Nitric-oxide synthases (NOS, EC 1.14.13.39) are heme-containing enzymes that catalyze the formation of nitric oxide from L-Arg. General cytochrome P-450 inhibitors and cytochrome P-450 isoform-selective substrates and inhibitors were used to characterize the activity of recombinant human inducible NOS (iNOS). Classical cytochrome P-450 ligands such as the mechanism-based inactivator 1-aminobenzotriazole did not inhibit iNOS. Of a panel of 30 human cytochrome P-450 isoform-selective substrates and inhibitors, only chlorzoxazone, a cytochrome P-450 2E1 (CYP2E1) substrate, showed any significant inhibition of iNOS activity. Chlorzoxazone was not a substrate for iNOS but was a potent competitive inhibitor with respect to L-Arg with $K_i = 3.3 \pm 0.7 \mu M$. The binding of chlorzoxazone to iNOS and human and rat liver microsomal cytochrome P-450 induced a high spin, type I spectra, which was reversed by imidazole. Although the binding of chlorzoxazone to iNOS and its inhibition of iNOS activity suggest some similarity between iNOS and CYP2E1 activity, other CYP2E1 substrates and inhibitors including zoxazolamine were not inhibitors of iNOS. Overall, iNOS activity is distinctly different from the major cytochrome P-450 enzymes in human liver microsomes.

Although the NOS isoforms catalyze the same reaction and require the same set of cosubstrates and cofactors, the primary sequences for the three mammalian enzymes, for example, are quite disparate except within defined regions associated with cofactor binding such as FAD, FMN, and NADPH. Two of the mammalian NOS isoforms are constitutively expressed, neuronal NOS (nNOS) and endothelial NOS (eNOS), and the third, inducible NOS (iNOS), is induced under cytokine stimulation within a large variety of cells. Nathan (6) and others (7, 8) have estimated that the primary sequences of the mammalian NOS isoforms average $53 \pm 2\%$ homology within a species. The conserved cofactor binding sites are contained within two separate structural domains of each subunit of homodimeric NOS (1, 2, 8). The N-terminal domain contains the oxygenase activity and binds heme, tetrahydrobiopterin, and the substrate L-Arg. The C-terminal domain has reductase activity (diaphorase) and binds FAD, FMN, and the nicotinamide cosubstrate. The calcium-dependent regulatory protein, calmodulin, binds within a region between the two domains and is thought to regulate electron transfer between the two NOS domains (9). For iNOS, calmodulin is tightly associated with the enzyme and represents a distinguishing feature for the inducible versus the constitutively expressed mammalian NOS isoforms (10, 11). The interaction between these two catalytic domains means that NOS isoforms have within a single protein the complete catalytic machinery of the two-protein microsomal electron transfer system, reminiscent of the bacterial fatty acid monooxygenase cycytochrome P-450BM-3 (12, 13).

The heme groups of all three mammalian NOS isoforms have been shown to be cytochrome P-450-type hemes by their CO difference spectra (14–21). Unlike most cytochrome P-450s, however, the heme groups of these NOS isoforms are high spin, yet both iNOS and nNOS can bind imidazole to give low spin heme-imidazole complexes (18, 21). The application of resonance Raman scattering (22) and magnetic circular dichromism (23) spectroscopic techniques for studying the heme of rat nNOS further indicate that the heme environment for NOS is distinct from other heme-containing enzymes such as cytochrome P-450CAM or fungal chloroperoxidase.

In order to further investigate the NOS heme domain and its binding specificity compared with that of other human P-450-containing enzymes, we examined a series of human cytochrome P-450 isoform selective and general substrates and inhibitors as potential inhibitors of recombinant human iNOS. We found that iNOS was poorly inhibited by compounds that are general cytochrome P-450 inhibitors, including 1-aminobenzotriazole. Isoform-specific cytochrome P-450 substrates and inhibitors were not recognized by iNOS, except for chlorzoxazone, a cytochrome P-450 2E1 (CYP2E1) substrate (24, 25).

The mechanism of inhibition of iNOS by chlorzoxazone was
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examined as well as the binding of chloroxazone to human and rat hepatic microsomal P-450s.

MATERIALS AND METHODS

Reagents and Proteins—N-Tris(hydroxymethyl)methyl-2-aminothiane-sulfonic acid (TES), L-arginine hydrochloride, NADPH, FAD, FMN, bovine serum albumin, bovine calmodulin, acetalaminophen (phenacetin), 1-aminobenzotriazole, NADP, β-naphtylamine, benzphetamine hydrochloride, caffeine, dapsone, dextromethorphan hydrobromide, monohydrate, disulfiram (tetraethyl-thiuramdisulfide), enoxacin, erythromycin, hemin, ketoconazole, lubrol PX, metoxypenamhydrochloride, (+)-naringenin, nifedipine, perphenazine, tacrine, tolbutamide, and warfarin, sodium salt were purchased from Sigma. 7,8-Benzoflavone (β-naphthaflavone), chloroxazone (5-chloro-2-hydroxybenzoxazole), diethylthiocarbamic acid, sodium salt, ellipticine, imidazole, 4-methylpyrazole, phenytoin (5,5-diphenylhydantoin), quinidine, and zoxazolamine were purchased from Aldrich. 6(5)-flavin mononucleotide (FMN) was purchased from Sigma. 7,8-Benzoflavone (β-naphthaflavone), chloroxazone (5-chloro-2-hydroxybenzoxazole), diethylthiocarbamic acid, sodium salt, ellipticine, imidazole, 4-methylpyrazole, phenytoin (5,5-diphenylhydantoin), quinidine, and zoxazolamine were purchased from Aldrich. 6(5)-flavin mononucleotide (FMN) was purchased from Sigma.

Rat liver microsomes were of 13.5 mg/ml, P-450 content of 0.3 nmol/mg) was a gift from Dr. Judy Rauch (Agouron Institute, La Jolla, CA). Human liver microsomes (HLM—purchased from Amersham Corp. Troleandomycin was purchased from Amersham Corp.. Troleandomycin was purchased from Amersham Corp.

Purification of iNOS was performed by immunoaffinity chromatography as described elsewhere (27). Cell lysates containing recombinant human constitutive NOS isoforms (nNOS and eNOS) were desalted and used without further purification. Human liver microsomes (HLM—purchased from Amersham Corp. Recombinant human NOS isoforms were cloned and expressed in the SB-baculovirus expression system according to a recent report (26).

RESULTS

Effects of general cytochrome P-450 inhibitors against iNOS—

A series of general cytochrome P-450 inhibitors and isoform-selective cytochrome P-450 substrates and inhibitors were tested as potential inhibitors of recombinant human iNOS. The evaluation of general cytochrome P-450 inhibitors included reversible inhibitors and mechanism-based inactivators such as 1-aminobenzotriazole (32, 33). All of these compounds have been reported to inhibit or inactivate many cytochrome P-450s and therefore are not regarded as selective toward any single class of cytochrome P-450. In addition to the use of cytochrome P-450-specific antisera (34), the identification of a particular cytochrome P-450 activity can be determined by using a panel of compounds whose inhibitory activity or metabolism is associated with specific cytochrome P-450 isoforms, e.g. 1A1 or 2E1 (30, 35–37). We have examined both general cytochrome P-450 inhibitors and a series of cytochrome P-450 isoform selective substrates and inhibitors against iNOS in order to characterize its activity compared with human cytochrome P-450s. Each compound was tested at 0.1 mM against 1 μM L-Arg either competitively or by first preincubating the compound with iNOS for 15 min in the presence of NADPH. Preincubation of compounds with enzyme in the presence of NADPH was used to evaluate possible mechanism-based inactivation of iNOS.

Evaluation of General Cytochrome P-450 Inhibitors against iNOS—

Human iNOS activity was not inhibited by any of the general cytochrome P-450 enzymes tested at 0.1 mM (Table I) even after preincubation with the enzyme in the presence of NOS cofactors and NADPH. 1-Aminobenzotriazole is a NADPH-dependent mechanism-based inactivator of many cytochrome P-450s and inactivates by alkylation of the heme group (38). This compound had no effect on iNOS activity. For hepatic microsomal cytochrome P-450s, the concentration of 0.1 mM 1-aminobenzotriazole was reported to be sufficient for the inactivation of enoyl activity (39). Its inhibitory activity is specific for cytochrome P-450-containing monoxygenases and does not affect other hemeproteins such as cytochrome b5 (40).

Likewise, the mechanism-based cytochrome P-450 inactivator benzylhydrazine (41) had no detectable inhibition of iNOS. The P-450 ligands cyanide and azide were poor inhibitors of iNOS at 0.1 mM. iNOS activity was unaffected by NaNO2 (up to 10 mM); however, KCN inhibited iNOS activity by greater than 75% at 5 mM. Apparently, iNOS binds low molecular weight anions

\[ y = \frac{V_{max} \cdot S}{K_m + \frac{1}{(V_{max} + 1/K_i)}} \]

\[ K_{d} = \frac{V_{max} \cdot S}{(1 + I/K_i)} \]

\[ K_{m} \] is the Michaelis constant, \( I \) is the inhibitor concentration, and \( K_{d} \) is the apparent inhibitor dissociation constant. The equilibrium dissociation constant, \( K_d \), for chloroxazone with iNOS was calculated after correction for the presence of imidazole, according to \( K_d = \frac{K_{d}}{1 + \frac{1}{(K_m)}} \), where \( I \) is the concentration of imidazole and \( K_m \) is the imidazole dissociation constant.

The inhibition of nitric-oxide synthase by chloroxazone was determined by fitting to the competitive inhibition equation:

\[ V = \frac{V_{max} \cdot S}{K_m + S} \]

\[ K_m \] is the Michaelis constant, \( S \) is the L-Arg concentration, \( V \) is the velocity, and \( V_{max} \) is the maximum velocity. The dissociation constant, \( K_i \), for chloroxazone concentration. IC_{50} and \( K_i \) determinations were made in either duplicate or triplicate and are given as average values with standard deviations. Apparent binding constants were determined from the difference spectra of the enzyme-inhibitor complex by fitting the change in absorbance to \( y = \frac{a}{b} (K_m + b) \).
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TABLE II
Effect of cytochrome P-450-selective substrates and inhibitors against iNOS

| CYP1A1 | Relative activity<sup>a</sup> |
|--------|-----------------------------|
| Inhibitors | 0.94 |
| Ellipticine | | |
| 7,8-Benzoflavone | 0.93 |

| CYP2C8, CYP2C9, CYP2C18 | Relative activity<sup>a</sup> |
|--------------------------|-----------------------------|
| Substrates |  |
| Benzphetamine | 1.00 |
| Phenytoin | 0.79 |
| Tolbutamide | 1.00 |
| Warfarin | 1.26 |
| Inhibitors |  |
| Sulfaphenazole | 0.97 |

| CYP2E1 | Relative activity<sup>a</sup> |
|--------|-----------------------------|
| Substrates |  |
| Acetaminophen | 1.01 |
| Chlorzoxazone | 0.10 |
| Zoxazolamine | 0.98 |
| Inhibitors |  |
| DDCA | 0.99 |
| Disulfiram | 0.96 |
| 4-Methyl pyrazole | 0.89 |

| CYP2A12 | Relative activity<sup>a</sup> |
|---------|-----------------------------|
| Substrates |  |
| Caffeine | 0.96 |
| Phenacetin | 0.94 |
| Tacrine | 0.97 |
| Inhibitors |  |
| Enoxacin | 1.01 |
| Furafylline | 0.88 |

| CYP2D6 | Relative activity<sup>a</sup> |
|--------|-----------------------------|
| Substrates |  |
| Debrisoquine | 0.92 |
| Dextromethorphan | 1.14 |
| Metoxophenate | 0.88 |
| Perphenazine | 0.74 |
| Propranolol | 0.85 |
| Inhibitors |  |
| Quinidine | 0.89 |

| CYP3A4 | Relative activity<sup>a</sup> |
|--------|-----------------------------|
| Substrates |  |
| Dapoxetine | 0.92 |
| Erythromycin | 0.93 |
| Nifedipine | 0.89 |
| Inhibitors |  |
| Ketoconazole | 1.01 |
| (+)-Naringenin | 1.05 |
| Troloxemocin | 0.89 |

<sup>a</sup> Determined in duplicate from relative values of L-[3H]citrulline formation at 0.1 mM compound against 1 μM L-Arg.
<sup>b</sup> DDCA, diethylthiocarbamic acid.

very weakly. Together, these results demonstrate the uniqueness of iNOS activity compared with human cytochrome P-450 enzymes and most likely reflects the affinity of iNOS for cationic groups such as L-Arg rather than hydrophobic aromatics and anions.

Evaluation of Cytochrome P-450 Isoform Selective Substrates and Inhibitors against iNOS—The identity of a particular hepatic cytochrome P-450 activity can be determined by the use of cytochrome P-450 isoform selective substrates and inhibitors (30, 35–37). Because these compounds are representative of substrates and inhibitors for the major human cytochrome P-450 families, they may be useful for determining if iNOS activity is similar to any human cytochrome P-450 activity. A panel of 30 compounds composed of human cytochrome P-450 isoform selective substrates and inhibitors were tested as inhibitors of iNOS. Table II summarizes the effect of these compounds against iNOS L-citrulline formation using 1 μM L-Arg. Only one compound, chlorzoxazone (5-chloro-2-hydroxybenzoxazolone) (Fig. 1) showed any significant inhibition of iNOS. Chlorzoxazone is a substrate for CYP2E1 (24, 25), undergoing 6-hydroxylation, although some minor 6-hydroxylation has also been reported with cytochrome P-450 1A1 (42–44). However, two other CYP2E1 substrates, zoxazolamine, structurally similar to chlorzoxazone (see Fig. 1), and acetaminophen, did not inhibit iNOS. Moreover, the CYP2E1 inhibitors: disulfiram, 4-methylpyrazole, and diethyldithiocarbamic acid, were not inhibitors of iNOS. Whereas the inhibition of iNOS by chlorzoxazone suggests that iNOS may have similar substrate affinity as CYP2E1 (or cytochrome P-450 4A1), it is unlikely because none of the other CYP2E1 (or cytochrome P-450 4A1) selective compounds were inhibitors of iNOS. Also it is noteworthy that debrisoquine, which contains a guanidino-group, was not an inhibitor of iNOS even though debrisoquine can be hydroxylated to N<sup>2</sup>-hydroxy-debrisoquine by rat liver microsomal P-450 (45). Overall, the lack of inhibitory activity of general and isoform-selective cytochrome P-450 substrates and inhibitors toward iNOS demonstrates that iNOS substrate and inhibitor binding is unique from that of human cytochrome P-450 enzymes.

Inhibition of iNOS by Chlorzoxazone—Chlorzoxazone was titrated against 1 μM L-Arg and inhibited iNOS L-citrulline production with an IC<sub>50</sub> value of 6.9 ± 1.8 μM. The inhibition of iNOS by chlorzoxazone was competitive with respect to L-Arg with K<sub>I</sub> = 3.3 ± 0.7 μM (Fig. 2). 6-Hydroxy-chlorzoxazone (see Fig. 1) was a less potent inhibitor of iNOS (relative activity = 0.52 ± 0.1 μM), but zoxazolamine did not inhibit iNOS even at 0.5 mM. Chlorzoxazone was also tested against recombinant human nNOS and eNOS as a measure of NOS isoform selectivity. IC<sub>50</sub> values for the inhibition of nNOS and eNOS by chlorzoxazone were 12.9 ± 0.8 and 8.5 ± 0.5 μM, respectively, against 1 μM L-Arg. Chlorzoxazone inhibited all three human NOS isoforms with nearly equal potency.

Although CYP2E1 catalyzes the 6-hydroxylation of chlorzoxazone, the likelihood of iNOS catalyzing the hydroxylation of a benzoxazole is remote because the substrate specificity of NOS isoforms is reported to be restricted to close L-Arg structural analogs such as L-homoarginine or N<sup>2</sup>-hydroxy-L-arginine (1, 3, 46). Although unlikely, this possibility was examined by incubating iNOS with chlorzoxazone under normal assay conditions with NADPH but without L-Arg. As a control we also incubated chlorzoxazone and NADPH with human hepatic microsomal P-450. The products were then analyzed by HPLC using authentic chlorzoxazone (6.4 min) and 6-hydroxy-chlorzoxazone (4.1 min) as standards. Fig. 3 shows representative HPLC traces for the products of chlorzoxazone with hepatic microsomal P-450 and iNOS. 6-Hydroxy-chlorzoxazone (4.1 min) was produced from chlorzoxazone (6.4 min) after incubation with hepatic microsomal P-450 (Fig. 3A). However, there was no detectable 6-hydroxy-chlorzoxazone or other products formed after 5 h of incubation of 0.5 mM chlorzoxazone with iNOS (at an enzyme concentration 10 times greater than for normal assays) (Fig. 3B). These results indicate that chlorzoxazone does not undergo any significant 6-hydroxylation by iNOS.
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**Reversible Type I Binding of Chlorzoxazone to iNOS—**Because the inhibition of iNOS by chlorzoxazone is competitive with respect to L-Arg, chlorzoxazone is most likely binding within the heme domain. Perturbation of the heme optical spectra upon ligand binding has been used to characterize the mode of binding of substrates and inhibitors within the active sites of hemeproteins (47–49). The binding of various compounds to hemeproteins can induce low spin or high spin transitions of the heme iron, which accounts for the observed shifts in the heme optical spectra. For membrane-bound cytochrome P-450s, the optical heme spectra can be complicated by the presence of detergents and other factors that can affect the spin state (49). Yet this method has been very useful for determining the binding of substrates and cofactors to soluble NOS isoforms (2, 18, 19). The iNOS substrate L-Arg and 1-Arg analog inhibitors such as N⁵-methyl-L-arginine induce type I perturbations of the NOS heme spectra (18, 21, 50) and therefore are termed type I ligands. Type I ligands bind near the heme group but do not coordinate directly to the heme iron, and their binding induces a high spin heme-iron transition (48, 49). Zoxazolamine, for instance, induces a type I spectral shift of the optical spectra of rat hepatic microsomal P-450 (51). Based on the structural similarity of chlorzoxazone and zoxazolamine, we predicted that chlorzoxazone might also bind as a type I ligand to iNOS. However, the structure of chlorzoxazone is not very similar to L-Arg but more closely resembles the aromatic nitrogen-containing NOS inhibitors: 1-phenylimidazole (21, 52–55) and 7-nitroindazole (56, 57). Imidazole and 1-phenylimidazole are type II ligands of iNOS, meaning that they bind as 6-axial ligands of the iron-heme group and induce a low spin heme-iron transition (21). These compounds have been reported as competitive inhibitors of iNOS with respect to L-Arg (21). The reversible binding spectra of 7-nitroindazole to iNOS has not yet been reported. Our attempts to measure the optical spectra of iNOS with 7-nitroindazole have been complicated by the strong absorbance of 7-nitroindazole, which interferes with the heme Soret band. In order to further characterize the nature of the binding of chlorzoxazone to iNOS, we measured the shift in the optical spectra induced by its addition to iNOS, hepatic cytochrome P-450s, and hemin.

We first examined the effect of increasing amounts of chlorzoxazone on the optical spectra of iNOS. This was performed by initially converting the native high spin heme of iNOS to low spin by the addition of the type II ligand, imidazole. The addition of chlorzoxazone then reversed the type II imidazole-induced spectra of iNOS, to give a type I difference spectra, which accounts for the observed shifts in the heme optical spectra. For membrane-bound cytochrome P-450s, the optical heme spectra can be complicated by the presence of detergents and other factors that can affect the spin state (49). Yet this method has been very useful for determining the binding of substrates and cofactors to soluble NOS isoforms (2, 18, 19). The iNOS substrate L-Arg and 1-Arg analog inhibitors such as N⁵-methyl-L-arginine induce type I perturbations of the NOS heme spectra (18, 21, 50) and therefore are termed type I ligands. Type I ligands bind near the heme group but do not coordinate directly to the heme iron, and their binding induces a high spin heme-iron transition (48, 49). Zoxazolamine, for instance, induces a type I spectral shift of the optical spectra of rat hepatic microsomal P-450 (51). Based on the structural similarity of chlorzoxazone and zoxazolamine, we predicted that chlorzoxazone might also bind as a type I ligand to iNOS. However, the structure of chlorzoxazone is not very similar to L-Arg but more closely resembles the aromatic nitrogen-containing NOS inhibitors: 1-phenylimidazole (21, 52–55) and 7-nitroindazole (56, 57). Imidazole and 1-phenylimidazole are type II ligands of iNOS, meaning that they bind as 6-axial ligands of the iron-heme group and induce a low spin heme-iron transition (21). These compounds have been reported as competitive inhibitors of iNOS with respect to L-Arg (21). The reversible binding spectra of 7-nitroindazole to iNOS has not yet been reported. Our attempts to measure the optical spectra of iNOS with 7-nitroindazole have been complicated by the strong absorbance of 7-nitroindazole, which interferes with the heme Soret band. In order to further characterize the nature of the binding of chlorzoxazone to iNOS, we measured the shift in the optical spectra induced by its addition to iNOS, hepatic cytochrome P-450s, and hemin.

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**Reversible Binding of Chlorzoxazone to Microsomal Cytochrome P-450—**Because chlorzoxazone is a CYP2E1 substrate, we were also interested in determining its reversible mode of binding to human and rat liver microsomal cytochrome P-450s. Human and rat hepatic microsome preparations containing chlorzoxazone hydroxylating activity were analyzed by optical spectroscopy and control sample without iNOS. Chromatograms are representative of duplicate experiments.

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2 S. K. Grant, unpublished results.
teract with the heme iron through the ring nitrogen lone pair electrons (37, 59). It is this interaction for imidazole that induces the low spin heme iron transition with its characteristic type II optical spectra (37, 49). Structurally, chlorzoxazone might be expected also to bind as a type II ligand, but this is not what we observed for iNOS and the hepatic cytochrome P-450s examined here. This raises the question as to whether chlorzoxazone has an intrinsically weak bond with heme iron that makes its ligation undetectable by optical spectroscopy under these conditions. To evaluate this possibility we used chlorzoxazone and imidazole binding to hemin as a model for their binding to hemeproteins. As expected, imidazole caused a type II spectral shift of human hepatic microsomal P-450 (1.0 mg/ml) with difference spectra absorbance maxima at 410 nm and minima at 424 nm and an isosbestic point at 418 nm. B, a type I spectra (difference spectra, $A_{412-425}$) was also observed upon addition of chlorzoxazone (0.2 mM) to rat hepatic microsomal P-450 (0.8 mg/ml) with difference spectra absorbance maxima at 412 nm and minima at 425 nm with an isosbestic point at 420 nm.
Fig. 6. Type II difference spectra for hemin with chlorzoxazone. The addition of increasing amounts of chlorzoxazone to hemin (6.7 μM) induced a type II spectral shift with absorbance maxima at 438 nm and minima at 370 nm and an isosbestic point at 410 nm. Representative spectra are shown: a–c, 0.1, 0.2, 0.4, 1.0, and 2.0 mM chlorzoxazone, respectively. Insert, reversible binding saturation curve for absorbance change (ΔA$_{370}$) versus chlorzoxazone concentration.

cules of either imidazole or chlorzoxazone to both sides of the porphyrin. This is different from what would be expected for iNOS and cytochrome P-450 enzymes where only a single molecule of chlorzoxazone or imidazole can bind axially because a thiolate ligand is also bound (16). These results demonstrate that chlorzoxazone can ligate with hemin to form a low spin heme iron complex. Therefore, although chlorzoxazone induces a low spin spectral shift for hemin, it binds specifically as a type I (high spin) ligand for iNOS and human and rat liver cytochrome P-450s.

**DISCUSSION**

Human cytochrome P-450s have a conserved heme binding motif but overall have disparate primary sequences (36, 37, 60–62). Yet despite the sequence diversity among cytochrome P-450s, these enzymes still maintain the structural and catalytic requirements for NADPH-dependent hydroxylation of their substrates. This conservation of catalytic motifs for a family of enzymes with diverse primary sequences is even more dramatic for the NOS isoforms. Indeed, the cofactor requirements for NOS activity makes the NOS family of enzymes one of the most complex of the hemeproteins. Our results further demonstrate that iNOS activity is distinct from human cytochrome P-450 enzymes as evaluated by a panel of general and isoform-selective cytochrome P-450 substrates and inhibitors. Based on the results of 30 isoform-selective compounds, iNOS activity would appear to be more closely related to CYP2E1 activity, because: (i) chlorzoxazone (a CYP2E1 substrate) was the only compound from this collection identified as a potent, competitive inhibitor of iNOS and (ii) chlorzoxazone is a type I ligand of iNOS similar to that for human and rat hepatic cytochrome P-450s.

Chlorzoxazone is a skeletal muscle relaxant with a long history of use in humans (24, 70). Its pharmacokinetic profile has been studied extensively, and it is metabolized primarily by CYP2E1 (24, 25, 42–44). Several researchers have suggested that chlorzoxazone 6-hydroxylation may be a useful *in vivo* marker for hepatic CYP2E1 activity (24, 70) by following the pharmacokinetics of blood chlorzoxazone and 6-hydroxychlorzoxazone levels after a dose of 250 mg of chlorzoxazone (70–72). Our results with chlorzoxazone and human NOS isoforms may have some implications for the use of this compound as a marker for CYP2E1. Although variability was reported for the disposition of chlorzoxazone within populations of volunteers, mean maximum blood concentrations of 5.0 ± 1.9 μg/ml (30 μmol) chlorzoxazone were measured 1–2 h after dosing (72, 73). At this blood concentration, chlorzoxazone might also inhibit NO biosynthesis. Given the multiple physiological roles of NO (1, 2, 8) as a neurotransmitter, vasodilator, and mediator in host defense, the use of chlorzoxazone should be exercised with caution in regard to its potential effects on NO biosynthesis.

There is a large pharmaceutical interest for the development of NOS inhibitors as potential therapeutics in a variety of human diseases including inflammation (74, 75) and cerebral ischemia (76, 77). The results presented here demonstrate that chlorzoxazone is a potent inhibitor of iNOS and to our knowl-
edge is the first report of a benzoxazole class of NOS inhibitor. Given that chlorzoxazone is a potent NOS inhibitor and is the first report of a benzoxazole class of NOS inhibitor.