Potent and Selective Inhibition of Human Nitric Oxide Synthases

INHIBITION BY NON-AMINO ACID ISO Thioureas*

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S-Ethylisothiourea was a potent competitive inhibitor of human nitric oxide synthase (NOS), with K, values of 17, 36, and 29 nM for the inducible (i), endothelial (e), and neuronal (n) isozymes, respectively. Unlike some potent inhibitors of NOS, no time dependence was observed. S-Ethylisothiourea was not a detectable substrate for sulfonic acid; ITU, isothiourea; Mes, 2-(N-morpholino)ethanesulfonic acid; Taps, 3-tris(hydroxymethyl)methylamino phenylphosphonic acid; Taps, 3-tris(hydroxymethyl)methylamino phenylphosphonic acid; Taps.

Although the three isozymes share the same catalytic mechanism, they are distinct from each other based on their regulation and primary sequences. A comparison of the human amino acid sequences deduced from the cDNAs of iNOS (Charles et al., 1993), nNOS (Nakane et al., 1993), and eNOS (Janssens et al., 1992) shows an overall 50–60% identity between the three sequences. Thus, with such diversity, the active sites of the three isozymes might be different enough so that selective inhibition could be achieved. The majority of NOS inhibitors thus far described have been amino acid analogs of the substrate L-arginine (Olken and Marletta, 1992, 1993; Furfine et al., 1993; Mayer et al., 1993; Narayanan and Griffith, 1994). The inhibition of NOS by L-NA (Furfine et al., 1993) has demonstrated that inhibition can be both extremely potent and selective for one isozyme. Non-amino acid analogs of L-arginine such as aminoguanidine (Misko et al., 1993) and alkylguanidines (Hasan et al., 1993) are inhibitors of NOS in whole tissue or animal studies. More interestingly, these compounds show some selectivity toward iNOS. However, their potency is weak compared to the amino acid analogs (e.g., L-NA).

We report here that isothioureas, non-amino acid analogs of L-arginine, are extremely potent inhibitors of the human NOS isozymes. More importantly, some of these compounds are selective for human iNOS. The most selective inhibitor was 190-fold more potent against iNOS than eNOS.

EXPERIMENTAL PROCEDURES

Materials

Calmodulin (from bovine brain), NADPH, FAD, L-citrulline, L-arginine, L-aminoguanidine, methylguanidine, 1-octanesulfonic acid, Mes, and Taps were purchased from Sigma (6R, 5R, 7R, 8R) tetrahydrobiopterin from RBI (Natick, MA); L-[1-14C]-Uarginine from Du Pont NEN; AG 50W-X8 cation-exchange resin was from Bio-Rad; and Ecolume scintillation liquid from ICN (Irvine, CA). L-[U-14C]-Ethylsulfoisolea-HII was a custom synthesis by Wizard Laboratories, Inc. (West Sacramento, CA). L-Nitroarginine was purchased from Aldrich. L-MA and L-NIO were synthesized by H. Hodson (Wellcome Research Laboratories, Beckenham, United Kingdom). The Hamilton PRP-1 guard column and 150 x 4.6 mm, 10-micron reverse phase column was from Hamilton Co. (Reno, NV).

NOS Isozyme Purification

Human iNOS was purified by the procedure of Sherman et al. (1993) from the human colorectal adenocarcinoma cell line DLD-1. eNOS was purified from human placenta (Garvey et al., 1994). Human brain nNOS was purified as described for bovine brain nNOS (Furfine et al., 1993). Mouse macrophage iNOS from γ-interferon, lipopolysaccharide-induced RAW 264.7 cells was purified by the procedure of White and Marletta (1993).

NOS Assay

The oxidation of L-arginine was monitored by the conversion of L-[1-14C]-L-arginine to L-[1-14C]-L-citrulline as described by Schmidt et al. (1991). Reaction mixtures contained 20 mM Hepes, pH 7.4, 2.5 mM dithiothreitol, 125 μM NADPH, 10 μM tetrahydrobiopterin, 10 μM FAD, and 0.2–10
Atlantic Microlab, Inc., Norcross, GA. Melting points were determined using a Thomas Hoover capillary melting point apparatus and are uncorrected. Silica gel chromatography was performed using EM Science PrepPak cartridges (10 mm) with a PEG-6000 eluent. Elemental analyses were performed by the Central Analytical Facility, University of Georgia.

V = V_{eq}/[S]^i + K_v (1 + [IVK_j])

Apparent $K_v$ values were obtained by measuring percent inhibition at 0.5 μM with at least three concentrations of inhibitor by and by assuming competitive inhibition. $K_v$ values for t-cysteine were 2.2 ± 0.3 μM (t-cysteine), 0.2 ± 0.1 μM (eNOS), and 1.6 ± 0.1 μM (nNOS). The selectivity of an inhibitor was defined as the ratio of $K_v$ values.

Synthesis of ITUs

General—The structure and purity of commercially purchased compounds included in Tables I-III were confirmed by 1H NMR, elemental analysis, and mass analysis. 1H NMR were obtained with a Varian Unity Plus 600 MHz spectrometer. Analytical samples were performed with EM Science Silica Gel 60 (200–400 mesh ASTM). Thin layer chromatography was performed with Analtech Silica Gel GP TLC plates (250 mm).

Method A—Equimolar quantities of an alkyl halide and thiourea in ethanol or 95% ethanol were heated to reflux from 1 to 24 h. Upon cooling, insoluble products were collected by filtration and dried under vacuum or recrystallized from an appropriate solvent.

Method B—Preparation of S,S'-(1,4-phenylenebis(methylene))bis-ITU dihydrobromide (37) is given as an example of Method B; other compounds, as indicated in Table III, were made by an analogous procedure. A solution of 10.0 g (51.5 mmol) of 1,4-phenylenediacetic acid in 200 ml of tetrahydrofuran was added dropwise to a 0 °C stirred suspension of 2.0 g (10.09 mmol) of the intermediate (50% ethyl acetate in hexanes) to provide 86% of diol as a clear, white crystalline solid (m.p. 231–233 °C). 1H NMR (200 MHz, D2O) δ 7.5–7.35 (m, 1H), 6.98 (d, 1H, J = 10 Hz), 4.38 (s, 2H), 3.38 (t, J = 6 Hz, 2H). Anal. Calcd: C 32.44 H 3.92 N 11.02 S 16.10. Found: C 32.55 H 3.96 N 11.02 S 16.14.

Reversed Phase Chromatography of [153C]-Ethyl-ITU Incubated with NOS

In a volume of 100 μl, 1 nmol of [153C]-ethyl-ITU (3 x 106 cpm) was incubated with placental eNOS (0.5 nmol/min) under standard assay conditions (in the absence of t-arginine). Control reactions were: no NOS or eNOS with no calcium/calmodulin. Samples were removed from the reaction mixture at time points up to 30 min, filtered through Ultrafree MC filters (Millipore) and applied to a Hamilton PRP-1 HPLC column. The column was developed with an isocratic system of 87.5% 1-octanesulfonic acid (20 μM, pH 2.25 with HCl), 12.5% acetonitrile at a flow rate of 1 ml/min. [153C]-Ethyl-ITU eluted at 10 min.

Difference Spectroscopy

Purified mouse iNOS was desalted through a Sephadex G-25 column in 20 mM Tris, pH 7.5, 1 mM dithiothreitol, 10% glycerol. It was then concentrated with Centriprep 30 (Amicon). The final concentration was 0.11 mg/ml (determined by the method of Bradford (1976) using the Pierce Coomassie Plus reagent and bovine serum albumin as a standard). UV visible spectra of mouse iNOS (~0.4 μM in 0.25 ml) in the absence or presence of ligands were recorded from 350 to 500 nm using a UV/Vis spectrophotometer (Kontron Instruments, Everett, MA) at 25 °C. Spectra of iNOS plus ligands were taken after adding 1 μl of a solution of ligand to the 250 μl of solution of iNOS; final concentrations of ligands were 1 μM S-ethyl-ITU, 100 μM t-arginine, or 2 μM S-ethylthiocitrulline. None of the ligands absorbed in the wavelengths examined. The difference spectra were calculated by subtracting the absolute spectra electronically.

Inhibition of Human iNOS in Whole Cells

DLD-1 cells (a human colorectal adenocarcinoma cell line; ATCC number CCL221) were grown and iNOS was induced with a combination of cytokines as described (Sherman et al., 1993). Inhibitors were added at the same time as cytokines. Culture supernatants were harvested at 18–24 h post-induction and stored at −20 °C for nitrite analysis (Sherman et al., 1993). The IC50 value was the concentration that decreased the amount of nitrite by 50% versus the control (cytokine with
Potent, Selective Inhibitors of Human NOS Isozymes

**FIG. 1. Steady state kinetics for inhibition of human iNOS by S-ethyl-ITU.** Panel A, initial velocities of L-citrulline formed versus concentration of L-arginine at the following concentrations of S-ethyl-ITU: 0 (circles), 8 nM (triangles down), 16 nM (squares), 32 nM (triangles up), and 64 nM (diamonds). The solid lines were calculated from the best fit of the parameters in Equation 1 (competitive inhibition model) to the data. Panel B, progress curves for human iNOS-catalyzed formation of L-citrulline without inhibitor (circles) or with 25 nM S-ethyl-ITU (triangles) at 0.5 L-arginine (no inhibitor). Experiments were performed in duplicate. The concentration of L-arginine in the medium was 1 mM, and the concentration inside the cells was not determined.

**RESULTS**

**S-Ethyl-ITU Is a Potent Inhibitor of NOS**—Simple alkyl isothioureas were potent inhibitors of the three human NOS isoforms. We initially characterized this inhibition by examining the pattern of inhibition of human iNOS by S-ethyl-ITU. When the concentration of L-arginine was varied at different fixed concentrations of inhibitor, S-ethyl-ITU was competitive with respect to L-arginine with a $K_I$ value of $19 \pm 1$ nM (Fig. 1A). S-ethyl-ITU was also a competitive inhibitor of human eNOS and nNOS, with $K_I$ values of $36 \pm 2$ and $29 \pm 3$ nM, respectively. Thus, based on its competitive inhibition and structural similarity to guanidine, we assumed that S-ethyl-ITU bound at the guanidine portion of the L-arginine site.

Unlike several NOS inhibitors (Olken and Marletta, 1992; Furfine et al., 1993, 1994), the progress curve for inhibited iNOS was linear (Fig. 1B), so that inhibition by S-ethyl-ITU was rapid on the time scale of manual mixing experiments. Preincubation of iNOS with S-ethyl-ITU in the absence of L-arginine for 25 min prior to initiation of the reaction with L-arginine did not cause additional inhibition. Therefore, inhibition by S-ethyl-ITU was in rapid equilibrium.

S-Ethyl-ITU was not a detectable substrate for eNOS. [14C]-ethyl-ITU (1.0 nmol) was incubated with an amount of human eNOS that would convert 0.2 nmol of L-arginine to L-citrulline/min. Samples were taken for up to 30 min, filtered to remove enzyme, and analyzed by high performance liquid chromatography. No new peaks were observed and [14C]-ethyl-ITU was quantitatively recovered, consistent with an absence of chemical modification during the incubation.

The dependence of $K_I$ on pH was examined. From pH 6.0 to 8.0 no change was observed in the potency of S-ethyl-ITU inhibition of human eNOS ($K_I$ value of $37 \pm 2$ nM). However, at pH 9.0, the $K_I$ value increased slightly to $75 \pm 2$ nM. Reactions were not carried out above pH 9.0 due to a drastic reduction in NOS activity and the lability of ITUs at basic pH. The measured $pK_a$ value for S-ethyl-ITU was 9.5; therefore, the protonated form of the inhibitor was the predominant species at all pH values studied. Because S-ethylisothiouronium ion was the predomi-
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Inhibition of human NOS isoymes by isothioureas

Inhibition constants were obtained by measuring percent inhibition with at least three concentrations of inhibitor as described under "Experimental Procedures," except where indicated. Values had a standard deviation of ±10% (n ≥ 3).

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* Purchased from A, Aldrich; B, Bader Fine Chemicals (Aldrich Sigma Library of Rare Chemicals); TCI, TCI America; RBl, Research Biochemicals International; L, Lancaster.

b See "Experimental Procedures."

c Determined by varying substrate at fixed concentrations of inhibitor as described under "Experimental Procedures." inhibition pattern was competitive.

nont species and no significant change in $K_i$ value was observed from pH 6.0 to 9.0. S-ethylisothiouronium ion most likely was the predominant species that bound to the enzyme.

Optical difference spectrophotometry has been used to characterize substrate-heme interactions in the rat brain NOS (McMillan and Masters, 1993). Due to insufficient amounts of human NOS isoymes, we purified mouse macrophase iNOS in order to determine if S-ethyl-ITU perturbs the heme environment. As with the human isoymes, S-ethyl-ITU was a potent competitive inhibitor of mouse iNOS, with a $K_i$ value of 5.2 ± 0.3 μM. The difference spectrum (in the region of heme absorption) for S-ethyl-ITU binding to mouse iNOS has a peak at ~385 nm, a trough at ~420 nm, and an isosbestic point at ~410 nm (Fig. 2) and was similar to that reported for L-arginine binding to rat nNOS (McMillan and Masters, 1993). The difference spectrum for L-arginine binding to mouse iNOS was a peak at ~385 nm, a trough at ~420 nm, and an isosbestic point at ~410 nm (Fig. 2) and was similar to that reported for L-arginine binding to rat nNOS (McMillan and Masters, 1993). The difference spectrum for L-arginine binding to mouse iNOS was similar (data not shown). In addition, the amide acid analog of S-ethyl-ITU, S-ethylthiocitrulline (Purfine et al., 1994), gave the same difference spectrum when bound to mouse iNOS (data not shown). These spectra are similar to the "type I" spectrum for substrates binding to cytochrome P450 (Schenkman et al., 1967) and are indicative of the ligands altering the environment of heme.

Structure-Activity Relationship of ITU Inhibition—Seventy isothioureas were either synthesized or purchased to develop the SAR for ITU inhibition of the three human NOS isoymes. A representative set of isothioureas is presented in Table I. Substitution on the sulfur was essential because thiourea (1) was not an inhibitor. ITUs that had small alkyl substitutions on sulfur were the most potent inhibitors. Maximal binding was observed with S-ethyl-ITU (3) and S-isopropyl-ITU (4), with a decrease in potency of at least an order of magnitude with alkyl chains that were smaller (methyl, 2), larger (tert-butyl, 5), or unbranched (propyl, 6).

Two other subsets of ITUs contained either a phenyl ring or a heteroatom/halogen. These inhibitors appeared to interact differently than the small alkyl ITUs. In one subset, a phenyl ring two carbons removed from sulfur (13) was critical for potent binding. A significant decrease in binding occurred with one less (11) or one more (15) carbon atom. This SAR could be reflective of a number of possible interactions with the phenyl ring, for example, π-π, π-cation, or hydrophobic interactions. A third subset was exemplified by S-amino (19), bromo (21), and hydroxy (22) alkyl-ITUs. Compared to S-butyl-ITU (8), these functionalities all increased binding by 20–40-fold, suggesting a possible third distinct interaction.

Methylation of an isothiourea nitrogen greatly reduced the potencies of S-methyl and S-ethyl-ITU (data not shown). The inhibition constants for the N-methyl compounds were on the average 500-fold greater than the values for ITUs. Furthermore, N,N'-disubstituted analogs were all weaker inhibitors (data not shown). In addition, N-methylguanidine was a weak inhibitor, with inhibition constants of 91, 57, and 68 μM against human iNOS, eNOS, and nNOS, respectively.

ITUs were generally 2–6-fold more potent against iNOS than eNOS. The greatest selectivity was observed with S-(3-methoxyphenethyl)ITU (14), which was 19-fold more potent against iNOS than eNOS. Compound 14 may derive its selectivity from interactions similar to those of bis-ITUs (see below).
Inhibition of NOS by 2-NH$_2$-Thiazoles—Cyclization of S-ethylisothiourea can be viewed as a 2-NH$_2$-thiazole or thiazoline ring system. Table II presents inhibition constants for a select number of such compounds tested as inhibitors against the three isozymes. Whereas thiazole and 2-NH$_2$-thiazole were at best weak inhibitors, substituted 2-NH$_2$-thiazoles were reasonably potent inhibitors of the three NOSs. In contrast, 2-NH$_2$-thiazoline itself was a fairly potent inhibitor. Inhibition by either 2-NH$_2$-4,5-dimethylthiazole or 2-NH$_2$-thiazoline was competitive with respect to L-arginine and in rapid equilibrium (data not shown). These compounds were not selective inhibitors of any of the human isozymes.

Potent, Selective Inhibition of Human iNOS by Bis-ITU—Bis-ITU represented a third class of ITU inhibitors (Table III). As with ITUs and 2-NH$_2$-thiazoles, bis-ITU were in rapid equilibrium and were competitive inhibitors with respect to L-arginine (data not shown). Assuming one of the two ITU moieties bound at the guanidine portion of the substrate site, the SAR of the bis-ITU linked by an alkyl chain (compounds 30-36) suggested that a second ITU site was five to seven carbons removed from the guanidine site.

More importantly, bis-ITU were selective inhibitors of iNOS (compared to eNOS), with many compounds showing 15-20-fold selectivity, with $S,S'-(1,3$-phenylenebis(1,2-ethanediy1))-bis-ITU (45) approaching 200-fold selective inhibition of iNOS. The structural requirement for this selectivity appeared to be an electron-rich region in the linker between the ITU moieties. An aromatic ring or an alkylene placed one to three carbons from the sulfur of the ITUs was a common feature in the highly selective compounds. In comparing compounds 6-8 and 13-15, the greatest potency and selectivity was achieved with the meta and para linkages on the phenyl ring. In both sets of compounds the ortho linkage led to poor nonelective inhibition. As mentioned above, S-(3-methoxyphenyl)ethylITU (14) appears to be an analog of $S,S'-(1,3$-phenylenebis(1,2-ethanediy1))-bis-ITU (45), where the second ITU functionality has been removed. Finally, the most potent inhibitor described in this report is $S$-(2-(5-((amidinothio)methyl)-2-thienyl)ethyl)ITU (60). Its $K_i$ value against human nNOS was 1.3 nM. In addition, this compound was 70-fold selective when compared with inhibition of human eNOS.

Comparison of Human and Mouse iNOS Inhibition by ITUs and Arginine Analogues—A direct comparison of human and mouse iNOS inhibition by a select group of ITUs and arginine analogs is presented as a reference (Table IV). Only relatively minor differences in the inhibition constants exist between the two enzymes (2-9-fold). Therefore, the potency of a compound against human iNOS can be approximated by its potency against the mouse enzyme. The differences that exist can be divided into two classes. Non-amino acid analogs were always more potent against mouse iNOS, and amino acid analogs were always more potent against human iNOS.

Inhibition of NOS by ITUs in Cultured Cells—To aid in predicting whether ITUs would be biologically active in animal studies, S-ethyl-ITU, $S,S'-(1,3$-phenethyl)bis-ITU, and $S,S'-(1,4$-phenethyl)bis-ITU were tested as inhibitors of cytokine-induced NO production in human cells (Table V). We used L-MA as a control, assuming that its cellular penetration would not be limiting. Relative to L-MA, each ITU was a markedly weaker inhibitor in whole cells than against isolated enzyme. Furthermore, the two bis-ITUs were significantly weaker inhibitors in whole cell studies than S-ethyl-ITU. These data suggested that cell permeability was as follows: L-MA > S-ethyl-ITU > bis-ITUs.

**DISCUSSION**

ITUs and bis-ITUs were potent inhibitors of human NOS, with the great majority being selective for the inducible isozyme. The most potent reversible inhibitor reported to date is L-NA ($K_i$ value of 15 nM against nNOS from bovine brain, Furfine et al. 1993). Some of the ITUs are at least as potent, if not more potent, as L-NA. The structural basis for the potency of the simple alkyl ITUs is suggested by the results presented here. First, S-ethyl-ITU was competitive with substrate and perturbed the environment of the heme in a manner similar to L-arginine, which suggested that it binds near the heme. Based on its structural similarity to guanidine, we assume that it binds in the guanidine portion of the substrate site. Second, the lack of pH dependence of the $K_i$ value and the lack of inhibition by the neutral thiourea are consistent with the isothiouronium form binding to the enzyme. Third, because S-methyl-ITU is

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*McMillan and Masters (1993) discussed that a large fraction (>80%) of purified rat nNOS (expressed in human kidney cells) contains a known ligand of heme that could be displaced by imidazole in the absence of l-arginine. Therefore, if the purified mouse RAW nNOS also has such a bound ligand, then the difference spectra presented in the present study may reflect binding to only a small fraction (<20%) of sites.*

*Two additional possibilities exist. One, both the neutral and protonated form of ITU bound NOS equally well. However, the neutral thiourea was less than 20,000 times as potent of an inhibitor as the protonated S-methyl-ITU. Although the methyl substituent or the different tautomeric form may contribute to this difference, the protonation state most likely plays a significant role in binding. Two, the neutral ITU was more weakly bound to NOS and the lack of pH dependence of $K_i$ was due to a compensatory change in concentration of neutral species of enzyme from pH 6 to 9. Because $K_i/V_{max}$ only increased 3-fold over this range (data not shown), the latter possibility appeared to be unlikely.*
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TABLE

Sources and determination of \( K_i \) values are described in Table I.

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<td>B</td>
<td>0.20</td>
<td>29</td>
</tr>
<tr>
<td>40</td>
<td>-(CH₂)₂-(1,4-C₆H₄)-CH₂-</td>
<td>B</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>41</td>
<td>-(CH₂)₂-C=CH₂-</td>
<td>B</td>
<td>6.2</td>
<td>45</td>
</tr>
<tr>
<td>42</td>
<td>-(CH₂)₅-C=CH₂-</td>
<td>B</td>
<td>0.47</td>
<td>6.9</td>
</tr>
<tr>
<td>43</td>
<td>-(CH₂)₉-(2,3-dimethyl)-1,4-Ph)-CH₂-</td>
<td>B</td>
<td>2.4</td>
<td>48</td>
</tr>
<tr>
<td>44</td>
<td>-(CH₂)₉-(1,2-Ph)-CH₂-</td>
<td>C</td>
<td>33</td>
<td>79</td>
</tr>
<tr>
<td>45</td>
<td>-(CH₂)₉-(1,3-Ph)-CH₂-</td>
<td>C</td>
<td>0.047</td>
<td>9.0</td>
</tr>
<tr>
<td>46</td>
<td>-(CH₂)₉-(1,4-Ph)-CH₂-</td>
<td>C</td>
<td>0.0074</td>
<td>0.35</td>
</tr>
<tr>
<td>47</td>
<td>-(CH₂)₉-(1,4-P)-CH₂-</td>
<td>C</td>
<td>3.8</td>
<td>39</td>
</tr>
<tr>
<td>48</td>
<td>-(CH₂)₉-(1,3-P)-CH₂-</td>
<td>C</td>
<td>0.50</td>
<td>8.6</td>
</tr>
<tr>
<td>49</td>
<td>-(CH₂)₉-(1,2-P)-CH₂-</td>
<td>C</td>
<td>0.16</td>
<td>2.3</td>
</tr>
<tr>
<td>50</td>
<td>-(CH₂)₉-(S-C₆H₅)-CH₂-</td>
<td>D</td>
<td>0.0077</td>
<td>0.09</td>
</tr>
</tbody>
</table>

\* Determined by varying substrate at fixed concentrations of inhibition as described under "Experimental Procedures," inhibition pattern was competitive.

TABLE IV

Comparison of inhibition constants for human and mouse iNOS
determination of \( K_i \) values is described in Table I.

<table>
<thead>
<tr>
<th>#</th>
<th>compound</th>
<th>non-amino acid analogs</th>
<th>human iNOS</th>
<th>human/mouse ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>S-methylITU</td>
<td>0.12</td>
<td>0.052</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>S-ethylITU</td>
<td>0.017</td>
<td>0.00052</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>S-benzylITU</td>
<td>5.6</td>
<td>0.63</td>
<td>8.9</td>
</tr>
<tr>
<td>17</td>
<td>S-dimethylaminoethylITU</td>
<td>2.7</td>
<td>0.39</td>
<td>6.9</td>
</tr>
<tr>
<td>27</td>
<td>2-NH₂,4,5-dimethylisoxazole</td>
<td>0.76</td>
<td>0.36</td>
<td>2.1</td>
</tr>
<tr>
<td>38</td>
<td>S,S'(1,3-phenylenebis(methylene))bisITU</td>
<td>1.5</td>
<td>0.32</td>
<td>4.7</td>
</tr>
<tr>
<td>39</td>
<td>S,S'(1,4-phenylenebis(methylene))bisITU</td>
<td>0.20</td>
<td>0.047</td>
<td>4.2</td>
</tr>
<tr>
<td>aminoacidoguanidine</td>
<td>110</td>
<td>32</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>amino acid analogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-MA</td>
<td>0.86</td>
<td>2.1</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>L-NA</td>
<td>0.67</td>
<td>4.4</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>L-NIO</td>
<td>0.34</td>
<td>1.0</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

An approximately 500-fold more potent inhibitor than methylguanidine, the sulfur atom may play a critical role in binding. Fourth, as described by the SAR of the simple alkyl-ITUs, an important interaction appears to be a good fit of the S-ethyl and S-isopropyl substituents, presumably into a small hydrophobic region.

A question that arises is the orientation of both the simple ITUs and the bis-ITUs within the active site of NOS. The active site minimally recognizes two parts of the substrate L-arginine: the amino acid and the guanidinium moieties (Fig. 3A). Assuming the isothiourea binds at the guanidinium site, the sulfur and its substituent could preferentially bind at the bridging nitrogen site or one of the two terminal nitrogen sites. Within the guanidinium site, two distinct pockets probably exist. One pocket would consist of the residues and cofactors (e.g., heme) that participate in the oxidation of one of the guanidinium's nitrogens. The other pocket would be formed by the residues that bind the remaining two nitrogens of the guanidinium. Thus, when one of the two terminal nitrogens is methylated (i.e., L-MA), the nitrogens are no longer equivalent and can bind in one of two orientations. The methylated nitrogen can be directed toward or away from where chemistry occurs. Because L-MA is oxidized exclusively on the methylated nitrogen (Olken and Marletta, 1993), L-MA probably binds with the methyl substituent pointing toward the site of chemical reaction. By analogy, we hypothesize that the ITUs bind in a similar manner (Fig. 3B).

In support of the sulfur not binding at the bridging nitrogen's site, the amino acid analog of ITU linked through an isothiouronium nitrogen is an extremely potent inhibitor (Purfine et al., 1994), but the analog linked through the sulfur is very weak.

The orientation of the bis-ITUs is more ambiguous. Again, if we assume that one of the isothioureas is binding at the guanidinium site, then the bis molecules could be directed toward any number of second sites (Fig. 3C). Based on the SAR, there actually appear to be two additional sites. One site binds the...
Potent, Selective Inhibitors of Human NOS Isozymes

TABLE V

<table>
<thead>
<tr>
<th>#</th>
<th>Compound</th>
<th>$K_i$, $\mu$M purified iNOS</th>
<th>$IC_{50}$, $\mu$M DLD-1 cells</th>
<th>$IC_{50}/K_i$ (relative to L-MA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>S-ethylITU</td>
<td>0.017</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>45</td>
<td>S,S'-(1,3-phenylenebis(1,2-ethanediyl))bisITU</td>
<td>0.047</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>46</td>
<td>S,S'-(1,4-phenylenebis(1,2-ethanediyl))bisITU</td>
<td>0.0074</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

The concentration of L-arginine in the medium was 1 mM. If the concentration of L-arginine was equivalent intracellularly, then, assuming competitive inhibition, the calculated $K_i$ value for L-MA would be $-0.1 \mu$M. This is evidence that the concentration of L-arginine intracellularly was significantly less than 1 mM.

Aminoguanidine, a selective inhibitor of rodent iNOS versus eNOS (Corbett et al., 1993; Miasko et al., 1995), fell into the non-amino acid analog class. Furthermore, aminoguanidine was selective for human iNOS; the inhibition constant for human eNOS was 8-fold greater (860 versus 110 $\mu$M). However, such an inefficient inhibition of iNOS is probably of little clinical use.

NOS inhibitors are known to increase blood pressure (Moncada et al., 1991) and have been used to reverse the hypotension observed in septic shock (Petros et al., 1994). Preliminary results have demonstrated that ITUs have these predicted effects in animals. S-Ethyl-ITU is a pressor agent in rats and dogs, and it reverses the hypotension observed in a sheep septic shock model. The effects of S-ethyl-ITU in the sheep model are similar to the effects of L-Arg$.^2$ Nitroarginine methyl ester in this model (Mayor et al., 1992). Subsequent to this work, we found that Smirk, Fastier, and colleagues (Smirk, 1941; Fastier and Reid, 1950) had demonstrated in the 1940's that simple ITUs were pressor agents in animals and that S-ethyl-ITU reversed the hypotension caused by spinal anesthesia (Smirk and McGeorge, 1942).

The potency and selectivity of the bis-ITUs as inhibitors of purified NOS are extremely encouraging for therapeutic use. However, their in vivo efficacy may be hindered by poor cellular penetration. Relative to L-MA, bis-ITUs did not show the expected potency against human iNOS in cultured cells, consistent with poor cellular penetration of these doubly charged molecules. Furthermore, the toxicity of bis-ITUs (Hendershot, 1958; Duzhak, 1968) may limit their therapeutic potential. We have confirmed that the most selective iNOS inhibitor, S,S'$^\prime$-(1,3-phenylene bis(1,2-ethanediyl))bis-ITU, is acutely toxic in mice and rats at doses as low as 10 mg/kg.

The selective inhibition of iNOS is proposed as a therapy for a variety of disease states: septic shock (Kilbourn et al., 1990; Wright et al., 1992), hypotension caused by cytokine therapy for cancer (Hibbs et al., 1992), and arthritis (McCarron-Francis et al., 1993). Minimally, the inhibitors should be selective for iNOS compared to eNOS to avoid hypertension. It is unclear how much selectivity for iNOS versus nNOS would be needed. The selective inhibition of nNOS could benefit neurodegenerative diseases such as stroke, where the desired selectivity would be nNOS versus eNOS. We have described inhibitors that are both potent and highly selective for either iNOS (90-fold) or nNOS (70-fold) as compared to eNOS. Minimally, these results demonstrate that the active sites (and surrounding region) of the human isozymes are different. Consequently, selective inhibitors of iNOS and nNOS can be designed.

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A. Tadepalli, W. Harrington, G. Grebe, and H. Berger, unpublished results.


J. Dillberger, J. Wilson, J. Wolberg, and E. Garvey, unpublished data.

Fig. 3. Model for substrate and ITUs binding to NOS. Panel A, binding pocket for L-arginine. Chemistry is assumed to occur at the heme site. Panel B, binding orientation of S-ethyl-ITU within L-arginine pocket. Panel C, binding orientation of a bis-ITU within L-arginine pocket. Arrow indicates a variety of possible orientations for the second ITU moiety.

Electron dense regions of the phenyl or thienyl rings or the triple bond of the alkyne. The other site binds the second isothiourea unit. The relationship between these second sites and the arginine binding site of NOS is undefined at present. Obviously, significant differences exist between the isozymes within these second sites. Thus, understanding how these molecules bind is paramount in designing highly selective inhibitors.

The majority of studies with iNOS inhibitors have used rodent iNOS (McCall et al., 1990; Grasso et al., 1990; Lambert et al., 1992; Okken and Marletta, 1992; Narayanar, and Griffith, 1994). Although the differences in $K_i$ values of a given compound between human and mouse iNOS were relatively small (2-9-fold different, Table IV), they were significant and can be divided into two classes. The ratio of $K_i$ values for human and mouse iNOS was greater than one for non-amino acid analogs and less than one for amino acid analogs.
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REFERENCES


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