1400W Is a Slow, Tight Binding, and Highly Selective Inhibitor of Inducible Nitric-oxide Synthase in Vitro and in Vivo*

(Received for publication, August 26, 1996, and in revised form, November 25, 1996)

Edward P. Garvey‡‡§, Jeffrey A. Oplinger‡, Eric S. Furfine‡‡, Rachel J. Kiff, Ferenc Laszlo‡, Brendan J. R. Whittle**, and Richard G. Knowles††‡

From the Divisions of Biochemistry and Medicinal Chemistry, Glaxo Welcome, Research Triangle Park, North Carolina 27709 and the Department of Pharmacology, Glaxo Wellcome Research, Beechenham, Kent BR3 3BS, United Kingdom

N-(3-(Aminomethyl)benzyl)acetamidine (1400W) was a slow, tight binding inhibitor of human inducible nitric-oxide synthase (iNOS). The slow onset of inhibition by 1400W showed saturation kinetics with a maximal rate constant of 0.028 s⁻¹ and a binding constant of 2.0 μM. Inhibition was dependent on the cofactor NADPH. L-Arginine was a competitive inhibitor of 1400W binding with a Kᵣ value of 3.0 μM. Inhibited enzyme did not recover activity after 2 h. Thus, 1400W was either an irreversible inhibitor or an extremely slowly reversible inhibitor of human iNOS with a Kᵥ value 7 nM. In contrast, inhibition of human neuronal NOS and endothelial NOS (eNOS) was relatively weaker, rapidly reversible, and competitive with L-arginine, with Kᵥ values of 2 μM and 50 μM, respectively. Thus, 1400W was at least 5000-fold selective for iNOS versus eNOS. This selectivity was similar to that observed in rat aortic rings, in which 1400W was greater than 1000-fold more potent against rat iNOS than eNOS. Finally, 1400W was greater than 50-fold more potent against iNOS than eNOS in a rat model of endotoxin-induced vascular injury. Thus, the potency and selectivity of 1400W inhibition of iNOS both in vitro and in vivo were far greater than of any previously described iNOS inhibitor.

Nitric oxide (NO)1 and l-citrulline are produced by the oxidation of L-arginine that is catalyzed by NO synthase (NOS). NOS utilizes molecular oxygen and a number of cofactors (NADPH, FAD, tetrahydrobiopterin, and heme) that aid in the generation or transfer of electrons (reviewed in Ref. 1). Three isoforms of NOS (iNOS, eNOS, and nNOS) are expressed in specific tissues to generate NO for specific physiological roles (reviewed in Ref. 2). In addition, each isoform has been implicated in specific diseases that are theoretically mediated by excess or inappropriate levels of NO (reviewed in Ref. 3). Therefore, much effort has been given to the design of selective inhibitors of NOS isoforms to be used as biological tools and more importantly as possible therapies to treat such diseases. Most inhibitors of NOS described to date have been analogs of the substrate l-arginine. However, the amino acid moiety is not a requirement for either potency or selectivity. We previously described bisisothioureas and bisisothioureas as potent and, in some examples, selective inhibitors of the human NOS isoforms (4). In particular, PBITU was 190-fold selective for human iNOS compared with eNOS and had a Kᵥ value of 47 nM against iNOS. This study extends our work on bisisothioureas by demonstrating that essentially absolute specificity between iNOS and eNOS can be achieved with 1400W. Further, unlike the bisisothioureas, 1400W was biologically active in vivo.

EXPERIMENTAL PROCEDURES

Materials—Calmodulin (from bovine brain), NADPH, FAD, l-citruline, l-arginine, Heps, endotoxin (Escherichia coli lipopolysaccharide 0111:B4, LPS) were purchased from Sigma; (6R)-5,6,7,8-tetrahydrobipterin from Research Biochemicals International (Natick, MA); l-[3,4-14C]arginine was from DuPont NEN; AG50W-X8 cation-exchange resin was from Bio-Rad; scintillation liquid from ICN (Irvine, CA); and L-octanesulfonic acid, sodium salt (HPLC reagent) was from J. T. Baker (Phillipsburg, NJ). The synthesis of 1400W (N-(3-aminoethyl)benzylacetamidine) will be described elsewhere. 1-[14C-ami dine]1400W was synthesized by J. Hill (Wellcome Research Labs). The Symmetry C₁₈ 5-μm column was from Waters (Milford, MA).

NOS Inhibitor Purification—Recombinant human iNOS (5), human placental eNOS (6), and human brain nNOS (7) were purified as described.

NOS Assay—The oxidation of l-[14C]arginine was monitored by the conversion of l-[14C]arginine to l-[14C]citrulline as described by Schmidt et al. (8). The enzyme was assayed at 37 °C in 20 mM Heps, pH 7.4, with 2.5 mM dithiothreitol, 125 μM NADPH, 10 μM tetrahydrobi- terin, 10 μM FAD, and 0.5–20 μM l-[14C]arginine (10,000 cpm). When eNOS or nNOS were assayed, calmodulin (10 μg/ml) and CaCl₂ (2.5 mM) were included. When time courses were measured, bovine serum albumin (1 mg/ml) was included.

NOS Inhibition in Vascular Tissue—eNOS inhibition was assayed as changes in tone (contraction) in rings of rat thoracic aorta with intact endothelium, as described previously (9). iNOS inhibition was similarly determined in aorta with endothelium removed and following a 6-h induction period in the presence of 100 ng/ml LPS (Salmonella typhosa LPS, Difo) to induce iNOS (10).

Endotoxin-induced Vascular Leakage in Rats—The effects of 1400W on plasma leakage were assessed in rats by determining the leakage of 113I human serum albumin from plasma into organs essentially as described (11, 12). 1400W (0.1–10 mg/kg, subcutaneous) was dissolved in isotonic saline and administered either concurrently with endotoxin or 3 h following LPS administration (E. coli LPS, 3 mg/kg intravenously). Plasma leakage was then assessed 1 or 5 h after delivery of 1400W. The intravascular volumes were subtracted, and the results were expressed as Δ μl g⁻¹ tissue.

Reverse Phase Chromatography of [14C]1400W Incubated with iNOS—[14C]1400W (15 μM) was incubated with iNOS (at a concentration that would convert 2 μM/min of 10 μM l-arginine), and the reaction was analyzed by HPLC at 10, 20, and 40 min. Reactions were as

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
described above for NOS except l-arginine was not included. Control reactions were without enzyme or without NADPH. 50-μl aliquots were filtered through Ultrafree MC filters and applied to a Waters Symmetry C_{18} HPLC column. The column was developed isocratically with 5 mM 1-octanesulfonic acid in 22% acetonitrile at a flow rate of 1 ml/min. 1400W was eluted from the column at 15 min.

Data Analysis—Ki values of eNOS and nNOS for 1400W were calculated from initial velocity studies in which l-arginine was varied from 0.4 to 10 μM at three concentrations of inhibitor. The parameters of the competitive inhibition equation (Equation 1) were fitted to the initial velocity data.

\[ V = V_{\text{max}} [S] / [S] + K_m (1 + [I]/K_i) \]  
(Eq. 1)

For iNOS, the binding of inhibitor (I) to an enzyme (E) was described by a two-step process.

\[ E + I \xrightarrow{k_2} E' \xrightarrow{k_{2-1}} E'' \]  
(Scheme 1)

To determine the kinetic parameters of 1400W binding to iNOS, the slow onset of inhibition was an apparent first-order process with 0.2–2.0 μM inhibitor. Because \( k_{2-1} \approx k_2[I] \), the parameters of the rate equation (Equation 2) were fitted to these data.

\[ k_{\text{obs}} = k_2[I]/K_i + [I] \]  
(Eq. 2)

If an inhibitor and substrate bind to the same site and if substrate binding is in rapid equilibrium, then the substrate will lower the value of the binding constant for the inhibitor (Ki) as defined by Equation 3, where \( [S] \) is the concentration of substrate, \( K_i \) is the inhibition constant for reduction of \( K_i \), and \( K_i' \) is \( K_i \) in the presence of competing substrate.

\[ K_i = K_i'/[1 + ([S]/K_s)] \]  
(Eq. 3)

ED_{50} values for 1400W protection against vascular leakage in the rat were obtained by fitting the logistic equation (Equation 4) to the data, where \( y \) is the determined value in the assay, \( M_{\text{ax}} \) is the maximal value, \( M_{\text{in}} \) is the minimum value, \( I \) is the dose of compound, and \( n \) is the slope (Hill coefficient).

\[ y = (M_{\text{ax}} - M_{\text{in}})(1 + ([I]/EC_{50})^n) + M_{\text{in}} \]  
(Eq. 4)

RESULTS

Progression from Bisothioureas to 1400W—Scheme 2 shows the progression of bisothioureas (4) to 1400W. The first advancement was achieved with the synthesis of \( N,N'-(1,3\text{-benzyl})\text{bisamidine} \). The chemical rationale for this alteration was the observation (13) that the guanidine of l-arginine can be replaced with an amidine moiety and retain, if not improve, binding. The second advancement was the replacement of one of the amidine moieties with the localized positive charge of an amine. The rationale for this substitution will be discussed below.

1400W Was a Potent Time-dependent Inhibitor of Human iNOS—Inhibition of human iNOS by 1400W was time-dependent. The first-order rate constant (\( k_{\text{obs}} \)) for the onset of inhibition of iNOS by 0.4 μM 1400W at 0.5 μM l-arginine was 0.0045 ± 0.0003 s\(^{-1}\) (Fig. 1). When the concentration of 1400W was varied, the dependence of \( k_{\text{obs}} \) on 1400W concentration suggested saturation kinetics (Fig. 2).\(^3\) The maximal rate constant (\( k_2 \)) was 0.028 ± 0.003 s\(^{-1}\). The apparent binding constant (apparent \( K_i \)) of 1400W was 2.3 ± 0.6 μM at an l-arginine concentration of 0.5 μM. The substrate l-arginine inhibited the binding of 1400W to iNOS with a \( K_a \) value of 3.0 ± 0.2 μM. This value is similar to the \( K_m \) value for l-arginine of 2.2 ± 0.2 μM. Thus, 1400W was competitive with l-arginine. The binding constant (\( K_i' \)) for 1400W of 2.0 ± 0.5 μM was calculated from Equation 3 using the apparent \( K_i \) at 0.5 μM.

\(^3\) Definitive evidence for saturation could not be obtained due to the decrease in amplitude and rapidity of binding at high concentrations of 1400W.

\[ \text{S,S'-(1,3-phenylenebis(1,2-ethanediyl))bisothiourea} \]

\[ \text{N,N'-(1,3-benzyl)bisamidine} \]

\[ \text{N-(3-(aminomethyl)benzyl)acetamide} \]

**Fig. 1. Time dependence of iNOS inhibition by 1400W.** Progress curves for l-citrulline formation with 0.5 μM L-[\(^{14}\)C]arginine in a standard assay ("Experimental Procedures") and 0 (squares) or 0.4 μM (circles) 1400W. The 0 μM inhibition control reaction data were normalized after measuring the reaction with 33% enzyme.

l-arginine and the \( K_a \) value for l-arginine.

Inhibition of human iNOS by 1400W did not reverse when iNOS was diluted into a reaction assay at 20 μM l-arginine after preincubation with 1400W (Fig. 3). The first-order rate constant for the loss of activity of uninhibited enzyme was \( (7.3 ± 0.6) \times 10^{-2} \) s\(^{-1}\). Therefore, if 1400W inhibition was slowly reversible, the dissociation rate constant (\( k_{\text{off}} \)) was estimated to be less than or equal to \( (7.3 ± 0.6) \times 10^{-3} \) s\(^{-1}\). Thus,
if 1400W were a slowly reversible inhibitor of iNOS, the overall binding constant ($K_i = k_{on}/k_{off}$) was less than or equal to 7 ns. Inactivation of iNOS was dependent on the presence of NADPH but not on the cofactors tetrahydrobiopterin or FAD (Table I). When NADPH was omitted, then the amount of inhibition observed after preincubation with inhibitor was only 11% greater than that due to residual inhibitor in the diluted reaction mixture.

1400W was not a substrate for iNOS. [14C]1400W (1.5 nmol) was incubated with an amount of iNOS that would convert 0.2 nmol of L-arginine to L-citrulline/min. The enzyme was removed by ultrafiltration, and the filtrate was analyzed by high performance liquid chromatography. No new peaks were observed, and [14C]1400W was quantitatively recovered, consistent with an absence of chemical modification during the incubation. Therefore, if 1400W was a substrate for iNOS, it had less than 0.2% activity of L-arginine. The low specific activity of [14C]1400W and the relatively low amount of enzyme prevented any attempt to detect a [14C]1400W-iNOS complex.

1400W Was a Rapidly Reversible and Relatively Inefficient Inhibitor of Human eNOS and nNOS—1400W inhibited human eNOS and nNOS inefficiently when compared with its inhibition of iNOS. Inhibition of eNOS by 1400W was competitive with L-arginine ($K_i = 50 \pm 2 \mu M$). In preincubation studies in the presence of all cofactors and calcium and calmodulin, no difference was observed between the rates of 1400W-treated and untreated eNOS when diluted into reaction assays containing 10 μM L-arginine and identical final concentrations of 1400W. Therefore, inhibition of eNOS by 1400W was rapidly reversible. Similar results were observed in studies of 1400W and human nNOS ($K_i = 2.0 \pm 0.3 \mu M$).

1400W Was Highly Selective for iNOS in Rat Tissue—Tissue-permeable nonselective NOS inhibitors cause contractions of endothelium-intact rat aortic rings (indicating inhibition of eNOS) as well as of endothelium-removed, LPS-treated tissue (indicating inhibition of iNOS) with similar concentration response curves. For example, L-NMMA inhibited eNOS in aortic rings with an EC50 value of 17 ± 3 μM and iNOS in the induced aortic rings with an EC50 value of 6.0 ± 1 μM (data not shown). As a demonstration of its poor cellular penetration, the previously described PBITU (4) failed to induce contraction in aortic rings (data not shown). In contrast, 1400W was a potent inhibitor of iNOS in aortic rings, with an EC50 value of 0.8 ± 0.3 μM. It had only a slight effect (17 ± 9%) on eNOS in aortic rings at the highest concentration tested (300 μM) (data not shown). Therefore, not only was 1400W active in this tissue assay for iNOS activity, it was at least 1000-fold selective for the inducible versus endothelial isozyme.

1400W Selectively Prevented Microvascularleakage in Rats—A nonselective NOS inhibitor such as L-NMMA has two distinct effects in the rat model of endotoxin-induced microvascular injury, depending on the time of administration (11). When given concurrently with endotoxin, L-NMMA causes a substantial increase in leakage from plasma to tissue. By contrast, when given 3 h after endotoxin, by which time iNOS is expressed, L-NMMA causes a dose-dependent decrease in vascular damage. These findings suggest that when such agents are given concurrently with LPS, the increased vascular injury reflects inhibition of eNOS, whereas the protective action of delayed administration reflects inhibition of iNOS.

The effects of 1400W administration on vascular leakage in the ileum are shown in Fig. 4. When administered at the same time as endotoxin at doses up to 10 mg/kg, subcutaneous, no exacerbation of acute leakage was detected. This was in contrast to the acute effect of L-NMMA, which caused greater than 400 μg/kg leakage into the ileum at a dose of 50 mg/kg (11). However, when administered 3 h after endotoxin, 1400W completely suppressed leakage into the ileum, with an ED50 value

![Graph](Image)

**Fig. 2.** Dependence of $k_{obs}$ for 1400W inhibition on the concentration of 1400W. Pseudo first-order rate constants were obtained as described in Fig. 1 at 0.1, 0.2, 0.4, 0.8, 1.2, and 2.0 μM 1400W. The solid line was calculated from the best fit of the parameters in Equation 2 to the data.

**Fig. 3.** iNOS activity after preincubation with 1400W. Preincubation in the presence (circles) and the absence (squares) of 5 μM 1400W under standard assay conditions (minus L-arginine) for 15 min at 37 °C followed by a 1:50 dilution into a standard assay reaction ("Experimental Procedures") with 10 μM L-[14C]arginine. The solid line for the control reaction was an exponential fit to the data and describes the inherent instability of iNOS. The solid line for the 1400W-treated reaction was linear fit to the data, and the slope was <99% of the control initial velocity.

### Table I

| Preincubation condition | Inhibition (% 10 min) | Inhibition (% 20 min) |
|------------------------|-----------------------|-----------------------|
| All cofactors          | 100                   | 99                    |
| -tetrahydrobiopterin   | 100                   | 100                   |
| -FAD                   | 100                   | 95                    |
| -NADPH                 | 28                    | 42                    |
| No preincubation       | 15                    | 31                    |
A Highly Selective and Potent Inhibitor of iNOS

Vascular leakage into the indicated organs was determined 5 h after concurrent administration of endotoxin (3 mg/kg, intravenous) with saline vehicle or 1400W (0.1–5 mg/kg, subcutaneous). The values shown were obtained by nonlinear least squares regression to data obtained from experiments with 4–12 rats.

Table II

| Organ | ED_{50} mg/kg | Maximal protection % |
|-------|---------------|----------------------|
| Ileum | 0.3 ± 0.1     | 99                   |
| Colon | 0.6 ± 0.1     | 100                  |
| Lung  | 0.4 ± 0.1     | 95                   |
| Liver | 0.3 ± 0.1     | 92                   |
| Kidney| 0.2 ± 0.1     | 54                   |
| Heart | 0.3 ± 0.1     | 94                   |

4962

FIG. 4. Effects of 1400W on acute (A) and delayed (B) vascular leakage in the ileum in response to LPS. Vascular leakage was measured 1 h (A) or 4 h (B) following the administration of LPS (3 mg/kg, intravenous) and 1 h following administration of 1400W (subcutaneous). The data shown are means ± S.E. from experiments with three to eight rats. *, p < 0.001 versus no 1400W control.

of 0.16 ± 0.03 mg/kg, subcutaneous. The ED_{50} value for l-NMMA was 25 mg/kg, subcutaneous (11). Therefore, 1400W was 150-fold more potent than l-NMMA in protecting this microvasculature. The protective effects of 1400W on LPS-induced vascular injury in the ileum were also observed when it was administered concurrently with LPS, and the leakage was evaluated after 5 h. Moreover, 1400W also dose-dependently reduced LPS-induced vascular leakage associated with iNOS induction in the colon, lung, liver, kidney, and heart (Table II). The maximal protection was close to 100% for all organs except the kidney (Table II).

DISCUSSION

1400W is the most selective inhibitor of purified human iNOS reported to date. Except for PBITU (4), all previously described selective inhibitors of iNOS such as isothioureas (4, 14, 15), aminoguanidine (16), cyclic amidines (17), and N-iminoethyl-L-ornithine (18) are at best 30-fold more potent against iNOS than eNOS. 1400W was greater than 5000- and 200-fold more potent against purified human iNOS than eNOS and nNOS, respectively. Furthermore, 1400W was at least 1000-fold selective in a tissue assay that indirectly measures iNOS and eNOS. Finally, 1400W potently (ED_{50} values = −0.3 mg/kg) reduced the delayed vascular injury in rats attributable to LPS-induced iNOS but failed to exacerbate acute vascular leakage when given concurrently with LPS. This in vivo selectivity is at least 100-fold greater than previously observed with such NOS inhibitors as aminoguanidine (12).

In comparison with bisisothioureas, not only did 1400W display greater selectivity, but it was active in biologically relevant assays of iNOS. The bisamidine analogue N,N'-[1,3-benzyl]bisamidine (Scheme 2) of 1400W shared a similar profile with 1400W against purified NOS isozymes and a similar tissue and in vivo profile with bisisothioureas.2 Thus, the chemical change likely to have improved the biological properties was the replacement of one of the delocalized positive charges with the localized positive charge of an amine. This substitution was an attempt to avoid the toxicities of bisisothioureas and bisamidines (discussed below), the rationale being partly derived from the work of Fastier and Smirk in the 1940s and 1950s (reviewed in Ref. 19). They hypothesized that many of the biological effects of isothioureas, guanidines, and amidines result from the delocalized positive charge mimicking solvated potassium and sodium ions and thus binding to receptors for these ions. In support of this, recent publications demonstrate that isothioureas are competitive antagonists of Na^+ in binding the renal Na,K-ATPase (20, 21).

We propose a minimal binding model for 1400W and NOS. 1400W binding was competitive with L-arginine. Because amidine is a structural analogue of guanidine, the amidine of 1400W most likely binds in the guanidine binding pocket of the substrate site. An analogue of 1400W with a carboxylate alpha to the amine was significantly less active than 1400W.4 Therefore, we suspect that the amine of 1400W does not bind in the amino acid site and consequently binds outside the immediate environment of the substrate site.

1400W was a time-dependent inhibitor of iNOS. The kinetics of binding are described by the two-step mechanism (Scheme 1). Clearly, most of the selectivity of iNOS inhibition was derived from interactions that develop slowly. The binding constant for the initial iNOS:1400W complex (K_{i}) was similar to the K_{i} value for 1400W against nNOS. Because this slow second step of iNOS inhibition was dependent on NADPH, chemistry catalyzed by iNOS could play a role in this step. Because electrons from NADPH oxidation eventually reduce heme iron (22), 1400W binding could alter this flow of electrons and induce inactivation of iNOS. It is not obvious why 1400W would not effect electron movement similarly in the constitutive isozymes.

1400W is one of many inhibitors of NOS that display time dependence. Inhibition of mouse iNOS by l-N^4-methylarginine (23) and by l-N^6-allylarginine (24), of mouse iNOS and bovine eNOS by aminoguanidine (16), of bovine nNOS by l-N^4-nitroarginine (25), and human nNOS by S-methyl- and S-ethyl-citrulline (26) are all time-dependent. The first three inhibitors listed were suggested to be irreversible, with the mechanism of inactivation by l-N^4-methylarginine partially explained by loss of heme (27). The latter three inhibitors were each shown to be fully reversible. Obviously, the proposed mechanisms for time-dependent inhibitors of NOS are varied and not completely understood. Of added interest, the time dependence is isozyme-specific for two inhibitors. l-N^4-Nitroarginine is slow binding on the two constitutive isozymes and rapidly reversible against iNOS (25). The binding of 1400W has the reciprocal properties. Therefore, in the development of isozyme-specific inhibitors, it is essential that detailed kinetic studies be performed with each isozyme.

As noted previously (4), some bisisothioureas are acutely toxic (28, 29). We had confirmed that PBITU was toxic in rodents at intravenous bolus doses as low as 10 mg/kg.5 Deaths

4 B. Shearer, J. Oplinger, E. Furfine, and E. Garvey, unpublished results.
5 J. Dillberger, J. Wilson, J. Wolberg, and E. Garvey, unpublished results.
occurred within seconds, suggesting a central nervous system or cardiovascular involvement, whereas transient convulsions were observed at slightly lower doses. $N,N'$-(1,3-Benzyl)bisamidine (Scheme 2) had the same toxicity profile as PBITU. Although 1400W showed a similar profile, such toxic effects were only observed at higher doses; rapid death in rats and mice required an intravenous bolus dose of 50 mg/kg.\(^5\) However, when 1400W was given as an intravenous infusion, rats tolerated a dose of 120 mg/day for a 7-day period.\(^5\) Thus, the acute lethal toxicity of 1400W appeared to occur only after exceeding a relatively high threshold plasma concentration of drug.

In theory, the therapeutic index for an iNOS inhibitor would reflect its selectivity of inhibiting iNOS versus eNOS. However, in practice, the therapeutic index for 1400W appears to reflect the dose that inhibits iNOS as compared with its no effect toxic dose (with the current assumption that toxicity is unrelated to inhibition of NOS). When given as a single subcutaneous dose in rats, 1400W reversed ($ED_{50} = \sim 0.3$ mg/kg) the delayed vascular injury that is attributable to LPS-induced iNOS. In comparison, when given as a single intravenous dose to normal rats, 1400W was tolerated up to 25 mg/kg (with no apparent effect on hemodynamics).\(^6\) Therefore, the window between biological activity and toxicity appears to be significant, although a true therapeutic index cannot be assigned due to the different modes of administration. Thus, 1400W holds promise as a much needed tool in the evaluation of the potential of iNOS inhibitors as novel therapeutic agents.

Acknowledgment—We thank Dave Porter for discussions and reading of the manuscript.

REFERENCES

1. Stuehr, D. J., and Griffith, O. W. (1992) \textit{Adv. Enzymol.} 65, 287–303
2. Moncada, S., Palmer, R. M., and Higgs, E. A. (1991) \textit{Pharmacol. Rev.} 43, 109–142
3. Gross, S. S., and Walin, M. S. (1995) \textit{Annu. Rev. Physiol.} 57, 737–769

\(^6\) A. Tadepalli, W. Harrington, and E. Garvey, unpublished results.