Implications for Isoform-selective Inhibitor Design Derived from the Binding Mode of Bulky Isothioureas to the Heme Domain of Endothelial Nitric-oxide Synthase*

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Nitric oxide produced by nitric-oxide synthase (NOS) is not only involved in a wide range of physiological functions but also in a variety of pathological conditions. Isoform-selective NOS inhibitors are highly desirable to regulate the NO production of one isoform efficiently to normal physiological functions from the uncontrolled NO production of another isoform that accompanies certain pathological states. Crystal structures of the heme domain of the three NOS isoforms have revealed a very high degree of similarity in the immediate vicinity of the heme active site illustrating the challenge of isoform-selective inhibitor design. Isothioureas are potent NOS inhibitors, and the structures of the endothelial NOS heme domain complexed with isothioureas bearing small S-alkyl substituents have been determined (Li, H., Raman, C.S., Martásek, P., Král, V., Masters, B.S.S., and Poulos, T.L. (2000) J. Inorg. Biochem. 81, 133–139). In the present communication, the binding mode of larger bisisothioureas complexed to the endothelial NOS heme domain has been determined. These structures afford a structural rationale for the known inhibitory activities. In addition, these structures provide clues on how to exploit the longer inhibitor substituents that extend out of the active site pocket for isoform-selective inhibitor design.

Nitrergic-containing NOS isoforms synthesize NO using L-arginine (L-Arg) as the substrate. First, L-Arg is converted to NO•⁻-hydroxy-L-arginine, and then in a second step NO•⁻-hydroxy-L-arginine is oxidized to l-citrulline and NO (1). The three NOS isoforms (nNOS, iNOS, and eNOS) identified in mammals share more than 50% sequence identity and have identical overall architecture. Each has a heme- and H₄B-containing N-terminal oxygenase domain, a FAD/FMN-containing C-terminal reductase domain, and a CaM-binding motif linking the two functional domains (2). The heme domain provides the site for L-Arg oxidation, while the FAD and FMN of the reductase domain shuttle electrons from NADPH to the heme (3). The cofactor, H₄B, is necessary for NO production and apparently plays an active redox role during the catalytic cycle (4–6).

NO contributes to a broad range of physiological functions in neurotransmission, control of platelet adhesion, vascular homeostasis, and cytotoxicity in the immune response (7). Accordingly, the different NOS isoforms are expressed in different tissues and are highly regulated transcriptionally or posttranscriptionally. nNOS and eNOS are expressed constitutively in neurons and endothelial cells among other cell types, but NO production is completely dependent on Ca²⁺/CaM binding (8, 9). However, iNOS activity is controlled at the transcriptional level and, once expressed, that isoform will produce NO at a high rate. In addition, iNOS is not regulated by CaM, but instead CaM is bound with high affinity and functions as a permanent subunit (10). Unregulated NO production is associated with various pathological conditions (11), e.g., ischemia-reperfusion injury in stroke, septic shock, and inflammatory disorder including arthritis. Pathology is due to nNOS and/or iNOS. Under such conditions, it is essential to block nNOS and/or iNOS but not eNOS because this last isoform is critical for maintaining proper vascular tone.

Because of the double-edged nature of NO in both basic physiological functions and various pathological conditions, the development of isoform-selective NOS inhibitors is highly desirable. Considerable effort has been directed toward developing NOS inhibitors (12, 13), and a vast majority of the existing NOS inhibitors were developed before any of the NOS crystal structures were available. Most of these inhibitors are L-arginine analogues and compounds bearing amidino or ureido functional groups that can simulate the guanidino group of L-Arg. Some of these inhibitors, indeed, show significant isoform selectivity (14).

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The development of NOS inhibitors has entered a new era now that crystal structures of the catalytic heme domain of three NOS isoforms (5, 15–17) (nNOS)² are available. These structures reveal a striking similarity in dimeric quaternary structure as well as in the heme and pterin binding sites. Given such close similarity, structure-based isoform-selective inhibitor design presents an especially challenging problem. A first step toward this goal requires a correlation between what is known about the inhibition of NOS by various inhibitors and the structure of these inhibitors complexed with the various inhibitors bound to the eNOS heme domain protein sample. We previously reported the structures of the eNOS heme domain complexed with isothioureas containing small alkyl side chains, and those structures reveal a striking similarity in dimeric quaternary structures. In this communication we report the studies provided a structural rationale for inhibition by this class of compounds (5, 18). In a comprehensive structure-activity-relationship study on isothiourea inhibitors, Garvey et al. (23) measured competitive binding against substrate using a model of ligands were built into the structures using TOM/FRODO (22), further structure refinements were carried out with CNS using the maximum likelihood target function. The data collection and refinement statistics are summarized in Table I. To illustrate the ligand binding, omit 2Fo – Fc electron density maps of all four complex structures are shown in Fig. 1. The maps were generated by running simulated annealing protocol in CNS at an initial temperature of 1000 K with the ligand of interest omitted during the calculation.

RESULTS AND DISCUSSION

Binding Mode of Bisisothioureas—Crystal structures of the eNOS heme domain complexed with S-alkyl-isothioureas bear a simple alkyl group (5, 18) show that the thiourea group occupies the site normally taken by the reactive guanidinium nitrogen of L-arginine with the sulfur occupying the site normally taken by the reactive guanidinium nitrogen of L-Arg (Fig. 2). The high potency of this class of isothiourea inhibitors is primarily attributed to the extensive nonpolar contacts between the small S-alkyl group and an apolar protein pocket adjacent to the substrate binding site (Fig. 2) (18). In a comprehensive structure-activity-relationship (SAR) study on isothiourea inhibitors, Garvey et al. (23) showed good potency comparable with the small S-alkyl-isothioureas. To understand how these larger inhibitors bind, we determined crystal structures of endothelial NOS heme domain complexed with 1,3-PBITU and 1,4-PBITU, respectively.

The initial difference electron density maps of both structures revealed the binding of inhibitor at the distal side of the heme plane. However, compared with 1,4-PBITU, the electron density of 1,3-PBITU is weaker indicating lower occupancy or higher thermal motion (Fig. 1). Given that similar concentration of inhibitors were used for crystallization setups of both complexes, the lower occupancy of 1,3-PBITU observed in the structure is consistent with its weaker binding affinity (Kᵣ = 9 μM) to eNOS than 1,4-PBITU (360 nM) (23). Alternatively, the weaker electron density also could be attributed to higher thermal motion.

As expected from the known binding mode of a single iso-
thiourea, the first ureido group of both bisisothioureas still fits
into the guanidino site, donating hydrogen bonds to both the
Glu-363 carboxylate side chain and the Trp-358 carbonyl oxy-
gen (Fig. 4). However, the remainder of the bisisothiourea
molecule does not adopt a favored extended conformation but
instead makes an unexpected twist into an “S” shape. The
energetic incentive for adopting the twisted conformation ap-
pears to be the positioning of the second ureido group close to
the heme propionate of pyrrole ring D for H-bonding interac-
tions (Fig. 4).

A comparison of both structures reveals differences in inhib-
itor conformation as well as inhibitor-protein contacts that
provide a structural basis for understanding why 1,4-PBITU
exhibits a 20-fold higher inhibition for all three NOS isoforms
compared with 1,3-PBITU (23). For example, in 1,4-PBITU
the alkyl group of the second ureido group is able to adopt a more
extended conformation than in 1,3-PBITU enabling the ureido
group to form an H-bond with the heme propionate. The alkyl
group in 1,3-PBITU must kink to H-bond with the heme pro-
pionate. The second ureido plane is, therefore, flipped almost
180° from 1,4-PBITU to 1,3-PBITU (Fig. 4). The phenyl rings in
each inhibitor also are oriented slightly differently. The phenyl
ring in 1,4-PBITU complex is approximately perpendicular to
the plane of the first ureido group, whereas in 1,3-PBITU
is tilted. The angles between the phenyl ring and first ureido
plane in 1,3-PBITU are 64° and 48° for two subunits compared
with 77° and 89° in 1,4-PBITU. As a consequence, all six carbon
atoms of the phenyl moiety in the 1,4-PBITU complex make
good van der Waals contacts with the side chain of Val-338, but
in 1,3-PBITU only C-1 and C-2 of the phenyl moiety are close
enough to contact Val-338 (Fig. 4).

The observed binding mode of the PBITU inhibitors provides
an excellent structural basis for interpreting the SAR studies
with substituents at the 1,2-positions (23) would result from
the crowding of two neighboring substituents and disposition of
the phenyl ring relative to Val-338.

Structure of the S-Ethyl-N-phenyl-isothiourea Complex—Al-
though small S-alkyl-isothioureas are potent NOS inhibitors,
they exhibit poor isoform selectivity (23). To improve isoform
selectivity, Shearer et al. (24) added N-substituents to S-ethyl-
isothiourea, and SENPITU was found to have submicromolar
binding affinity for all three isoforms, a good scaffold for SAR
studies. To provide a structural basis for potential isoform-
selective inhibitors built upon this scaffold, we determined the
crystal structure at 1.93 Å of eNOS heme domain with SEN-
PITU bound.

SENPITU, as with S-ethyl-isothiourea, binds at the active
site using both the phenyl-substituted nitrogen and the unsub-
substituted nitrogen of its thioureido function to H-bond with
Glu-363 while making nonpolar contacts to the protein sur-
roundings with its S-ethyl and N-phenyl groups (Fig. 2). Meth-
ylation of either of the two ureido nitrogen atoms abolishes
the NOS inhibition (24), implying that the H-bond-donating ability
of the ureido group is important for binding. The crystal struc-
ture reveals that the pocket bordered by the carboxylate of
Glu-363 and the carbonyl of Trp-358 is too narrow to accom-
modate even a monomethyl substitution on the ureido nitro-
gens if the bifurcate hydrogen bonds between enzyme and
ligand are kept in place. This is consistent with the observa-
tions based on binding affinity assays of a series of L-Arg ana-
logues. (25) In those cases, the tightly bound L-Arg analogues
always place an unsubstituted -NH₂ or =NH₂ in the
distal guanidinium nitrogen pocket. This pocket favors the
H-bond donating nitrogen over =S or =O group and exhibits
very little affinity for -CH₃.

The N-phenyl group of SENPITU in the active site is ori-
ented such that the aromatic ring adopts an angle of 153°
relative the ureido plane (Fig. 2). Similar to the situation
observed in the PBITU structures, this phenyl ring makes close
van der Waals contacts (3.4 Å) with the Val-338 side chain.
These nonpolar interactions are quite important since replac-
ing the phenyl ring by a pyridyl group resulted in much poorer
binding (24). Interestingly, to avoid steric clashing with the
N-phenyl ring, the S-ethyl group in SENPITU complex adopts
a different conformation from that observed in the S-ethyl-
isothiourea complex structure. In the SENPITU complex
the bridging carbon in the ethyl group makes a tight contact (3.1 Å)
with the carbonyl oxygen of Pro-336, whereas in S-ethyl-isoth-
ourea it is the terminal carbon of the ethyl group that is in
the proximity of Pro-336 (Fig. 2).

With reasonably tight binding to all three isoforms of NOS,
SENPITU becomes a useful scaffold to further explore isoform
selectivity because substituents can be readily added to various
positions on the phenyl ring. Although compounds with sub-
stituents on all three positions (2-, 3-, and 4-) of the phenyl ring
showed similar binding affinity, the 4-position substituents
have a much greater effect on isoform selectivity (24). Among
a few dozen compounds, which have been characterized, medi-
ium-sized apolar substituents at the 4-position of the phenyl
ring significantly elevate the Kᵢ value toward iNOS by one
order of magnitude while leaving Kᵢ to eNOS unchanged.
The derivative with a 4-trifluoromethyl (4-CF₃) substituent exhib-
ts a 115-fold selectivity for nNOS over iNOS. By examining the
structure of the respective NOS active sites, it is clear that the
4-position substituent would make direct contact with the
heme propionates. Support for this view stems from the much
poorer inhibitory potency observed when the 4-position has a
carboxylate substituent creating a repulsive clash with the
heme propionates. However, exactly how these contacts with
heme propionates lead to isoform selectivity will remain difficult to envision until the structures of the selective inhibitor bound to different NOS isoforms become available.

Not surprisingly, the amidino analogue of N-phenyl-isothiourea, N-phenyl-acetamidine (Fig. 3), also proved to be a good scaffold for SAR studies (26). It is slightly different from the N-phenyl-isothiourea in that substituents on the 3-(meta)-, rather than 4-(para)-, position of the phenyl ring of acetamidine showed better selectivity toward nNOS. Among the functional groups tested, 3-aminomethyl was the best choice because it presumably hydrogen bonds to a heme propionate. Once again, when the amidino methyl group is replaced with a slightly bulkier nonpolar moiety, such as 2-furanyl or 2-thienyl, the potency of the inhibitor improved dramatically (26). This is because the amidino methyl group does not make any direct contact with the protein whereas 2-furanyl or 2-thienyl can most likely protrude into the hydrophobic pocket defined by Pro-336, Val-338, and Phe-355 (Fig. 2) (18). However, the 2-pyridyl group causes a decrease in binding affinity indicating the incompatibility of a more polar group in this hydrophobic protein environment lining one side of the active site.

Extended N-Substituted Bisisothioureas—Crystal structures of both endothelial (5, 16) and inducible NOS's (15–17) revealed a deep, wide open access channel that connects the bulk solvent to both the heme and pterin binding sites. In a substrate-bound form, the amino acid moiety of L-Arg is confined within a pocket, which is not part of the substrate access channel but specifically recognizes the L-enantiomer of Arg. As we have seen in the present work, any non-amino acid-based inhibitor that does not have a functional group capable of binding in the chirality-specific pocket must extend out into the space leading toward the open channel.

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substrate access channel. The surface residues expected to interact with the ureido of the ligand are labeled. The availability of NOS isoforms with different substrate access channels provides some modest insights into the possibility of achieving isoform selectivity by exploiting differences along the substrate access channel. Shown in Table II are amino acid variations that are potentially useful for the inhibitor design. For instance, Ser-248 in eNOS (Ser-477 in nNOS) is replaced by Ala-262 in iNOS. A functional group from the ligand, e.g. amino or carboxylate, that is capable of making a hydrogen bond with the Ser side chain may exhibit better selectivity toward eNOS (or nNOS). Similarly, the substitution of Leu-107 in eNOS (Leu-337 in nNOS) by Thr-121 in iNOS could be utilized in designing an iNOS-specific inhibitor by introducing a hydrogen bonding partner from the ligand to Thr-121.

A high degree of primary sequence variation exists also in the region between the conserved Ser residue (334 in rat nNOS, 104 in bovine eNOS, and 119 in human iNOS), which makes a hydrogen bond with Hb, and the first α-helix (α1) in NOS. Unfortunately, this region constitutes a surface loop that is disordered in both the eNOS and nNOS crystal structures. In the human iNOS structure this loop is ordered and lies along the substrate access channel (17). The amino acid substitutions on this loop can, therefore, be utilized in isoform-selective inhibitor design. Lys-123 in iNOS protrudes into the substrate access channel. This residue is replaced by Arg-109 in eNOS and Ser-339 in nNOS and should be reachable by the ligand, assuming the loop in eNOS or nNOS adopts a conformation similar to that observed in iNOS.

Reported Bulky NOS Inhibitors That Lend Support to This Approach—The available data indicate that exploiting amino

\[ \text{TABLE II} \]

Amino acid substitutions among NOS isoforms along the substrate access channel

|                  | Val-106 | Leu-107 | Arg-109 | Gln-112 | Ser-248 | His-373 | Asp-480 |
|------------------|---------|---------|---------|---------|---------|---------|---------|
| Bovine endothelial NOS |         |         |         |         |         |         |         |
| Rat neuronal NOS   | Met-336 | Leu-337 | Ser-339 | Thr-342 | Ser-477 | Ser-602 | Asp-709 |
| Human inducible NOS | Met-120 | Thr-121 | Lys-123 | Thr-126 | Ala-262 | Gln-387 | Glu-494 |

Distance from Cg of the residue to heme iron (Å)

|                  | 15.8    | 16.1    | 20.4    | 17.2    | 14.2    | 21.8    | 19.8    |
|------------------|---------|---------|---------|---------|---------|---------|---------|

- Distances are measured from the human iNOS heme domain structure (PDB code 1NSI).

**Note:**

4 B. R. Babu and O. W. Griffith, unpublished observations.

5 C. S. Raman, H. Li, P. Martásek, B. S. S. Masters, and T. L. Poulos, manuscript in preparation.
acid differences along the access channel may provide a useful approach. BN 80933, a bulky nNOS selective inhibitor described by Chabrier et al. (28), possesses an N-phenyl-thiopheneamidine scaffold (Fig. 3) that anchors at the substrate guanidino binding site. An antioxidant moiety, vitamin E or Trolox, is linked to the scaffold by a piperidine molecule. The bulky antioxidant moiety must be accommodated by the open channel. An attempt to model the molecule in the eNOS heme domain structure reveals a few potential contacts that may explain its isoform selectivity. The carbonyl group between the piperidine and vitamin E moiety could be in the vicinity of Ser-248, the vitamin E ring system being in contact with Leu-107 while the hydroxyl group from vitamin E can reach as far as Asp-480. These three contact points are exactly where the amino acid substitutions occur from the constitutive NOS (eNOS and nNOS) to iNOS (Table II), which could be the reason for the selection against iNOS. Selectivity for nNOS over eNOS presumably dependents on other, unfavorable interactions with eNOS not present in nNOS.

Another similar example is the nNOS-specific inhibitor, ARL17477 (Fig. 3) (29) and its analogues (30), which have an identical NOS active site binding scaffold as that in BN 80933 but with a chlorophenyl group attached to it through a small NH-containing alkyl chain. Again, the bulky functional group could provide isoform selection by interacting with amino acids along the substrate access channel. Similarly, a series of N\textsuperscript* nitroarginine-containing dipeptide amide compounds developed in Silverman’s laboratory showed impressive isoform selectivity (31). Considering the length of the dipeptides, the second residue should be able to extend into the substrate access channel. The nitroarginine moiety provides good binding affinity to the NOS active site while the second residue imposes the selectivity.

Conclusion—The high degree of structural similarity in the active sites among the three NOS isoforms provides the rationale for the lack of isoform selectivity among simple amino acid-based or other small isothiourea- or amidine-based inhibitors. However, bulky inhibitors often do exhibit better selectivity. Based on our current state of structural information, we can conclude that a good isoform-selective inhibitor consists of three components. The first is a structural scaffold that provides a guanidino, amidino, or ureido group that donates hydrogen bonds to the glutamate located in the NOS active site. A small hydrophobic group such as alkyl or thienyl is often part of the scaffold to provide additional non-polar interaction with the protein surroundings opposite to the location of the glutamate residue involved in H-bonds to guanidino of thioreido nitrogen atoms. The second is an isoform-selectivity conferring functional group bearing hydrogen bonding capability that can reach into the substrate access channel remote from the active site. The functional group imposes isoform selection by taking advantage of amino acid substitutions along the channel. The third is a linker serving as a spacer between the scaffold and the functional group. The linker should have the appropriate length and flexibility to allow the functional group to reach hot spots along the channel for isoform-specific interactions.

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