Kirk KL, Haufe G. J. Med. Chem 2004;47:5860. [PubMed: 15537343]
23. Other solvents such as THF and DMF did not give satisfactory yields.
24. Lakshmipathi P, Crévisy C, Grée R. J. Comb. Chem 2002;4:612. [PubMed: 12425606]
25. Hevel JM, Marletta MA. Methods Enzymol 1994;233:250. [PubMed: 7516999]
26. Fang J, Silverman RB. Anal. Biochem 2009;390:74. [PubMed: 19362065]

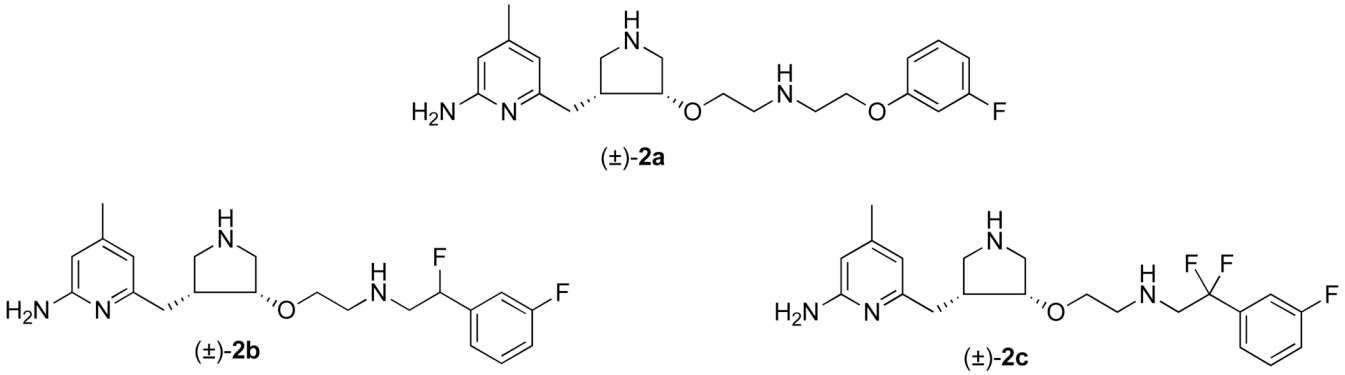
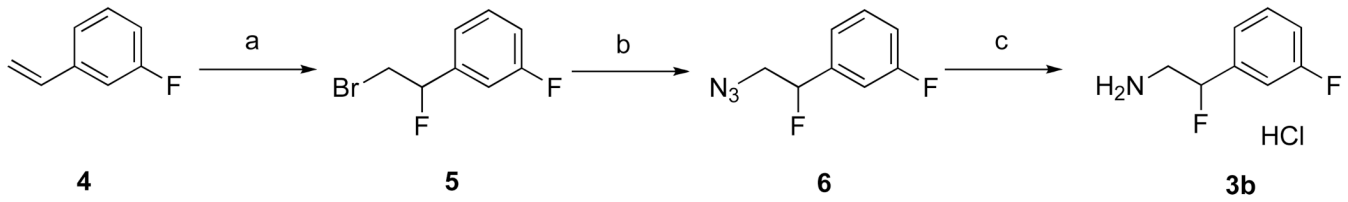
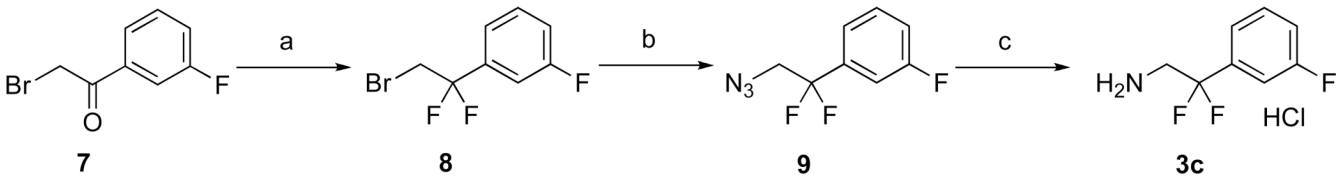


Figure 1.
Chemical structures of **2a**–**c**.



Scheme 1.

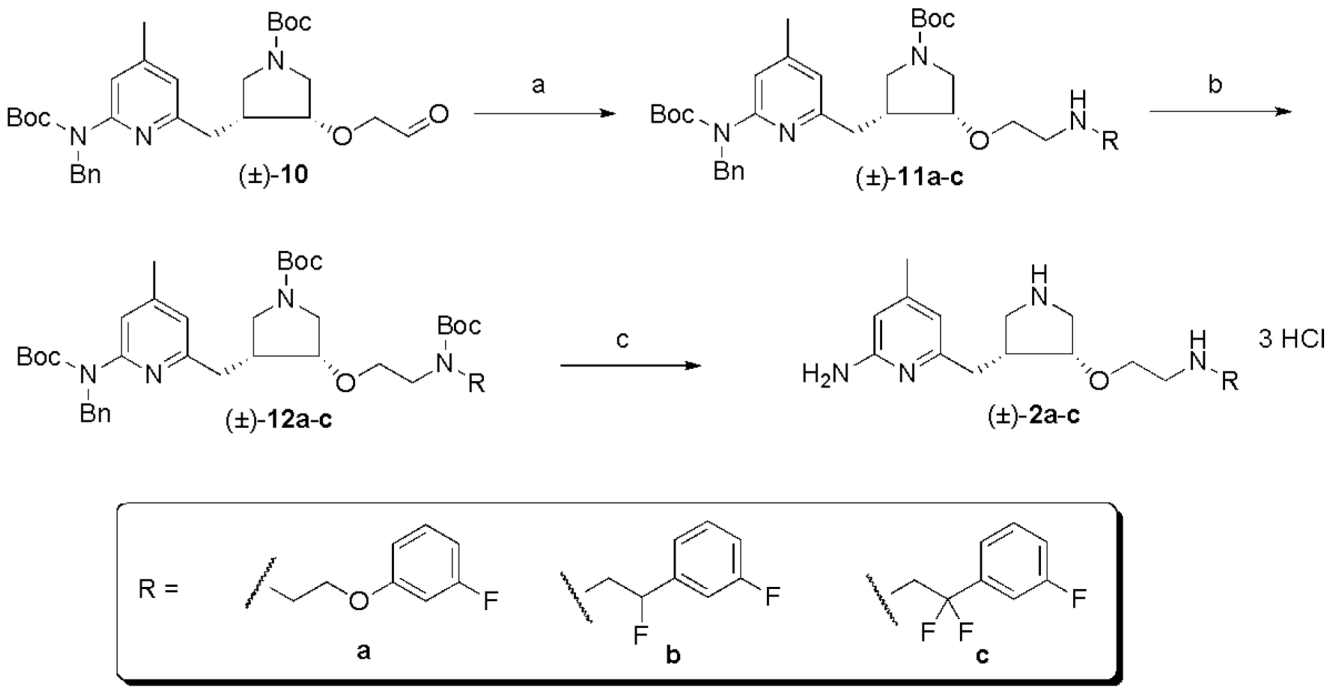
Reagents and conditions: (a) NBS, $Et_3N{\cdot}3HF$, CH_2Cl_2, 0 °C to r.t., 12 h, 85%; (b) NaN_3, DMSO, 65 °C, 4 h, 85%; (c) $Pd(OH)_2/C$, H_2, EtOH/1 N HCl (2:1), r.t., 24 h, 98%.



7 **8** **9** **3c**

Scheme 2.

Reagents and conditions: (a) DAST (neat), rt, 7 days, 70%; (b) NaN_3, DMSO, 110 °C, 1 h, 88%; (c) $Pd(OH)_2/C$, H_2, EtOH/1 N HCl (2:1), rt, 24 h, 91%..



Scheme 3.

Reagents and conditions: (a) (i) **3a**–**c**, THF, r.t., 5 min, (ii) $NaHB(OAc)_3$, r.t., 3 h; (b) $(Boc)_2O$, Et_3N, MeOH, r.t., 12 h, 48–60% for two steps; (c) $Pd(OH)_2/C$, H_2, 2:1 EtOH/HCl (12 N), r.t., 500 psi, 40 h, 85–91%.

Table 1

Physiochemical properties of inhibitors **1** and **2a**–**c**.

Compound	M_w	pK_a[a]
1	372	8.94
2a	388	8.12
2b	390	7.32
2c	408	5.56

[a] Lipophilicity data were calculated with ACD/LogD version 7.0, Advanced Chemistry Development, Inc., Toronto, Canada.

Table 2

K_i[a] values of inhibitors for rat nNOS, bovine eNOS, and murine iNOS.

Compound	nNOS (μM)	eNOS (μM)	iNOS (μM)	selectivity[b]	
				n/e	n/i
1[19]	0.015	31	9.5	2100	630
2a	0.086	21	18	240	210
2b	0.036	36	13	1000	360
2c	0.080	62	52	780	650

[a]The K_i values were calculated based on the directly measured IC_{50} values, which represent at least duplicate measurements with standard deviations of ±10%.

[b]The ratio of K_i (eNOS or iNOS) to K_i (nNOS).

Table 3

IC_{50} values of inhibitors in purified enzyme assay and cell-based assay.

Compound	IC_{50} (μM)[a]	$IC_{50(cell)}$ (μM)[a]	$IC_{50(1)}$/ $IC_{50(x)}$	$IC_{50(1)(cell)}$/ $IC_{50(x)(cell)}$
1	0.13	9	-	-
2b	0.31	10	0.42	0.9
2c	0.70	19	0.19	0.47

[a]The IC_{50} values represent at least duplicate measurements with standard deviations of ±10%.