0026-895X/00/051026-09$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:1026–1034, 2000

Vol. 58, No. 5
67/861095
Printed in U.S.A.

The Inhibitory Potency and Selectivity of Arginine Substrate Site Nitric-Oxide Synthase Inhibitors Is Solely Determined by Their Affinity toward the Different Isoenzymes

RAINER BOER, WOLF-RÜDIGER ULRICH, THOMAS KLEIN, BERIT MIRAU, SABINE HAAS, and ILKA BAUR

Byk Gulden Pharmaceuticals, Konstanz, Germany

Received February 4, 2000; accepted July 28, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

We have investigated various nitric oxide (NO) synthase inhibitors for their affinity and selectivity toward the three human isoenzymes in radioligand binding experiments. Therefore, we developed the new radioligand [^{3}H]2-amino-4-picoline to measure binding of these compounds to the three human NO synthase (NOS) isoenzymes. Aminopicoline is a potent and nonselective inhibitor of all three isoforms. [^{3}H]2-amino-4-picoline bound saturably and with high affinity to human NOSs. Affinity constants (K_D values) of 59, 111, and 136 nM were obtained for the inducible, neuronal, and endothelial NOS isoforms (iNOS, nNOS, eNOS). Binding of [^{3}H]2-amino-4-picoline was competitive with the substrate arginine. From all the inhibitors tested, AMT (2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride) showed the highest affinity and no selectivity. L-NIL [L-N^6-(1-Iminoethyl)lysine hydrochloride] and aminoguanidine were moderately iNOS-selective while L-NA (N^G-nitro-L-arginine) and L-NAME (N^G-nitro-L-arginine methyl ester hydrochloride) showed selectivity toward the constitutive isoforms. High iNOS versus eNOS selectivity was found for 1400W, whereas several isothiourea derivatives and 1400W displayed moderate n- versus eNOS selectivity. To relate the affinity of these compounds to their inhibitory potency, we measured the inhibitory potency under almost identical conditions using a new microtiter plate assay. The inhibitory potency of selective and nonselective NOS inhibitors was almost exactly mirrored by their affinity toward the different isoenzymes. Highly significant correlations were obtained between the potency of enzyme inhibition and the inhibition of [^{3}H]2-amino-4-picoline binding for all three isoenzymes. These data show that the potency and selectivity of NOS inhibitors are solely determined by their affinity toward the different isoforms. Furthermore, these data identify the new radioligand [^{3}H]2-amino-4-picoline as a very useful radiolabel for the investigation of the substrate binding site of all three isoforms.

Nitric-oxide synthases are enzymes responsible for the generation of nitric oxide using arginine and NADPH as substrates. Nitric oxide is an important signaling molecule that, besides acting as a signal transducer, exerts a variety of regulatory and cytostatic functions (Murad, 1998). Two classes of nitric-oxide synthases exist in higher animals and are conserved between species (Michel et al., 1996). The constitutively expressed neuronal and endothelial isoforms are tightly regulated by Ca^{2+}/calmodulin and are involved mostly in housekeeping functions such as blood pressure regulation and memory formation. The inducible isoform is activated transcriptionally after stimulation of various cells by proinflammatory signals and once expressed is active over longer time periods without any major short-term regulation. This activity profile leads to high and cytotoxic NO levels necessary for an effective immune defense against invading pathogens. The inducible isoform has evoked great attention, as this isoform is involved in a number of pathophysiologies such as septic shock, inflammatory conditions of the joint, intestine, and CNS (Hobbs et al., 1999). Great efforts have been made to develop selective inhibitors of the inducible isoform for the treatment of these pathophysiologies (Hobbs et al., 1999). Some nonselective compounds have already entered clinical trials (for review see Kilbourn et al., 1997; Grover et al., 1999). Many experimental compounds are used to study the function of NOS isoforms in physiology and pathophysiology. It seems clear that for the treatment of the above-mentioned pathophysiologies a selective inhibition of

ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; AG, aminoguanidine; BH-4, 6*R*9–5,6,7,8-tetrahydro-L-biopterin dihydrochloride; AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride; DPI, diphenyleneiodonium chloride; L-NMMA, N^G-monomethyl-L-arginine; S-et-ITU, *S*-ethylisothiourea hydrobromide; S-me-ITU, *S*-methylisothiourea hydrobromide; S-et-TFMP-ITU, *S*-ethyl-*N*-[4-(trifluoromethyl)-phenyl]-isothiourea hydrochloride; S-aet-ITU, *S*-(2-aminoethyl)-isothiourea; S-me-TC, *S*-methyl-L-thiocitrulline; L-NIL, L-N^6-(1-iminoethyl)lysine hydrochloride; L-NIO, L-N^5-(1-iminoethyl)ornithine; 1,3-PB-ITU, *S*,*S*′-(1,3-phenylene-bis(1,2-ethanediyl))bis-isothiourea; 1,4-PB-ITU, *S*,*S*′-(1,4 -phenylene-bis(1,2-ethanediyl))bis-isothiourea; L-PA, N^ω-propyl-L-arginine; L-VNIO, [L-N^5-(1-imino-3-butenyl)-ornithine; 1400W, [*N*-(3-aminoethyl)benzyl)-acetamidine hydrochloride; L-NA, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride; i-, n-, and eNOS, inducible-, neuronal-, and endothelial NO synthase.

the inducible isoform is absolutely necessary and that nonselective inhibitors will deteriorate these conditions due to concomitant inhibition of constitutive isoforms. Inhibition of the constitutive endothelial isoform results in severe hypertension. This seems to be related to increased organ damage in animal models of septic shock (Billiar and Harbrecht, 1997; Schwartz et al., 1997; Titheradge, 1999).

Radioligand binding experiments are valuable tools in the investigation of drug/target interactions. Up to now the use of this technique to investigate the interaction of inhibitors with the arginine binding site of NO synthases (where most inhibitors bind) was limited to the constitutive isoenzymes. The only available radioligand [^{3}H]nitroarginine has, due to its selectivity profile (high selectivity for e- and nNOS versus iNOS), sufficient affinity to be used as a suitable radioligand for the two constitutive isoforms (Klatt et al., 1994). But the low affinity for the inducible isoform prohibits its use for the characterization of the substrate binding site of inducible NOS. Radiolabeled [^{3}H]tetrahydrobiopterin has been successfully used as a radioligand for iNOS (Gorren et al., 1996; Alderton et al., 1998), but this compounds binds at a different epitope of the enzyme and shows complex kinetic interactions with NO synthases (Klatt et al., 1994), thus limiting its use for the investigation of the arginine binding site. Therefore we developed a radiolabeled inhibitor which is nonselective toward the three isoforms and is useful as a radioligand for the substrate binding site of iNOS. Using this new radioligand we characterized the most often used NOS inhibitors for their affinity and selectivity toward the NOS isoforms. In addition to substrate and product analogs such as L-NIL, L-NIO, L-NMMA (Moore et al., 1994), and thiocitrullines (Furfine et al., 1994; Narayanan et al., 1995), we also included various isothiourea derivatives (Southan et al., 1995; Shearer et al.,1997), heterocycles (Nakane et al., 1995), aminoguanidine (AG) (Misko et al., 1993), 1400W (Garvey et al., 1997), and other structures. (Griffith and Gross, 1996; Macdonald, 1996; for reviews see Moore and Handy, 1997).

To relate the measured affinities and selectivities of NOS inhibitors with the inhibitory potencies and selectivities at the enzymatic level, we decided to measure the inhibitory potency and selectivity of NOS inhibitors in a very similar assay system.

Although most of the inhibitors used in our study are already reported in the literature, a direct comparison of compounds described by different laboratories is difficult due to the use of various assay systems, assay conditions, and species. Additionally, the reported potencies and selectivities of these compounds are not absolutely clear, and variable values are reported for one and the same compound. For example for aminoguanidine IC_{50} values between 5 μM (Wolff et al., 1998) and 168 μM (Moore et al., 1996) were reported for the inducible isoform. Similar discrepancies are obvious for L-NIL (Moore et al.,1996; Wolff et al., 1998). Certain isothiourea derivatives are used as selective iNOS inhibitors by some investigators (Gunderson et al., 1997; Chen et al., 1998; Wang et al., 1998), although being described as nonselective compounds by others (Garvey et al., 1994; Macdonald, 1996). Similar confusion regarding compound selectivities exists for L-NAME (Cochran et al., 1999) or L-NA (Resta et al., 1999) both selective inhibitors of the constitutive isoforms (Nakane et al., 1995; Moore et al., 1996). Therefore we felt the need to determine these parameters again, using the same source of enzyme, assay conditions, and species. We developed a fast and reliable microtiter plate screening system to characterize these NOS inhibitors with respect to selectivity and potency.

Experimental Procedures

Materials

[^{3}H]Arginine (60–80 Ci/mmol) was bought from Amersham/Pharmacia (Cardiff, UK); 6*R*9–5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH-4) was obtained from Schircks Laboratories (Jona, CH); and 2-amino-4-picoline (2-AP) was from Biotrend/RBI (Köln, Germany). [^{3}H]2-AP was synthesized by bromine/tritium exchange from 2-amino-3,5 dibromo-4-methyl-pyridine at Amersham/Pharmacia. A specific radioactivity of 64 Ci/mmol was obtained.

2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride (AMT), diphenylene-iodonium chloride (DPI), N^G-monomethyl-L-arginine (L-NMMA), *S*-ethylisothiourea hydrobromide (S-et-ITU), *S*-methylisothiourea hydrobromide (S-me-ITU), *S*-ethyl-*N*-[4-(trifluoromethyl)phenyl]-isothiourea hydrochloride (S-et-TFMP-ITU), *S*-(2-aminoethyl)-isothiourea (S-aet-ITU), *S*-methyl-L-thiocitrulline (S-me-TC), L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL), L-N^5-(1-iminoethyl)-ornithine (L-NIO), *S*,*S*′-(1,3-phenylene-bis(1,2-ethanediyl))bis-isothiourea (1,3-PB-ITU), N^ω-propyl-L-arginine (L-PA), L-N^5-(1-imino-3-butenyl)-ornithine (L-VNIO), and *N*-(3-aminoethyl)benzyl)acetamidine hydrochloride (1400W) were from Alexis (Grünberg, Germany). N^G-Nitro-L-arginine (L-NA), N^G-nitro-L-arginine methyl ester hydrocloride (L-NAME) were from Tocris Cookson (Langford, UK). All other chemicals were from commercial suppliers with the highest grade of purity.

The human isoforms of NO synthases were obtained from Vasopharm (Würzburg, Germany) after transfection of Sf9 cells with the respective human cDNAs via the baculovirus system. The cytosolic fraction of homogenized Sf9 cells was used for all experiments.

Methods

Measurement of NO Synthase Activity. The enzyme reaction was performed in 96-well microtiter F-plates (655101; Greiner, Frickenhausen, Germany) in a total volume of 100 μl in the presence of 100 nM calmodulin, 226 μM $CaCl_2$, 477 μM $MgCl_2$, 5 μM flavin-adenine-dinucleotide (FAD), 5 μM flavin mononucleotide (FMN), 0.1 mM NADPH, 7 mM glutathione, 10 μM BH-4, and 100 mM HEPES, pH 7.2. Arginine concentrations were 0.1 μM for enzyme inhibition experiments and varied from 0.78 μM to 200 μM for the determination of K_M values. [^{3}H]arginine (150,000 dpm) was added to the assay mixture for enzyme inhibition studies. For K_M value measurements, 1×10^6 dpm were added to the highest concentration (200 μM) and diluted serially. Enzyme reaction was started by the addition of 4 μg of crude enzyme preparation, and the reaction mixture was incubated for 45 to 60 min at 37°C. Enzyme reaction was stopped by adding 10 μl of 2 M MES buffer pH 5,0. 50 μl of the incubation mixture were transferred into a MADP N 65 filtration microtiter plate (Millipore, Eschborn, Germany) containing 50 μl of AG-50W-X8 cation exchange resin (Bio-Rad, München, Germany). The resin in the sodium-loaded form was pre-equilibrated in water and 70 μl (corresponding to 50 μl dry beads) were pipetted under heavy stirring with an eight-channel pipette into the filtration plate. Alternatively a simple column loader device (Millipore) could be used to transfer 50 μl of dried beads into the microtiter plates. After pipetting 50 μl of the enzyme reaction mixture onto the filtration plates, the plates were placed on a filtration manifold (Porvair, Shepperton, UK) and the flowthrough was collected in Pico scintillation plates (Packard, Meriden, CT). The resin in the filtration plates is washed with 75 μl of water (1×50 μl and 1×25 μl), which is also collected in the same plate as the sample. The total flowthrough of 125 μl is mixed with 175 μl of Microscint-40 scintillation cocktail (Packard), and the scintillation plate is sealed with TopSeal P-foil (Packard). Scintillation plates were counted in a TopCount (Packard) or Micro-

beta (Wallac, Turku, Finland) scintillation counter. The expensive filtration plates were used several times after disposal of the used and dried ion exchange resin. For the measurement of NOS inhibitors, increasing concentrations of inhibitors were included in the incubation mixture.

Radioligand Binding Experiments. Radioligand binding experiments were performed in 96-well microtiter F-plates (Greiner) in a total volume of 100 μl in the presence of 2 mM $CaCl_2$, 10 μM BH-4, 1 mM dithiotreitol, and 50 mM TRIS/HCl, pH 7.4. In most experiments 100 μM NADPH was included. For radioligand competition experiments approximately 250,000 dpm corresponding to a concentration of 18 nM were included. For saturation isotherms up to 2×10^6 dpm were used for the highest ligand concentration. Nonspecific binding was determined in the presence of 10 μM AMT. The assay mixture was incubated for 45 to 60 min at 37°C and then filtered over GF-C glass fiber filter mats (Whatman, Maidstone, UK) with a cell harvester (Skatron, Lier, Norge). Filters were automatically rinsed with 5 ml of ice-cold incubation buffer without NADPH and BH-4. Filters were punched into 3.5-ml scintillation vials and were counted after adding 3.5 ml of UltimaGold scintillation cocktail (Packard). For radioligand competition experiments, a constant amount of [^{3}H]2-AP was incubated in the presence of increasing concentrations of inhibitors.

Nonlinear regression analysis was used to determine kinetic constants (K_M values) for enzyme activity and affinity constants (K_D values) for [^{3}H]2-AP binding. IC_{50} values were calculated from enzyme inhibition and binding inhibition data. For all calculations the program Prism 3.0 (GraphPad, Sorrento Valley, CAL) was used.

Results

Potency and Selectivity of 2-AP. The inhibitory potency of 2-AP at the three isoenzymes was determined leading to pIC_{50} values of 7.27 ± 0.09, 7.30 ± 0.15 and 7.21 ± 0.06 nM for i-, n-, and eNOS (n = 3; Fig. 1). This corresponds to IC_{50} values of 54, 50, and 62 nM for i-, n-, and eNOS. The slope values of the inhibition curves were 1.00 (iNOS), 1.02 (nNOS), and 0.95 (eNOS) and were not significantly different from unity (Fig. 1), indicating a homogeneous population of enzyme and simple Michaelis-Menten behavior. The very similar IC_{50} values obtained at the three isoenzymes identify 2-AP as a potent but nonselective NO synthase inhibitor.

The mechanism of action of 2-AP was determined on the inducible isoform. 2-Amino-4-picoline (Fig. 2) is an arginine competitive inhibitor of the inducible and most probably also constitutive NO synthases (not determined). Measurement of iNOS activity at increasing arginine concentrations in the absence and presence of 0.3 and 1 μM 2-AP resulted in a shift of the K_M value from 10 μM in the absence of inhibitor to 41 (0.3 μM) and 123 μM (1 μM). V_{max} values were 7.45 pmol/mg in the absence and 7.43 and 7.28 pmol/mg in the presence of compound and were not significantly different from one another (Fig. 2). Calculation of the K_I value for 2-AP at the inducible isoform according to Michaelis-Menten kinetics gave a value of 76 nM.

Affinity of [^{3}H]2-AP. Radioligand saturation experiments were performed with [^{3}H]2-AP at the three isoenzymes. Binding of [^{3}H]2-AP was time- and concentration-dependent at all three isoforms. Nonspecific binding as determined in the presence of a several hundred-fold excess of AMT (10 μM) was 10 to 30% of total binding at a protein concentration between 10 and 30 μg/ml. The resulting saturation isotherms were monophasic for all isoforms, therefore obeying mass action law. Dissociation constants (K_D values) of 59 ± 16 (n = 6), 111 (n = 2), and 136 ± 30 (n = 3) nM were obtained for i-, n-, and eNOS (Fig. 3). The presence of a constant arginine concentration of 10 or 30 μM in [^{3}H]2-AP saturation experiments resulted in a reduction of [^{3}H]2-AP affinity without a significant change in maximal binding (data not shown). To obtain a good comparability between catalytic activity and binding activity we routinely performed the radioligand binding experiments in a similar buffer system as used for the measurement of enzymatic activity (see below) yielding catalytically active enzyme in our radioligand binding assays. Omission of NADPH, necessary for catalytic activity from the binding buffer, resulted in a [^{3}H]2-AP K_D value of 57 nM for iNOS. Therefore binding of NADPH or catalytic activity is not necessary for the binding of [^{3}H]2-AP. Association of [^{3}H]2-AP to and dissociation from inducible NO synthase was fast at 37°C, permitting the calculation of kinetic constants.

In radioligand competition experiments performed at the inducible, neuronal, and endothelial isoform, increasing concentrations of unlabeled 2-AP resulted in dose-dependent inhibition of [^{3}H]2-AP binding. IC_{50} values of 72, 68, and 112 nM were obtained for i-, n-, and eNOS. Calculation of the radioligand-independent affinity constants (K_I values) according to Cheng and Prusoff (1973) resulted in K_I values of

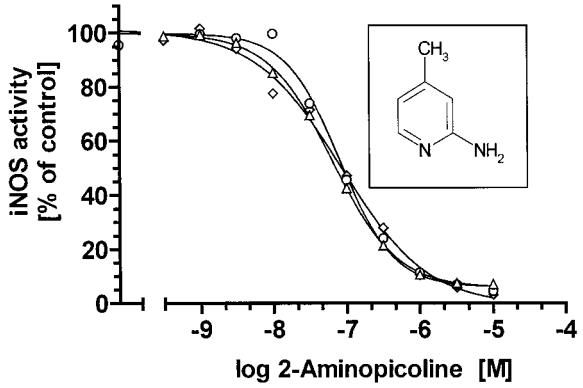

Fig. 1. Inhibition of human inducible (△), neuronal (◇), and endothelial (○) NO synthases by 2-AP. pIC_{50} values in molar concentrations of 7.20, 7.07, and 7.09 were obtained for i-, n-, and eNOS. The inset shows the structure of 2-AP.

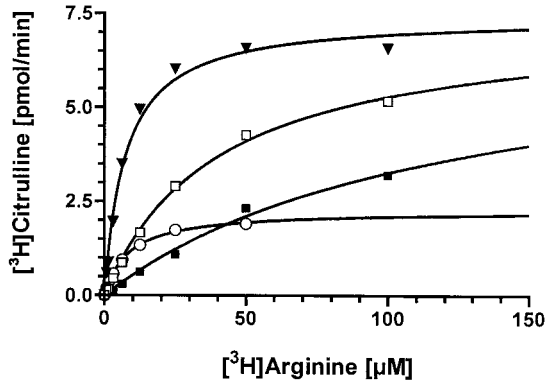

Fig. 2. Substrate dependence of inducible NOS activity in the absence (▼) or presence of 300 nM 2-AP (□), 1 μM 2-AP (■), or 100 nM DPI (○). Calculated V_{max} values were 7.45 pmol/min in the absence of inhibitor, 7.43 pmol/min in the presence of 300 nM 2-AP, 7.28 pmol/min in the presence of 1 μM 2-AP, and 2.28 pmol/min in the presence of DPI. The following K_M values were obtained for arginine: 7.44 μM in the absence of inhibitor, 40 and 123 μM in the presence of 300 nM and 1 μM 2-AP, and 8.67 in the presence of 100 nM DPI.

55, 65, and 98 nM for i-, n-, and eNOS, which are in good agreement with the radioligand saturation experiments. From the radioligand competition data with nonradioactive 2-AP, a saturation curve was constructed for the inducible enzyme and a calculated K_D value of 73 nM is obtained from the data (Fig. 4), again in good agreement with the above-mentioned values.

These data show that [3H]2-AP is a suitable, high affinity radioligand for inducible and constitutive NO synthase isoforms. The compound binds competitively with arginine to the arginine substrate binding site of NO synthases.

Affinity of NOS Inhibitors to NO Synthase Isoenzymes. The affinity of NOS inhibitors was determined for i-, n-, and eNOS in radioligand competition experiments with [3H]2-AP as radioligand. All compounds inhibited [3H]2-AP binding in a dose-dependent manner with slope values around unity. Table 1 shows the $-\log IC_{50}$ and slope values for all inhibitors tested, and the calculated selectivity ratios obtained from binding data. AMT showed the highest affinity and no selectivity between isoforms with IC_{50} values around 10 nM. 1400W, 1,3-, and 1,4-PB-ITU displayed somewhat lower affinity but were the most i-/eNOS-selective compounds with selectivity factors around 60. Isothiourea derivatives with small substituents at the sulfur atom displayed intermediate (*S*-aminoethyl-) to high affinity (*S*-ethyl-, *S*-methyl-) but no selectivity. L-NMMA, L-NA, L-NAME, and PA showed selectivity for the constitutive isoforms of NO synthases. Moderate n- versus eNOS selectivity is found for 1,3-PB-ITU, 1,4-PB-ITU, and S-et-TFMP-ITU with selectivity ratios >10. Representative radioligand competition curves for AMT, 1400W, AG, L-NAME, L-NMMA, and S-et-ITU at the inducible isoform are shown in Fig. 5. DPI bound with very low affinity ($IC_{50} < 100\ \mu M$) to all three isoforms.

Microtiter Plate [3H]Citrulline Assay of NO Synthases. For the measurement of NO synthase activity we developed a robust and reliable microtiter plate assay for the measurement of radiolabeled [3H]citrulline from the NOS substrate, [3H]arginine. [3H]Arginine is converted enzymatically to [3H]citrulline and NO. In our assay the enzymatic reaction took place in a reaction microtiter plate. For the determination of [3H]citrulline, excess substrate is removed by binding to a cation exchange resin. This cation exchange step was performed in a separate microtiter filtration plate and the flowthrough (noncharged citrulline) was collected in a third microtiter plate. In principle the whole procedure could also be reduced to one microtiter plate by pipetting the cationic exchange resin into the reaction wells and by retracting between 20 and 50 μl from the supernatant after settlement of the exchange resin. This procedure resulted in an increased signal to noise ratio (10-fold instead of 25-fold). Due to a noncharged impurity of the [3H]arginine, which did

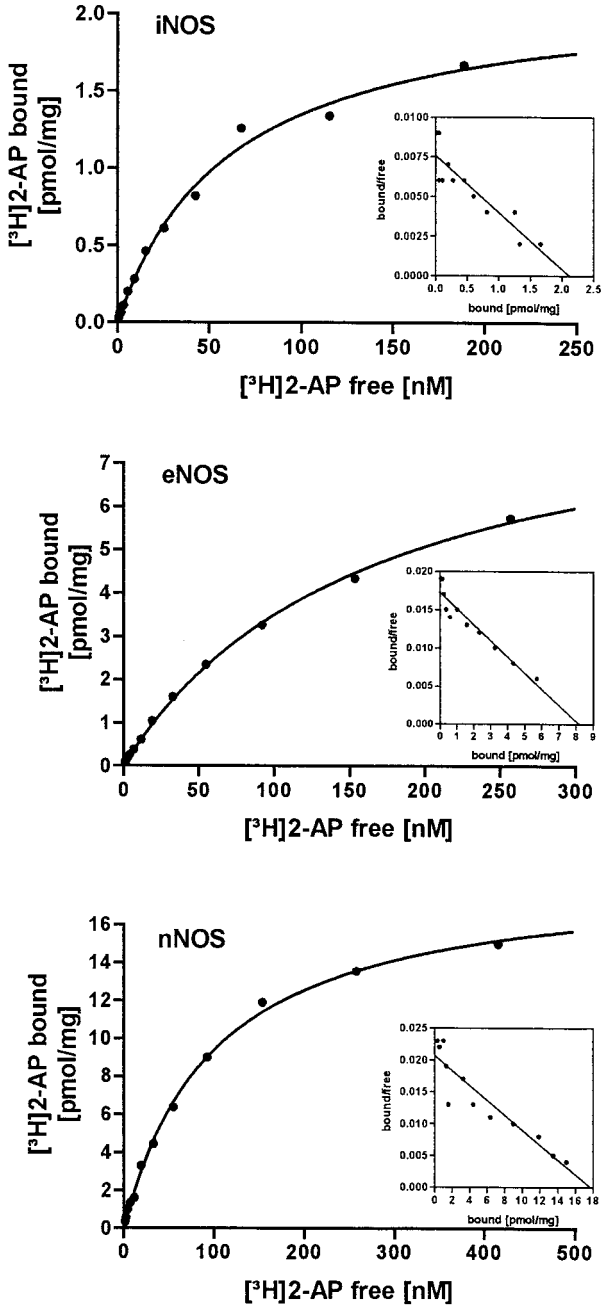

Fig. 3. Saturation isotherms of [3H]2-AP binding to human inducible NO synthase (top), endothelial NO synthase (middle), and neuronal NO synthase (bottom). Affinity constants (K_M values) of 62, 100, and 166 nM were obtained for i-, e-, and nNOS.

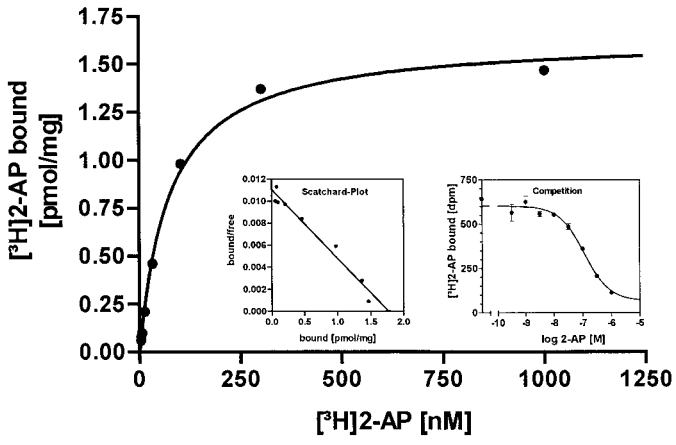

Fig. 4. Saturation isotherm of [3H]2-AP binding to inducible NO synthase calculated from a radioligand competition experiment performed in the presence of increasing concentrations of nonlabeled 2-AP. The insets show the transformed Scatchard plot (left) and the underlying radioligand competition curve for 2-AP (right). The calculated K_D value was 76 nM. The pIC_{50} value in molar concentrations for half-maximal inhibition of [3H]2-AP binding by 2-AP was 6.95 corresponding to an IC_{50} value of 112 nM.

not bind to the resin during the ion exchange step and eluted together with [^{3}H]citrulline, it was necessary to purify the radiolabeled [^{3}H]arginine by ion exchange chromatography on AG × 8 resin before use. The purified [^{3}H]arginine allowed the use of high [^{3}H]arginine concentration (up to 1 × 10^6 dpm at 100 μM) with an acceptable signal to noise ratio of 3.

All three isoenzymes converted [^{3}H]arginine to [^{3}H]citrulline in a dose-dependent and saturable manner obeying monophasic Michaelis-Menten kinetics. The K_M values were 5.68 ± 1.87 μM (Fig. 4), 2.84 ± 0.98 μM, and 3.30 ± 1.01 μM for i-, n-, and eNOS (n between 5 and 10). The reaction was linear up to 45 min followed by a decline in activity due to substrate depletion and substrate instability.

Potency and Selectivity of NOS Inhibitors. To determine the inhibitory potency of NOS inhibitors, we measured [^{3}H]citrulline generation by the human isoenzymes in the presence of increasing inhibitor concentrations. An arginine concentration of 0.1 μM was chosen for all experiments. All compounds tested displayed monophasic inhibition curves with slope values near unity. A very similar picture of inhibitory activity as found in radioligand binding experiments emerged with regard to potency and selectivity from these measurements. The pIC_{50} values for selective and nonselective NOS inhibitors are given in Table 2. Highest potency with no selectivity between isoforms is found for AMT. IC_{50} values vary from 4 nM for nNOS to 5 nM for iNOS and 11 nM for eNOS. Somewhat lower potency with no selectivity is found for the substrate analogs L-NMMA, L-NIO, L-VNIO, and for S-me-TC. A similar potency with low, respectively high iNOS versus eNOS selectivity is found for L-NIL and 1400W. Both compounds show a moderate iNOS versus nNOS selectivity. Subtype-selective substrate analogs with 10- to 30-fold selectivity for the constitutive isoforms (n- and eNOS) are L-NA and L-NAME. L-NAME is 30- to 100-fold less potent than L-NA. In the structural group of isothioureas, IC_{50} values in the range between 1 and 0.1 μM are seen for the nonselective *S*-ethyl- and *S*-methyl-isothiourea. Among the phenylene-bis-isothioureas, the 1,3- and 1,4-substituted derivatives show high iNOS versus eNOS selectivity with small or no iNOS versus nNOS selectivity. Some of these compounds (1,3-PB-ITU, 1,4-PB-ITU, and S-et-TFMP-ITU) therefore showed moderate to high selectivity between the constitutive isoforms. High nNOS versus iNOS selectivity (>100) is seen with S-et-TFMP-ITU. AG is a weak and moderately i- versus eNOS-selective compound.

DPI is a nonarginine competitive inhibitor with high potency (IC_{50} values about 0.1 μM) and almost no selectivity. By measuring Michaelis-Menten kinetics of arginine substrate dependence on enzyme turnover the allosteric inhibitory nature of DPI could be demonstrated. The presence of 0.1 μM DPI led to a reduction of the maximal turnover rate

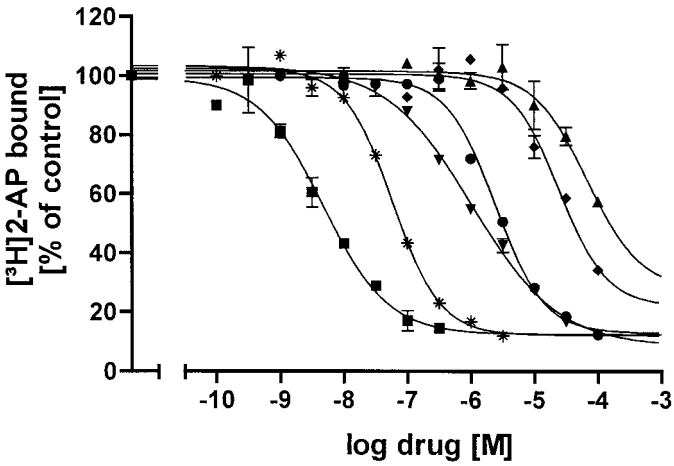

Fig. 5. Competition of selected NOS inhibitors with [^{3}H]2-AP binding to human inducible NO synthase. The following pIC_{50} values in molar concentrations were obtained: 8.41 (AMT, ■), 7.20 (S-et-ITU, ∗), 5.97 (1400W, ▼), 5.55 (L-NMMA, ●), 4.44 (L-NAME, ♦) and 4.16 (AG, ▲,). Each data point is the mean of two measurements. The calculated standard deviation (SD) is shown as error bars.

TABLE 1
Affinity and selectivity of NO synthase inhibitors at human isoenzymes
Selectivity factors were rounded to the next unit.

Compounds	Affinity			Selectivity		
	iNOS	nNOS	eNOS	i-/eNOS	i-/nNOS	n-/eNOS
	pIC_{50} value ± S.D.				*ratio*	
Substrate analogs						
L-NA	5.5 ± 0.1	7.33 ± 0.02	7.04 ± 0.05	0.03	0.02	2
L-NAME	4.5 ± 0.1	6.12 ± 0.02	5.82 ± 0.02	0.05	0.02	2
L-PA	4.0 ± 0.1	5.4 ± 0.1	5.06 ± 0.03	0.1	0.04	2
L-NMMA	5.58 ± 0.04	6.6 ± 0.1	6.5 ± 0.1	0.1	0.1	1
L-NIO	5.8 ± 0.1	6.02 ± 0.03	6.00 ± 0.01	1	1	1
L-VNIO	6.1 ± 0.1	6.4 ± 0.1	5.85 ± 0.04	2	0.5	4
L-NIL	5.7 ± 0.2	5.4 ± 0.2	5.11 ± 0.04	4	2	2
S-me-TC	6.7 ± 0.1	7.4 ± 0.1	6.94 ± 0.03	1	0.2	3
Isothioureas						
S-me-ITU	6.3 ± 0.1	6.29 ± 0.02	6.22 ± 0.04	1	1	1
S-et-ITU	7.25 ± 0.05	6.91 ± 0.04	6.78 ± 0.04	3	2	1
S-aet-ITU	5.1 ± 0.2	4.84 ± 0.02	4.54 ± 0.03	4	2	2
S-et-TFMP-ITU	<4.0	5.44 ± 0.01	4.3 ± 0.1	<0.5	<0.04	14
1,3 PB-ITU	6.3 ± 0.1	5.63 ± 0.04	4.5 ± 0.1	63	5	13
1,4 PB-ITU	7.0 ± 0.1	6.49 ± 0.01	5.24 ± 0.01	58	3	18
Miscellaneous						
1400W	6.1 ± 0.1	5.5 ± 0.1	4.31 ± 0.02	62	4	15
AMT	8.1 ± 0.2	8.08 ± 0.03	7.6 ± 0.2	3	1	3
DPI	<4.0	<4.0	<4.0			
AG	3.9 ± 0.2	3.2 ± 0.2	3.1 ± 0.1	6	5	1
2-AP	7.1 ± 0.2	7.17 ± 0.02	6.95 ± 0.03	1	1	2

V_{max} from 7.45 pmol/min in the absence to 2.28 pmol/min in the presence of compound without a significant change in K_M values (Fig. 2). Selectivity ratios calculated from measured IC_{50} values at the three isoenzymes are also given in Table 2.

Correlation of Enzymatic Activity with Radioligand Binding. For all three isoenzymes, the inhibitory potency of the above-mentioned inhibitors (except DPI, see *Discussion*) was compared with the respective affinity of these inhibitors to the different isoforms. Excellent correlations between binding affinity and inhibitory potency were found for each isoform (Fig. 6). Correlation coefficients (r^2) were 0.93, 0.81 and 0.97 for i-, n-, and eNOS. The slope values of 0.97 for iNOS, 0.90 for nNOS, and 1.00 for eNOS indicate that the affinity of inhibitors for the respective isoenzymes directly translates into inhibitory potency and selectivity.

Discussion

We have characterized and compared the binding of several of the most commonly used NOS inhibitors at the arginine substrate site with respect to affinity and selectivity at the three human isoenzymes. Therefore we developed [^{3}H]2-AP, a new radioligand with high enough affinity to allow the measurement of direct binding to NO synthases.

Before radiolabeling we investigated the inhibitory potency and selectivity of 2-AP. 2-AP is an arginine competitive and nonselective inhibitor of all three NO synthase isoforms. IC_{50} values between 50 and 70 nM were obtained. This compound has been described as a potent, nonselective, and arginine-competitive inhibitor of NO synthases by others (Faraci, 1996). Our results on enzyme activity obtained with the modified microtiter plate assay are in good agreement with their data. The high potency of 2-AP made it a good candidate for radiolabeling and for use in radioligand binding experiments.

[^{3}H]2- AP binds with high affinity to all three isoforms. The K_D value of 59 nM for iNOS as measured in radioligand saturation experiments is in excellent agreement with the calculated K_I value of 55 nM from radioligand competition experiments.

Radioligand binding data for [^{3}H]2-AP correspond well to enzyme inhibition data. Half-maximal binding to the inducible enzyme is seen in the same concentration range (K_D or K_I values of 50 to 80 nM) as half-maximal enzyme inhibition (K_I = 76 nM.)

With [^{3}H]2-AP as radioligand we investigated the affinity and selectivity of several NOS inhibitors to the three isoforms in binding competition experiments. Published literature data from various sources showed that the rank order of affinities for NOS inhibitors only roughly mirrors the rank order of inhibitory potencies in enzymatic assays. A valid correlation of our affinity values with inhibitory potencies could not be performed from reported data, due to the use of various experimental systems, different assay conditions and species (Nakane et al.,1995; Wolff et al., 1998) by different investigators. For example Cowart et al. (1998) used 10 μM, Hagen et al. (1998) used 70 μM, and Wolff et al. (1998) used 0.1 μM arginine in their systems. Most of the reported compounds are arginine-competitive, and IC_{50} values are directly dependent on the arginine concentration used in the respective assay system.

Additionally, the selectivities of NOS inhibitors are not totally clear from literature data because one and the same compound is sometimes used as selective or nonselective inhibitor by different investigators. A profound characterization of inhibitors used to differentiate the effects of NO derived from these different isoforms is of extreme importance, especially for NO synthases, where the constitutive isoforms are recognized as protective housekeeping enzymes and the inducible isoform is involved in several pathophysiologies such as septic shock, rheumatoid arthritis, inflammatory diseases of the lung, intestine myocard, and kidney.

TABLE 2

Potency and selectivity of NO synthase inhibitors at human isoenzymes

Selectivity factors were rounded to the next unit.

Compounds	Potency (log IC^{50}-value)			Selectivity		
	iNOS	nNOS	eNOS	i-/e-NOS	i-/n-NOS	n-/e-NOS
	pIC_{50} value ± S.D.				*ratio*	
Substrate analogs						
L-NA	5.8 ± 0.1	7.5 ± 0.2	7.1 ± 0.2	0.05	0.02	3
L-NAME	4.3 ± 0.1	5.7 ± 0.2	5.6 ± 0.1	0.05	0.05	1
L-PA	4.6 ± 0.2	6.3 ± 0.2	5.7 ± 0.1	0.08	0.02	4
L-NMMA	5.9 ± 0.2	6.0 ± 0.2	6.5 ± 0.2	0.3	0.8	0.3
LCM;0NIO	6.6 ± 0.1	6.3 ± 0.2	6.2 ± 0.2	3	2	1
L-VNIO	6.15 ± 0.02	6.3 ± 0.1	5.84 ± 0.06	2	0.7	3
L-NIL	6.2 ± 0.1	5.3 ± 0.2	5.06 ± 0.05	14	8	2
S-me-TC	7.2 ± 0.1	8.1 ± 0.6	7.27 ± 0.04	0.9	0.1	7
Isothioureas						
S-me-ITU	6.7 ± 0.2	6.7 ± 0.2	6.5 ± 0.2	2	1	2
S-et-ITU	7.2 ± 0.2	6.8 ± 0.3	7.0 ± 0.1	2	2	0.6
S-aet-ITU	5.2 ± 0.2	4.9 ± 0.1	4.6 ± 0.1	4	2	2
S-et-TFMP-ITU	4.0 ± 0.2	6.1 ± 0.1	4.7 ± 0.1	0.2	0.008	25
1,3 PB-ITU	6.5 ± 0.2	6.0 ± 0.3	4.4 ± 0.1	125	3	40
1,4 PB-ITU	7.6 ± 0.2	7.7 ± 0.1	5.7 ± 0.1	80	0.8	100
Miscellaneous						
1400W	6.9 ± 0.1	5.6 ± 0.1	4.6 ± 0.1	200	20	10
AMT	8.3 ± 0.2	8.4 ± 0.2	7.9 ± 0.2	3	0.8	3
DPI	7.4 ± 0.1	7.17 ± 0.01	6.55 ± 0.03	7	2	4
AG	4.5 ± 0.2	4.3 ± 0.2	3.57 ± 0.04	9	2	5
2-AP	7.3 ± 0.1	7.3 ± 0.2	7.2 ± 0.1	1	1	1

Therefore we decided to reevaluate the inhibitory potencies and selectivities of these compounds at all three isoenzymes. NO synthase activity is normally tested by either measuring methhemoglobin formation from oxyhemoglobin, by using the Griess reaction for measurement of nitrite or by determining the conversion of radiolabeled arginine to citrulline. The oxy-/methhemoglobin assay has been adapted to the microtiter format (Dawson and Knowles, 1998), but is relatively insensitive and susceptible to quenching by colored compounds. The radiolabeled citrulline assay was the most reliable and sensitive assay for our purposes. This procedure normally uses a laborious ion exchange step to retain excess of radiolabeled arginine from generated citrulline. We adapted this assay to a 96-well microtiter format to allow an increased throughput of compounds.

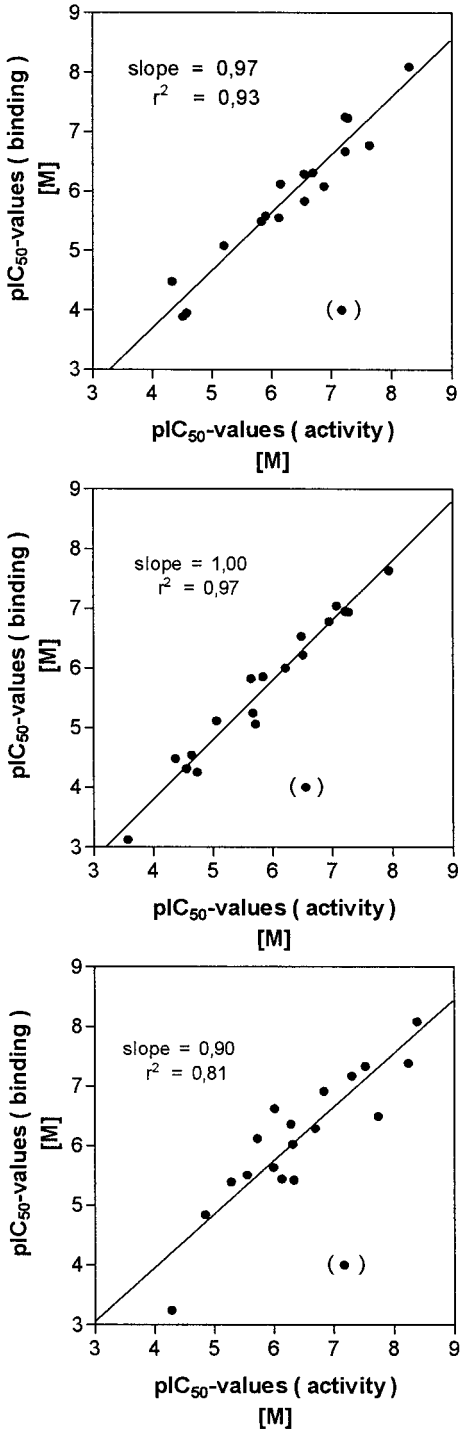

Fig. 6. Correlation of pIC_{50} values for NO synthase inhibitors obtained in radioligand binding experiments with [^{3}H]2-AP with pIC_{50} values obtained in enzyme inhibition experiments at human inducible NO synthase (top), human endothelial NO synthase (middle), and human neuronal NO synthase (bottom). Correlation coefficients (r^2) were 0.93 (top), 0.97 (middle), and 0.81 (bottom). Slope values were 0.97 (top), 1.0 (middle), and 0.90 (bottom). DPI in brackets was omitted from the calculation of correlation coefficients and slope factors.

We used the affinity data obtained from [^{3}H]2-AP binding to elucidate the relationship between inhibitor affinity and inhibition of enzymatic activity obtained under almost identical assay conditions. Only the noncompetitive (with respect to arginine) and flavin site inhibitor DPI showed a marked deviation between enzyme inhibition and radioligand binding data. IC_{50} values of approximately 100 nM were found for enzyme inhibition, while [^{3}H]2-AP binding was inhibited only at concentrations >100 μM. This and the allosteric mode of inhibition of iNOS activity indicates that DPI interacts with a binding site different from the substrate site (most probably the flavine, the BH-4, or NADPH site) and that the allosteric interaction between both sites is weak. Binding of DPI to its binding site does not influence the structure of the arginine substrate site as reflected by the unchanged binding of [^{3}H]2-AP at concentrations where a greater than 90% inhibition of enzyme activity is seen. The inhibitory mechanism of DPI therefore lies most probably in the interference with electron transport from NADPH to arginine, which is necessary for catalytic activity but is not necessary for binding of inhibitors at the substrate site. This is in agreement with reported properties of DPI (Stuehr et al., 1991). For these reasons DPI was omitted from the correlations between binding and catalytic function.

As can be seen from the correlation at the three isoenzymes, the measured inhibitory potency and the calculated selectivity of compounds are solely determined by their affinity to the different isoforms. A somewhat higher scatter in the correlation data is found for the neuronal isoenzyme. The reasons for this are not clear and, if relevant at all, most probably do not result from technical or assay related processes (they are exactly the same for all three isoforms) but may lie in structural peculiarities of the neuronal isoform. It has been shown that the neuronal isoform contains structural motives such as the PIN binding domain (Jaffrey and Snyder, 1996) and the PDZ binding domain (Brenman et al., 1996) not found in the other two isoenzymes. Interaction of PIN or other proteins with these sites may influence enzyme activity and radioligand binding in a slightly different manner.

A close correlation between inhibition of binding and inhibition of enzymatic function can be assumed from kinetic considerations, but for example, a clear split between both parameters is found for phoshodiesterase IV isoforms (Christensen et al., 1996). Aside from the above conclusion, enzyme inhibition data clearly confirm our potencies and selectivity ratios obtained in radioligand competition experiments. Both systems together lead to a profound and comparable characterization of NOS inhibitors with regard to selectivity and potency.

From the inhibitors tested, AMT was clearly the most potent but nonselective NOS inhibitor in both systems. We

could not reproduce the reported iNOS selectivity of this compound seen by others.(Nakane et al., 1995). By far the most i-/eNOS-selective compound is 1400W with a selectivity ratio >100 (enzyme inhibition data).

Varying potencies and selectivities are found for the different isothiourea derivatives with some representatives showing high n- versus eNOS selectivity. Our results clearly show that S-aet-ITU, S-et-ITU, and S-me-ITU, three commonly used isothiourea derivatives, are almost nonselective and should not be used in experiments aiming at a selective inhibition of iNOS (Gundersen et al.,1997; Wang et al., 1998; Chen et al., 1999). The observed effects of S-me-ITU on blood pressure cited by Moore (Moore and Handy, 1997) are easily explained by eNOS inhibition due to the nonselective nature of this inhibitor.

For L-NMMA a weak selectivity is observed in both assay systems in favor of the endothelial isoform. Clinical trials with this compound have been stopped due to an increase in mortality in the treatment group. The weak eNOS selectivity might in part explain the outcome of that study.

In our hands, aminoguanidine, although iNOS-selective, is an extremely weak iNOS inhibitor in vitro and complete inhibition of iNOS could only be expected at high, almost millimolar concentrations. However, we could not exclude the possibility of improved potency in vivo due to pharmacokinetic processes. Our aminoguanidine data are in agreement with those reported by Moore et al. (1996).

The potencies of NOS inhibitors at the isolated enzymes do not exactly reflect potency at the cellular level or in isolated organ systems. With regard to potency in general, there is a rough congruence between data reported in this article and data from cellular systems or isolated organ models, in that AMT is the most potent and aminoguanidine the least potent compound (Faraci et al., 1996; Eltze et al., 1998, 1999). For the other compounds, the rank order of potency at cellular or isolated organ systems is similar but not identical with the values obtained at isolated enzymes. A complete congruence cannot be expected as the cellular uptake of different structural classes of compounds varies due to their very different physicochemical properties and/or their affinity to cellular cationic transport proteins involved in the uptake of charged amino acids and inhibitors. Nevertheless, we found an excellent agreement between our selectivity ratios reported in this study and the selectivity ratios determined in isolated organ models for iNOS (LPS-treated rat aorta), nNOS (rabbit corpus cavernosum, rat gastric fundus), and eNOS (rat aorta) for a limited set of the above-mentioned NOS inhibitors (Eltze et al., 1998, 1999).

Taken together these data identify [^{3}H]2-AP as a valuable tool to investigate the binding parameters of NOS inhibitors at the arginine substrate binding site of all three isoforms. Arginine site competitive or allosteric inhibitors such as DPI can easily be identified and characterized. Furthermore, by using radioligand binding experiments, catalytically inactive fragments of NO synthases such as the oxygenase domains successfully used in crystallization studies (Crane et al., 1997; Raman et al., 1998; Fischmann et al., 1999) can be characterized with respect to the preservation of the structural integrity of the binding site. Also binding of inhibitors in the absence of cosubstrates such as NADPH and structural changes in the binding site can be monitored. The omission of NADPH resulted in very similar K_D values of [^{3}H]2-AP binding demonstrating that the occupation of the NADPH binding site does not influence the structure of the substrate binding site. Another important use of this technique lies in the evaluation of consequences of amino acid exchange in the arginine binding site on substrate or inhibitor binding parameters.

References

Alderton WK, Boyhan A and Lowe N (1998) Nitroarginine and tetrahydrobiopterin binding to the heme domain of neuronal nitric oxide synthase using a scintillation proximity assay. *Biochem J* **332:**195–201.

Billiar T and Harbrecht BG (1997) Resolving the nitric oxide paradox in acute tissue damage. *Gastroenterology* **113:**1405–1407.

Brenman JE, Chao DS, Gee-SH, McGee AW, Craven E, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC and Bredt DS (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. *Cell* **84:**757–767.

Chen LW, Hsu CM, Wang JS, Chen JS and Chen SC (1998) Specific inhibition of iNOS decreases the intestinal mucosa peroxynitrite level and improves the barrier function after thermal injury. *Burns* **24:**699–705.

Cheng Y and Prusoff WH (1973) Relationship between the inhibitory constant (KI) and the concentration of an inhibitor that causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Christensen SB, DeWolf WE, Ryan MD and Torphy TJ (1996) Molecular aspects of inhibitor interaction with PDE4, in *Phosphodiesterase Inhibitors* (Schudt C, Dent G and Rabe KF eds) pp185–207, Academic Press Ltd., London.

Cochran JB, Genovese F, Ogura S, Teti G and Cook JA (1999) Effect of nitric oxide donors and nitric oxide synthase inhibitors in neonatal rat endotoxic shock. *Biochem Pharmacol* **15:**687–691.

Cowart M, Kowaluk EA, Daanen JF, Kohlhaas KL, Alexander KM, Wagenaar FL and Kervin JF (1998) Nitroaromatic amino acids as neuronal nitric oxide inhibitors. *J Med Chem* **41:**2636–2642.

Crane BR, Arvai AS, Gachhui R, Wu C, Gosh DK, Getzoff ED, Stuehr DJ and Tainer JA (1997) The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science (Wash DC)* **278:**425–431.

Dawson J and Knowles RG (1998) A microtiter-plate assay of human NOS-isoforms, in *Methods in Molecular Biology* (Titheradge MA, ed) pp 237–242, Humana Press, New Jersey.

Eltze M, Grebe T, König H, Haas S, Mirau B, Baur I, Klein T, and Boer R (1999) The inducible:neuronal nitric oxide synthase (i/nNOS) selectivity of NOS inhibitors in rat aorta versus rabbit corpus cavernosum or rat gastric fundus correlates with human i- and nNOS. *Naunyn-Schmiedeberg's Arch Pharmacol* **359:**R48.

Eltze M, Klein T, Grebe T, Haas S, Mirau B and Boer R (1998) Potency and selectivity of inhibitors of inducible and endothelial nitric oxide synthase in rat aorta correlate with human isoenzymes. *Naunyn-Schmiedeberg's Arch Pharmacol* **357:**R49.

Faraci WS, Nagel AA, Verdries KA, Vincent LA, Hong X, Nichols LE, Labasi JM, Salter ED and Pettipher ER (1996) 2-Amino-4-methylpyridine as a potent inhibitor of inducible NO synthase activity in vitro and in vivo. *Br J Pharmacol* **119:**1101–1108.

Fischmann TO, Hruza A, Niu XD, Fossetta JD, Lunn CA, Dolphin E, Prongay AJ, Reichert P, Lundell DJ, Narula SK and Weber PC (1999) Structural characterization of nitric oxide synthase isoforms reveals striking active site conservation. *Nat Struct Biol* **6:**233–242.

Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA and Garvey EP (1994) Potent and selective inhibition of human nitric oxide synthases. *J Biol Chem* **269:**26677–26683.

Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BRJ and Knowles RG (1997) 1400W is a slow, tight binding and highly selective inhibitor of inducible nitric oxide synthase in vitro and in vivo. *J Biol Chem* **272:**4959–4963.

Garvey EP, Oplinger JA, Tanouri GJ, Sherman PA, Fowler M, Marshall S, Harmon MF, Paith JE and Furfine ES (1994) Potent and selective inhibition of human nitric oxide synthases. *J Biol Chem* **269:**26669–26676.

Gorren ACF, List BM, Schrammel A, Pitters E, Hemmens B, Werner E, Schmidt K and Mayer B (1996) Tetrahydrobiopterin-free neuronal nitric oxide synthase: Evidence for two identical highly anticooperative pteridine binding sites. *Biochemistry* **35:**16735–16745.

Griffith OW and Gross SS (1996) Inhibitors of nitric oxide synthases, in *Methods in Nitric Oxide Research* (Feelisch M and Stamler JS eds) pp 187–208, John Wiley & Sons Ltd., New York.

Grover R, Zaccardell D, Colice G, Guntupalli K, Watson D and Vincent JL (1999) An open-label dose escalation study of the nitric oxide synthase inhibitor NG-methyl-L-arginine hydrochloride (546C88), in patients with septic shock. *Crit Care Med* **27:**913–922.

Gundersen Y, Corso CO, Leiderer R, Dörger M, Lilleaasen P, Aasen AO and Messmer K (1997) Use of selective and nonselective nitric oxide synthase inhibitors in rat endotoxemia: Effects on hepatic morphology and function. *Shock* **8:**368–372.

Hagen TJ, Bergmanis AA, Kramer SW, Fok KF, Schmelzer AE, Pitzele BS, Swenton L, Jerome GM, Kornmeier CM, Moore WM, Branson LF, Connor JR, Manning PT, Currie MG and Hallinan EA (1998) 2-Iminopyrrolidines as potent and selective inhibitors of human inducible nitric oxide synthase. *J Med Chem* **41:**3675–3683.

Hobbs AJ, Higgs A and Moncada S (1999) Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu Rev Pharmacol Toxicol* **39:**191–220.

Jaffrey SR and Snyder SH (1996) PIN: An associated protein inhibitor of neuronal nitric oxide synthase. *Science (Wash DC)* **274:**774–777.

Kilbourn RG, Szabo C and Traber DL (1997) Beneficial versus detrimental effects of

nitric oxide synthase inhibitors in circulatory shock: Lessens learned from experimental and clinical studies. *Shock* **7:**235–246.
Klatt P, Schmidt K, Brunner F and Mayer B (1994) Inhibitors of brain nitric oxide synthase. *J Biol Chem* **269:**1674–1680.
Macdonald JE (1996) Nitric oxide synthase inhibitors. *Annu Rep Med Chem* **31:**221–230.
Michel T, Xie QW and Nathan C (1996) Molecular biological analysis of nitric oxide synthases, in *Methods of Nitric Oxide Research* (Feelisch M and Stamler JS eds) pp 161–175, John Wiley & Sons Ltd., New York.
Misko PM, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, McDaniel ML, Williamson JR and Currie MG (1993) Selective inhibition of inducible nitric oxide synthase. *Eur J Pharmacol* **233:**119–125.
Moore PK and Handy RLC (1997) Selective inhibitors of neuronal nitric oxide synthase—is no NOS really good NOS for the nervous system? *TIPS* **18:**204–211.
Moore WM, Webber RK, Fok KF, Jerome GM, Kornmeier CM, Tjong FS and Currie MG (1996) Inhibitors of human nitric oxide synthase isoforms with the carbamidine moiety as a common structural element. *Bioorg Med Chem* **4:**1559–1564.
Moore WM, Webber RK, Jerome GM, Tjong FS, Misko TP and Currie MG (1994) L-N^6-(1-iminoethyl)lysine: A selective inhibitor of inducible nitric oxide synthase. *J Med Chem* **37:**3886–3888.
Murad F (1998) Nitric oxide signalling: Would you believe that a simple free radical could be a second messenger? *Recent Prog Horm Res* **53:**43–59.
Nakane M, Klinghofer V, Kuk JE, Donnelly JL, Budzik GP, Pollock JS, Basha F and Carter GW (1995) Novel potent and selective inhibitors of inducible nitric oxide synthase. *Mol Pharmacol* **47:**831–834.
Narayanan K, Spack L, McMillan K, Kilbourn RG, Hayward MA, Masters BS and Griffith OW (1995) S-Alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity in vivo. *J Biol Chem* **270:**11103–11110.
Raman CS, Li H, Martasek P, Kral V, Masters BS and Poulos TL (1998) Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pteridine function involving a novel metal center. *Cell* **95:**939–950.
Resta TC, O'Donaughy TL, Earley S, Chicoine LG and Walker BR (1999) Unaltered vasoconstrictor responsiveness after iNOS inhibition in lungs from chronically hypoxic rats. *Am J Physiol* **276:**L122–130.
Schwartz D, Mendonca M, Schwartz I, Xia YX, Satriano J, Wilson CB and Blantz RC (1997) Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. *J Clin Invest* **100:**439–448.
Shearer BG, Lee S, Franzmann KW, White HAR, Sanders DCJ, Kiff RJ, Garvey ED and Furfine ES (1997) Conformationally restricted arginine analogues as inhibitors of human nitric oxide synthase. *Bioorg Med Chem Lett* **7:**1763–1768.
Southan GJ, Szabo C and Thiemermann C (1995) Isothioureas: Potent inhibitors of nitric oxide synthases with variable isoform selectivity. *Br J Pharmacol* **114:**510–516.
Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R and Nathan CF (1991) Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenylene-iodonium and its analogs. *FASEB J* **5:**98–103.
Titheradge MA (1999) Nitric oxide in septic shock. *Biochim Biophys Acta* **1411:**437–455.
Wang D, Yang XP, Liu YH, Carretero OA and LaPointe MC (1999) Reduction of myocardial infarct size by inhibition of inducible nitric oxide synthase. *Am J Hypertens* **12:**174–182.
Wang Y, Lawson JA and Jaeschke H (1998) Differential effect of 2-aminoethylisothiourea, an inhibitor of the inducible nitric oxide synthase, on microvascular blood flow and organ injury in models of hepatic ischemia/reperfusion and endotoxemia. *Shock* **10:**20–25.
Wolff DJ, Lubeskie A, Gauld DS and Neulander MJ (1998) Inactivation of nitric oxide synthases and cellular nitric oxide formation by N^6-imino-L-lysine and N^5-iminoethyl-L-ornithine. *Eur J Pharmacol* **350:**325–334.

Send reprint requests to: Dr. Rainer Boer, Byk Gulden Pharmaceuticals, Byk Gulden-Str. 2, D-78467 Konstanz, Germany. E-mail: rainer.boer@byk.de