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Novel 2-aminobenzothiazoles as selective neuronal nitric oxide synthase inhibitors

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Abstract—A series of substituted 2-aminobenzothiazole compounds have been synthesized and evaluated as nitric oxide synthase (NOS) inhibitors. Compound **14** shows activity in the nM range and is selective for the human neuronal NOS isoform. We have also evaluated the compounds against the rat NOS isoforms. For some of the compounds, there are significant differences in NOS inhibitory activities between the human and rat enzymes. For example, compound **10b** has nM activity against the rat nNOS while low μM activity against the human nNOS.

Nitric oxide (NO) has diverse roles both in normal and pathological processes including the regulation of blood pressure, in neurotransmission, and in the macrophage defense systems.[1] NO is synthesized by three isoforms of nitric oxide synthase (NOS), two of which, one in the endothelial cells (eNOS) and one in the neuronal cells (nNOS), are constitutive while the one in macrophage cells is inducible (iNOS).[2] These enzymes are homodimeric proteins that catalyze a five-electron oxidation of L-arginine, yielding NO and citrulline. The role of NO produced by each of the NOS isoforms is quite unique. Overstimulation or overproduction of individual NOS isoforms plays a role in several disorders including septic shock, arthritis, diabetes, ischemia-reperfusion injury, pain, and various neurodegenerative diseases.[3–5] NOS inhibitors can be therapeutic in many disorders, but preservation of physiologically important nitric oxide synthase function requires the development of isoform-selective inhibitors. In particular, when designing nNOS inhibitors, selectivity for nNOS relative to eNOS is of paramount importance in order to avoid adverse cardiovascular effects.[6]

A number of different classes of NOS inhibitors have been reported in the literature. Early NOS inhibitors were structural analogs of the natural substrate L-arginine, which lack isoform selectivity.[7] However, some later generations of L-arginine analog NOS inhibitors are potent and selective.[8,9] In addition, potent and selective non-amino acid NOS inhibitors have been reported.[10–14] The general pharmacophore model for a NOS inhibitor that targets the arginine binding site is a guanidine isosteric group and a basic site attached to a central linker (Fig. 1). The minimum requirement is an isostere of the substrate's guanidine group to make a bidentate interaction with a conserved glutamic acid residue of the enzyme.

In our research on designing selective nNOS inhibitors for treating CNS disorders, we envisioned using a bicyclic heterocyclic scaffold as a central linker. In this report, we present novel substituted 2-aminobenzothiazole inhibitors of NOS that are potent and selective for the neuronal isoform.[15]

Our initial focus was to determine if the 2-aminobenzothiazole scaffold is tolerated in the NOS active site. We

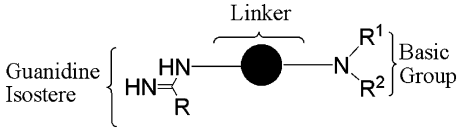

Figure 1. NOS inhibitor pharmacophore model.

Keywords: Nitric oxide synthase; Nitric oxide; Inhibitor; 2-Aminobenzothiazole.
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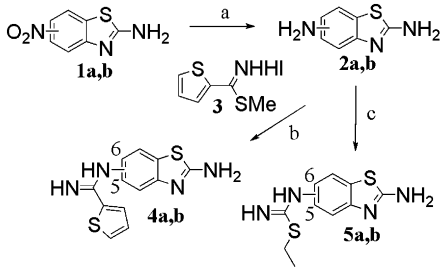
synthesized a few analogs containing guanidine isosteric groups with no other substitution on the bicyclic heterocyclic ring. The syntheses of 5- and 6- substituted 2-aminobenzothiazoles are outlined in Scheme 1. Reduction of the commercially available nitro compounds furnished the anilines. These anilines provided a handle for attaching the guanidine isostere. Two common guanidine isosteric groups were employed: a thiophene amidine and an *S*-ethyl isothiourea.

The inhibitory activities of these compounds[16] against all three human isoforms of NOS were measured by following the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline. The observed NOS IC_{50} values and the selectivity for nNOS ratios, defined as IC_{50} (eNOS)/IC_{50} (nNOS) and IC_{50} (iNOS)/IC_{50} (nNOS), are shown in Table 1.

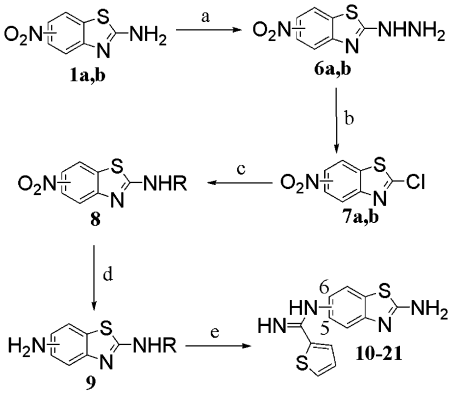
The data indicated that compounds with the thiophene amidines (**4a** and **4b**) displayed better NOS inhibitory activities over the *S*-ethyl isothioureas (**5a** and **5b**). Also, compounds **4a** and **4b** showed excellent selectivities for nNOS over iNOS (400- and 90-fold, respectively). Similar inhibitory activities against nNOS and eNOS were obtained regardless of whether the guanidine isostere is attached at the 5- or 6-position of the 2-aminobenzothiazole scaffold. Compounds with the 6-substituted guanidine isostere are slightly more active against iNOS than their 5-substituted counterpart, however, all of the compounds displayed poor inhibitory activity against this isoform.

In an effort to improve nNOS potency and selectivity for nNOS over eNOS, we explored substitution of the aminobenzothiazole by tethering different groups from the 2-position. These analogs were synthesized according to Scheme 2. The 2-hydrazinylnitrobenzothiazoles **6a**,**b** were obtained from the corresponding nitroaminobenzothiazole compounds by treating with hydrazine at elevated temperature.[17] These compounds were then converted to the 2-chloronitrobenzothiazoles **7a**,**b** in refluxing thionyl chloride.[18] Treatment of the 2-chloronitrobenzothiazoles with a series of amines in H_2O or DMF with K_2CO_3 at 90 °C gave the corresponding 2-amino substituted nitrobenzothiazoles of general structure **8**. These compounds were reduced with $SnCl_2$ in refluxing ethanol to furnish the anilines with the general structure **9**. Since the thiophene amidine proved to be more potent and selective for nNOS than the corresponding isothioureas, it was used as a guanidine isostere for all subsequent compounds. Thus, this group was coupled to the anilines to give the final compounds **10**–**21**.

The inhibitory activities and selectivities of the 2,5- and 2,6-di-substituted aminobenzothiazoles against the human NOS isozymes are presented in Table 2. In general, introduction of the sidechains did not have any detrimental effects on the inhibitory activity against all three NOS isoforms. Similar inhibitory activities against nNOS were obtained regardless of whether the thiophene amidine is attached at the 5- or 6-position of the 2-substituted aminobenzothiazole scaffold. However, for eNOS and iNOS, the 6-substituted thiophene amidines showed an increase in activity when compared to their corresponding 5-substituted analogs. In a similar fashion to the mono-substituted analogs, all compounds displayed poor inhibitory activity against iNOS.



Scheme 1. Reagents and conditions: (a) $SnCl_2{\cdot}2H_2O$, EtOH, reflux; (b) EtOH, rt; (c) (i) benzoyl isothiocyanate, THF; (ii) 1 N NaOH, THF; (iii) EtI, K_2CO_3, THF.



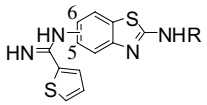
Scheme 2. Reagents and conditions: (a) NH_2NH_2HCl, NaOH, ethylene glycol, 145 °C; (b) $SOCl_2$, reflux; (c) RNH_2, H_2O, 90 °C or DMF, K_2CO_3, 90 °C; (d) $SnCl_2{\cdot}2H_2O$, EtOH, reflux; (e) EtOH, rt.

Table 1. Inhibition of human NOS isozymes by 2-aminobenzothiazoles

Compound	Subst.	nNOS IC_{50}[a] (μM)	eNOS IC_{50}[a] (μM)	iNOS IC_{50}[a] (μM)	eNOS/nNOS[b]	iNOS/nNOS[c]
4a	5	0.2	2.3	79	13	395
4b	6	0.2	2.9	25	18	90
5a	5	4.6	12	97	3	21
5b	6	1.4	9.2	46	7	32

[a] Inhibitory activity for human NOS was measured by monitoring the conversion of [^{14}C]-Arg to [^{14}C]-Cit. For each compound, the percent inhibition was determined in duplicate at 8–12 different concentrations.
[b] Selectivity against nNOS over eNOS is the ratio of eNOS IC_{50}/nNOS IC_{50}.
[c] Selectivity against nNOS over iNOS is the ratio of iNOS IC_{50}/nNOS IC_{50}.

Table 2. Inhibition of human NOS isozymes by di-substituted aminobenzothiazoles



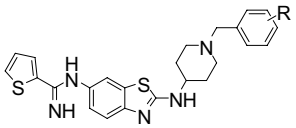
Compound	Subst.	R	nNOS IC_{50}[a] (μM)	eNOS IC_{50}[a] (μM)	iNOS IC_{50}[a] (μM)	eNOS/nNOS[b]	iNOS/nNOS[c]
10a	5	2-(Pyridin-2-yl)ethyl	1.1	6	139	5	126
11a	5	2-Morpholinoethyl	1.3	12	141	9	108
12a	5	1-Benzylpiperidin-4-yl	0.4	16	48	40	123
13a	5	1-(4-Fluorobenzyl)piperidin-4-yl	0.8	13	91	16	118
14	5	(±)-2-(1-Methylpyrrolidin-2-yl)ethyl	0.03	1.6	15	53	483
10b	6	2-(Pyridin-2-yl)ethyl	1.3	0.9	13	1	10
11b	6	2-Morpholinoethyl	2.0	1.5	37	1	18
12b	6	1-Benzylpiperidin-4-yl	0.5	6	14	12	28
13b	6	1-(4-Fluorobenzyl)piperidin-4-yl	0.2	8	14	40	70
15	6	2-(1*H*-Imidazol-5-yl)ethyl	0.3	0.7	6	2	19
16	6	4-Bromophenethyl	4.4	2.1	75	0.5	17
17	6	Tetrahydro-2*H*-pyran-4-yl	2	16	64	8	32

[a] Inhibitory activity for human NOS was measured by monitoring the conversion of [^{14}C]-Arg to [^{14}C]-Cit. For each compound, the percent inhibition was determined in duplicate at 8–12 different concentrations.
[b] Selectivity against nNOS over eNOS is the ratio of eNOS IC_{50}/nNOS IC_{50}.
[c] Selectivity against nNOS over iNOS is the ratio of iNOS IC_{50}/nNOS IC_{50}.

The most potent and selective nNOS inhibitor obtained was with the racemic sidechain 2-(1-methylpyrrolidin-2-yl)ethyl, compound **14** (nNOS IC_{50} = 0.03 μM, 53- and 483-fold selectivity against eNOS and iNOS, respectively). No attempts were made to separate the enantiomers. This compound contains a basic group with a 3-carbon linker separating the basic nitrogen from the 2-amino group on the heterocyclic core. In general, a 3-carbon linker from the 2-aminobenzothiazole scaffold to a basic site provided potent nNOS inhibitors in the sub-micromolar range (compounds **12a**,**b**, **13a**,**b**, **14**, and **15**). Although compounds **10a** and **10b** contain this structural requirement, these compounds did not give sub-micromolar activity in the nNOS inhibition assay. One possible reason for this could be the reduced basicity of the pyridine group as compared to an alkyl substituted nitrogen. Further evidence for the importance of the basicity of the nitrogen comes from comparing the nNOS inhibitory activity of the imidazole analog **15** (nNOS IC_{50} = 0.3 μM) with that of the pyridine analog **10b** (nNOS IC_{50} = 1.3 μM). For these analogs, one would expect the imidazole to be more basic than the pyridine. Also, compounds **16** and **17** do not contain basic groups in the sidechain and as a result weaker activities were observed.

As a sub-series, the *N*-benzylpiperidine analogs (Table 2, compounds **12a**,**b** and **13a**,**b**) gave potent and selective nNOS inhibitory activities. We focused our attention on optimizing this series by exploring substitution on the phenyl ring. Keeping the amidine group at the 6-position of the heterocyclic scaffold, a few analogs were synthesized according to Scheme 2 and evaluated for NOS inhibitory activity (Table 3). Compounds containing the 3- and 4-methoxyphenyl groups, **18** and **19**, respectively, proved to be potent nNOS inhibitors. In addition, comparing iNOS inhibitory activities for these two compounds revealed that the position of the methoxy group is crucial for activity. This could be attributed to a steric clash between **18** and the iNOS enzyme. For nNOS and eNOS, it is interesting that an ester **20** is tolerated at the 4-position of the phenyl ring but not an

Table 3. Inhibition of human NOS isozymes by substituted benzylpiperidine aminobenzothiazoles



Compound	R	nNOS IC_{50}[a] (μM)	eNOS IC_{50}[a] (μM)	iNOS IC_{50}[a] (μM)	eNOS/nNOS[b]	iNOS/ nNOS[c]
18	3-OMe	0.1	3.8	>100[d]	27	>1000
19	4-OMe	0.2	5.7	9	28	45
20	4-CO_2Me	0.3	5.2	>100[d]	18	>300
21	4-CO_2H	5.1	59	>100[d]	12	>20

[a] Inhibitory activity for human NOS was measured by monitoring the conversion of [^{14}C]-Arg to [^{14}C]-Cit. For each compound, the percent inhibition was determined at 8–12 different concentrations.
[b] Selectivity against nNOS over eNOS is the ratio of eNOS IC_{50}/nNOS IC_{50}.
[c] Selectivity against nNOS over iNOS is the ratio of iNOS IC_{50}/nNOS IC_{50}.
[d] No inhibition at the highest test concentration of 100 μM.

Table 4. Inhibition of rat NOS isozymes by di-substituted aminobenzothiazoles

Compound	nNOS IC_{50}[a] (μM)	eNOS IC_{50}[a] (μM)	iNOS IC_{50}[a] (μM)	eNOS/nNOS[b]	iNOS/ nNOS[c]
10b	0.05	3.4	25	63	500
11b	0.8	9.1	44	12	55
12b	0.09	12.5	36	139	400
15	4.8	4.6	26	1	5
16	4.7	5.2	NT	1	ND
18	6.7	1.3	19	0.2	3

NT, not tested.

ND, not determined.

[a] Inhibitory activity for rat NOS was measured by monitoring the conversion of [^{14}C]-Arg to [^{14}C]-Cit. For each compound, the percent inhibition was determined in duplicate at 8–12 different concentrations.

[b] Selectivity against nNOS over eNOS is the ratio of eNOS IC_{50}/nNOS IC_{50}.

[c] Selectivity against nNOS over iNOS is the ratio of iNOS IC_{50}/nNOS IC_{50}.

acid **21** which is probably due to electrostatic repulsion. Excellent selectivity for nNOS against iNOS was obtained for compounds **18** and **20**.

Since there are species differences among the NOS enzymes and we would like to evaluate our compounds in rat models of disease states, a few compounds have been tested for inhibitory activity in the rat NOS assays (Table 4). In a similar fashion to the human enzymes, inhibition was determined by measuring their effect on the conversion by NOS of [^{3}H]L-arginine into [^{3}H]L-citrulline. While there are differences between the rat and human NOS enzymes, we were somewhat surprised by the dramatic differences in nNOS activities and selectivities of some of the compounds. For example, compound **10b** (human nNOS IC_{50} = 1.3 μM vs rat nNOS IC_{50} = 0.05 μM) and compound **12b** (human nNOS IC_{50} = 0.5 μM vs rat nNOS IC_{50} = 0.09 μM) showed the largest differences in nNOS inhibitory activities between the two species. In the case of **10b**, there was no selectivity for the human nNOS over eNOS while there was a 60-fold selectivity for the rat nNOS over eNOS. Similarly, compound **12b** was much more selective (140-fold) for the rat nNOS over eNOS. In addition compounds **10b** and **12b** showed excellent selectivity (500-fold and 400-fold, respectively) for rat nNOS over iNOS. It should be noted that while the inhibitory activities can vary by as much as 2 orders of magnitude for nNOS (compound **10b**) in the two species, such is not the case for eNOS and iNOS inhibition. Interestingly, **18** showed a dramatic loss of nNOS potency but an increase in its eNOS activity.

In summary, we have designed and synthesized a series of 2-aminobenzothiazole inhibitors that are selective for human and rat nNOS. Compound **14** was the most potent and selective for human nNOS over eNOS and iNOS, while compound **12b** was the most selective for rat nNOS over eNOS. We have shown that by introducing substituents at the 2-position of the heterocyclic scaffold we were able to design nNOS selective compounds by as much as 50-fold over eNOS for the human enzymes. We have also shown that for a few compounds significant differences in NOS inhibitory activities among the rat and human enzymes can occur. We are currently evaluating some of our selective compounds in rat models of disease states in which nNOS is thought to play a role and will present the results in due course.

Acknowledgments

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