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Novel Potent and Selective Inhibitors of Inducible Nitric Oxide Synthase

MASAKI NAKANE, VERED KLINGHOFER, JANE E. KUK, JENNIFER L. DONNELLY, GERALD P. BUDZIK, JENNIFER S. POLLOCK, FATIMA BASHA, and GEORGE W. CARTER

Abbott Laboratories, Abbott Park, Illinois 60064

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SUMMARY

We have identified two novel potent and selective inhibitors of inducible nitric oxide synthase, *S*-ethylisothiourea and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine. K_i values of 14.7 nM for *S*-ethylisothiourea and 4.2 nM for 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine were obtained with partially purified preparations of inducible nitric oxide synthase. These compounds demonstrate about 1000-fold greater potency than prototypical inhibitors, and the inhibitions are 10–40-fold more selective for murine inducible nitric oxide synthase, compared with the rat neuronal and bovine endothelial isoforms of nitric oxide synthase. These compounds also potently inhibit the nitric oxide synthase activity in intact J774 mouse macrophages. The inhibition is competitive with the substrate L-arginine and reversible in both enzymatic and intact cell assays. These potent and selective inhibitors of inducible nitric oxide synthase may have potential therapeutic applications in the treatment of inflammatory and autoimmune diseases.

NO is an ubiquitous paracrine substance that can also act as an intracellular second messenger in various cells and tissues (1–3). NO is synthesized from L-arginine by NO synthase (EC 1.14.13.39), with molecular oxygen being a co-substrate. Three isoforms of NO synthase, which render many cells capable of synthesizing NO, have been described (4, 5). Isoform I, classically found in neuronal cells, and isoform II, present in "activated" macrophages, are mostly soluble, whereas isoform III, from endothelial cells, is myristoylated and found predominantly in the particulate fraction. The activities of isoforms I and III are regulated by Ca^{2+} and calmodulin, whereas the activity of isoform II is Ca^{2+} independent. Although isoform I and isoform III seem to be physiologically important, inappropriate induction of isoform II is suggested to be involved in inflammatory and autoimmune diseases (4, 5).

Therefore, it would be valuable to develop potent and selective inhibitors of isoform II for potential therapeutic use. We report here the identification and characterization of novel potent and selective inhibitors, EIT and AMT (Fig. 1), of isoform II NO synthase.

Materials and Methods

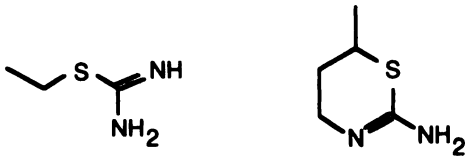
Chemicals. L-NNA and L-aminoguanidine were purchased from Sigma Chemical Co. (St. Louis, MO), and L-NIO was obtained from Research Biochemicals (Natick, MA). L-NMA was synthesized and purified in our laboratory.

EIT was synthesized by a modification of a reported method (6). A slurry of thiourea (22.8 g, 300 mmol; Aldrich, Milwaukee, WI) and diethyl sulfate (25.5 g, 165 mmol; Aldrich) was heated to 130°, at which point a vigorous reaction ensued and the temperature rose to 170°. The heating was continued for 2 hr at this temperature, and the reaction mixture solidified. The reaction mixture was cooled to room temperature, treated with 30% ethanol/ether, and transferred to a filter funnel. The solid was washed with ether (200 ml) and recrystallized from ethanol to afford 23.9 g (47% yield) of the desired EIT-1/2 hydrogen sulfate salt. The characterization of the product was by melting point measurement and NMR spectrometry.

Following a reported preparation method (7), AMT was synthesized starting from 1-(3-butenyl)-2-thiourea (8, 9). A mixture of 2.0 g of 1-(3-butenyl)-2-thiourea and 35 ml of 12 M hydrochloric acid was heated in a closed pressure tube at about 95–100° for 3.5 hr. Solvents were removed by distillation under reduced pressure. The residue was treated with aqueous potassium hydroxide solution and the mixture was extracted with ether. The ether extract was washed with saturated sodium chloride solution, dried with anhydrous magnesium sulfate, and evaporated to dryness by distillation under reduced pressure. The compound was obtained as an oil, which was converted to its hydrochloride salt and has properties consistent with those reported previously (7).

Enzyme preparation. Rat brain NO synthase (isoform I) was prepared as described (10), with slight modifications. Frozen rat brains were homogenized in 5 volumes of ice-cold buffer (50 mM

ABBREVIATIONS: NO, nitric oxide; EIT, *S*-ethylisothiourea; AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine; BAE, bovine aortic endothelial; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; L-NIO, *N*-iminoethyl-L-ornithine; L-NMA, N^G-methyl-L-arginine; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.



EIT (S-ethylisothiourea)

AMT (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine)

Fig. 1. Structures of EIT and AMT.

Tris·HCl, pH 7.5, containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) and centrifuged at 105,000 × *g* for 60 min. The resultant supernatant was used as an isoform I enzyme preparation.

RAW 264.7 macrophage NO synthase (isoform II) was prepared as described (11). RAW 264.7 cell suspension cultures were activated with 100 ng/ml LPS from *Escherichia coli* serotype 055:B5 (Sigma) and 3 units/ml interferon-γ (Sigma) and were harvested 18 hr later by centrifugation. The cell pellet was homogenized in 5 volumes of ice-cold buffer (50 mM Tris·HCl, pH 7.5, containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) and centrifuged at 105,000 × *g* for 60 min. The resultant supernatant was used as an isoform II enzyme preparation. For enzyme kinetic studies, the supernatant was further purified using 2′,5′-ADP-Sepharose column chromatography, as described (10).

BAE cell NO synthase (isoform III) was prepared as described (12), with slight modifications. BAE cells were cultured to confluence in roller bottles, scraped off, and washed in phosphate-buffered saline. The cell pellet was homogenized in 5 volumes of ice-cold buffer (50 mM Tris·HCl, pH 7.5, containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) with 20 mM CHAPS and was centrifuged at 105,000 × *g* for 60 min. The resultant CHAPS extract was used as an isoform III enzyme preparation. CHAPS, up to a final concentration of 0.5 mM, did not interfere with the NO synthase assay described below.

Assay of NO synthase. NO synthase activity was measured as the conversion of [^{3}H]arginine to [^{3}H]citrulline, as described (10), with slight modifications. Samples (50 μl) were incubated in 50 mM HEPES, pH 7.4, with 10 μM L-[2,3,4,5-^{3}H]arginine (9.8 GBq/mmol), 1 mM NADPH, 2 mM $CaCl_2$, 30 nM calmodulin, 3 μM 5,6,7,8-tetrahydrobiopterin, 2 μM FAD, and 2 μM FMN, in a final volume of 100 μl. The reaction was carried out for 45 min at 25° and terminated by addition of 0.5 ml of stop buffer (20 mM HEPES, pH 5.5, containing 2 mM EDTA and 2 mM EGTA). The incubates were applied to 1-ml columns of Dowex AG50WX-8 (Na^+ form) and eluted three times with 0.5 ml of the stop buffer. All of the liquid was pooled and radioactivity was determined by liquid scintillation counting. Under these conditions, the enzyme activity was linear up to 60 min (data not shown).

Intact cell assay. J774 mouse macrophages were activated with LPS (300 ng/ml) and interferon-γ (3 units/ml) for 18 hr. The cells were exposed to the compound in basal Eagle medium (without phenol red and with 0.1 mM L-arginine and 10 mM HEPES; Life Technologies, Grand Island, NY) for 2 hr, the medium was replaced by fresh medium with the compound, cells were incubated for 1 hr, the medium was replaced again by fresh medium with compound, and nitrite accumulation in the medium during the next 3 hr was measured by a fluorometric assay with 2,3-diaminonaphthalene (13). For reversibility studies of inhibitors, the cells were exposed to the compound for the first 2 hr, the compound was removed from the medium during the second (1-hr) and third (3-hr) incubations, and nitrite accumulation in the medium during the last 3-hr incubation was measured as outlined above.

Results

Inhibition of NO synthase *in vitro*. During the screening of various compounds, we found two potent inhibitors of isoform II NO synthase, EIT and AMT (Fig. 1). As shown in Fig. 2, EIT and AMT inhibited isoform II NO synthase activity much more potently than did various reference inhibitors (L-NMA, L-NNA, L-NIO, and L-aminoguanidine). IC_{50} values for EIT and AMT were 13 nM and 4 nM, respectively, indicating that these compounds are about 1000-fold more potent than the prototypical NO synthase inhibitors (Table 1). In addition, EIT and AMT were 10–40-fold more selective for murine isoform II versus rat isoform I and bovine isoform III (Table 1). With the exception of L-aminoguanidine, the reference inhibitors did not show such selectivity (Table 1).

Using a partially purified preparation of murine isoform II NO synthase from RAW 264.7 macrophages, we investigated the nature of the inhibitory activities of these compounds. From the double-reciprocal plots shown in Fig. 3, both EIT and AMT were found to be competitive inhibitors of L-arginine binding to the enzyme. The K_m of L-arginine was 18 μM under these conditions. We obtained K_i values of 14.7 nM for EIT and 4.2 nM for AMT, which were derived from replots of the slope of each double-reciprocal plot versus the corresponding inhibitor concentration (data not shown). In addition, preincubation of isoform II NO synthase with cofactors and EIT or AMT did not result in loss of enzyme activity over 1 hr, suggesting that the inhibition is reversible (data not shown).

Inhibition of isoform II NO synthase in intact cells. The synthesis of NO, measured as nitrite accumulation, by activated J774 cells was significantly inhibited by the prototypical NO synthase inhibitors, in a concentration-dependent manner. Of the four prototypical inhibitors L-NIO was the most potent, and the effects of L-NIO and L-aminoguanidine seemed to be irreversible in intact cells (the ratio of values obtained with and without washing was about 5) (Table 2). On the other hand, EIT and AMT more potently inhibited the synthesis of NO by activated J774 cells, showing IC_{50} values of 82 nM and 15 nM, respectively. Moreover, the inhibition could be easily washed out (the ratios of values obtained with and without washing were >366), indicating that EIT and AMT are reversible inhibitors in intact cells (Table 2).

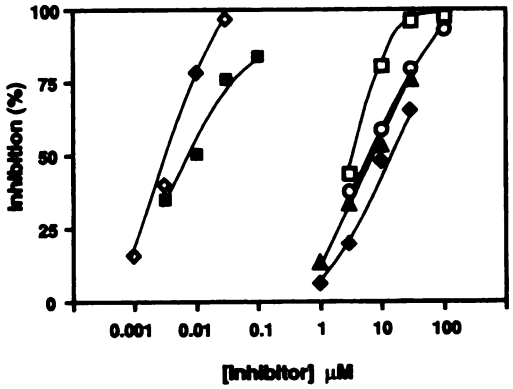


Fig. 2. Effects of L-NMA (○), L-NNA (▲), L-NIO (□), L-aminoguanidine (◆), EIT (■), and AMT (◇) on isolated murine isoform II NO synthase activity. Each *point* is the mean of duplicate determinations.

TABLE 1

Potency of NO synthase inhibitors in enzyme assays with the three isoforms

Values are IC_{50} values obtained by measuring percentage inhibition at four concentrations of inhibitor and are the means ± standard deviations of at least triplicate determinations.

	Inhibitor IC_{50}		
	Isoform I (rat)	Isoform II (murine)	Isoform III (bovine)
		μM	
L-NMA	3.85 ± 0.39	6.15 ± 1.12	3.78 ± 1.90
L-NNA	0.55 ± 0.08	7.71 ± 1.70	0.84 ± 0.26
L-NIO	1.92 ± 0.93	0.32 ± 0.04	4.62 ± 0.64
L-Aminoguanidine	>200	12.3 ± 0.45	>1000
EIT	0.25 ± 0.05	0.013 ± 0.005	0.37 ± 0.02
AMT	0.034 ± 0.011	0.0036 ± 0.0005	0.15 ± 0.06

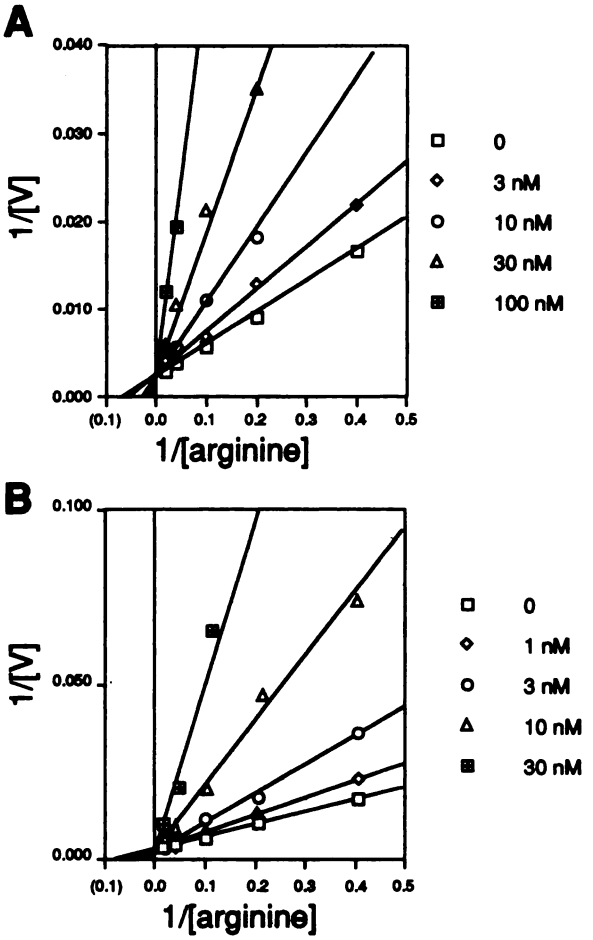


Fig. 3. Double-reciprocal plots of murine isoform II NO synthase activity in the presence of the inhibitors EIT (A) and AMT (B). Isoform II NO synthase was partially purified from the supernatant of activated RAW 264.7 macrophages by 2′,5′-ADP-Sepharose column chromatography, as described in Materials and Methods. The *points* are the average of duplicate determinations. This experiment was repeated twice, with similar results.

Discussion

Characterization and molecular cloning of the NO synthases from different cell types suggest that at least three distinct isoforms exist, i.e., isoform I, which is constitutive and typically found in neuronal cells; isoform II, which is inducible and found in cytokine-activated cells; and isoform III, which is constitutive and typically found in endothelial cells. Although all of the isoforms of NO synthase contain heme, utilize the co-substrates L-arginine and molecular oxygen, and require NADPH, 5,6,7,8-tetrahydrobiopterin, Ca^{2+}-calmodulin, FAD, and FMN as cofactors, the binding affinities of substrates and cofactors vary significantly between isoforms (4, 5). This study utilizes isoforms of NO synthase from various species. The binding affinities of substrates and cofactors do not appear to vary significantly between species for the same isoform; however, there is the possibility that this may introduce a variable in the present study. On the other hand, the two inhibitors that have been identified in this study have been characterized with the three human isoforms of NO synthase from recombinant sources (14) and have shown characteristics similar to those reported here.

TABLE 2

Potency and reversibility of NO synthase inhibitors in intact cell assays of activated J774 cells

Values are IC_{50} values obtained by measuring percentage inhibition at four concentrations of inhibitor and are the means of at least triplicate determinations.

Compound	Inhibitor IC_{50}		Reversibility ratio (with/without washing)
	Without washing	With washing	
	μM		
L-NMA	9.45 ± 3.99	[a]	
L-NNA	27.3 ± 4.6	[a]	
L-NIO	0.472 ± 0.189	2.53 ± 0.97	5.36
L-Aminoguanidine	5.80 ± 3.37	26.8 ± 4.4	4.63
EIT	0.082 ± 0.026	>30	>366
AMT	0.015 ± 0.005	24.4 ± 9.7	1627

[a] No inhibition after washing.

There have been a number of inhibitors of NO synthase reported. L-NMA and L-NIO were found to inhibit the various isoforms of NO synthase with equal potency (Table 1) (15–17). L-NNA is a potent inhibitor of isoforms I and III, while being a relatively poor inhibitor of isoform II (Table 1) (15, 17, 18). L-Aminoguanidine and L-canavanine inhibit isoform II in macrophages, while having little or no effect on isoforms I and III (Table 1) (2, 19, 20).

L-NMA and L-NNA have been suggested to bind to NO synthase in a competitive and reversible manner (21), whereas other investigators reported that L-NNA irreversibly inhibited isoform I NO synthase (22) and L-NMA also has been shown to be an irreversible inhibitor (23, 24). Others have more recently found that the inhibition of isoform I NO synthase by L-NNA is not irreversible but slowly reversible (25). These findings suggest that L-NMA and L-NNA do not simply compete with L-arginine for the substrate binding site on NO synthase. These compounds also produce time-dependent enzyme inactivation, and L-NMA has been reported to be a substrate for NADPH-dependent hydroxylation by NO synthase (23, 24). L-NIO has been shown to be a potent irreversible inhibitor of NO synthase in phagocytic cells (26), but the mechanism of this inhibition is unclear.

Among the prototypical inhibitors, L-NIO was the most potent inhibitor of NO generation in J774 cells and L-NNA only weakly inhibited NO synthesis (Table 2), whereas all four inhibitors showed similar IC_{50} values for isoform II in the enzymatic assay (Table 1). We used J774 cells for the intact cell assay because intact RAW 264.7 mouse macrophages displayed no inhibition of NO generation with L-NMA (data not shown). The intact cell assay may be influenced not

only by the potency of inhibitors but also by the transport of the compounds into the cells. L-NIO is transported rapidly into intact cells, whereas L-NMA and L-NNA are transported slowly, suggesting that the transport of the inhibitors is not via simple diffusion (26). L-NMA and L-NIO are reported to be transported into cells through the cationic transport system y^+ (arginine transporter), whereas L-NNA utilizes the leucine transporter (27, 28). Moreover, treatment of J774 cells with LPS selectively increased the transport of L-NMA and L-NIO through the induction of the cationic transport system y^+, whereas transport of L-NNA was unaffected (29). This may explain why L-NMA and L-NIO remained potent in the intact cell assay, whereas L-NNA lost potency (Tables 1 and 2). We observed that EIT and AMT also lost some potency in the intact cell assay, compared with the enzymatic assay (Tables 1 and 2), indicating that these two inhibitors may not use the arginine transporter. We used 10-fold higher arginine concentrations in the intact cell assay than in the enzyme assay. This might be another reason why some compounds, especially competitive inhibitors of arginine, lost some potency in the intact cell assay.

We tried to determine the selectivity of the compounds for isoforms of NO synthase in the intact cell assay using neuronal (N1E115) cells and endothelial (BAE) cells. Unfortunately, basal nitrite production by these cells was below the detection limit of the assay. Most likely, due to the Ca^{2+} dependence of the NO synthases (isoforms I and III) in these cells, with physiological concentrations of Ca^{2+} N1E115 and BAE cells do not produce enough nitrite to be detected. Even after stimulation of the cells with ATP or calcium ionophore (29), the production of nitrite was transient and below detection limits with this assay.

These potent, selective, and reversible inhibitors of isoform II NO synthase should prove to be valuable pharmacological tools for probing the roles of isoform II NO synthase in experimental models. After this paper was submitted, Garvey *et al.* (31) reported the selective and potent inhibition of isoform II NO synthase by isothioureas.

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Send reprint requests to: Masaki Nakane, D-4NB, AP10, Abbott Laboratories, Abbott Park, IL 60064.