Comprehensive In Vitro Analysis of Voriconazole Inhibition of Eight Cytochrome P450 (CYP) Enzymes: Major Effect on CYPs 2B6, 2C9, 2C19, and 3A

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Voriconazole is an effective antifungal drug, but adverse drug-drug interactions associated with its use are of major clinical concern. To identify the mechanisms of these interactions, we tested the inhibitory potency of voriconazole with eight human cytochrome P450 (CYP) enzymes. Isoform-specific probes were incubated with human liver microsomes (HLMs) (or expressed CYPs) and cofactors in the absence and the presence of voriconazole. Preincubation experiments were performed to test mechanism-based inactivation. In pilot experiments, voriconazole showed inhibition of CYP2B6, CYP2C9, CYP2C19, and CYP3A (half-maximal [50%] inhibitory concentrations, <6 μM); its effect on CYP1A2, CYP2A6, CYP2C8, and CYP2D6 was marginal (<25% inhibition at 100 μM voriconazole). Further detailed experiments with HLMs showed that voriconazole is a potent competitive inhibitor of CYP2B6 (K<sub>i</sub> < 0.5), CYP2C9 (K<sub>i</sub> = 2.79 μM), and CYP2C19 (K<sub>i</sub> = 5.1 μM). The inhibition of CYP3A by voriconazole was explained by noncompetitive (K<sub>i</sub> = 2.97 μM) and competitive (K<sub>i</sub> = 0.66 μM) modes of inhibition. Prediction of the in vivo interaction of voriconazole from these in vitro data suggests that voriconazole would substantially increase the exposure of drugs metabolized by CYP2B6, CYP2C9, CYP2C19, and CYP3A. Clinicians should be aware of these interactions and monitor patients for adverse effects or failure of therapy.

Voriconazole, a derivative of fluconazole, belongs to the second generation of triazole antifungal drugs and has improved potency and a spectrum of antifungal activity that is expanded compared with the potency and activity of fluconazole (56, 59). Currently, orally or intravenously administered voriconazole is considered the first-line therapy for invasive aspergillosis (39, 56, 59). In addition, voriconazole is widely used for the management of patients infected with a broad range of other fungal pathogens, particularly patients who are intolerant of or who developed resistance to other conventional antifungal therapies (59).

However, despite its proven efficacy, the goal of optimal therapy with voriconazole is made difficult by the occurrence of clinically important drug-drug interactions. Several clinical studies and case reports have documented that voriconazole substantially reduces the clearance of several drugs, including warfarin (49), phenytoin (48), midazolam (53), diazepam (52), immunosuppressant drugs (cyclosporine, sirolimus, and tacrolimus) (46, 47), efavirenz (37), methadone (36), ibuprofen (27), diclofenac (26), fen-tanyl and alfentanil (54), oxycodone (22), and omeprazole (47). Considering the mechanisms of clearance of the drugs affected (3), many of these drug-drug interactions appear to be attributable to pharmacokinetic changes that can be understood in terms of inhibition of the cytochrome P450 (CYP) system. Indeed, in vitro studies by Niwa et al (41, 43) have documented that voriconazole inhibits CYPs 2C9, 2C19, and 3A, while its effect on the activity of other CYPs (CYPs 1A2, 2D6, and 2E1) was marginal. However, the mechanisms of inhibition were not addressed, and quantitative information that allows accurate prediction of voriconazole drug interactions in vivo was not provided in those studies because 50% inhibitory concentrations (IC<sub>50</sub>) obtained by using only a single substrate probe concentration were estimated. In addition, some drug-metabolizing CYPs (e.g., CYP2A6, CYP2C8, and CYP2B6) have not been studied. Comprehensive inhibitory analyses that encompass all major drug-metabolizing CYPs are important because not all pharmacokinetic drug interactions involving voriconazole can be explained by the CYPs studied so far. For example, voriconazole has recently been shown to slow the elimination of efavirenz (37), a drug mainly metabolized by CYP2B6 (9, 66).

The purpose of the present study was to determine the inhibitory potency of voriconazole with eight different major drug-metabolizing CYP isoforms in vitro. Inhibition constants (K<sub>i</sub> values) were estimated for those isoforms that were markedly inhibited by voriconazole in pilot experiments to guide predictions of drug interactions in vivo.

MATERIALS AND METHODS

Chemicals. Voriconazole, efavirenz, 7-hydroxyco-tramcinolone, 8-hydroxyefavirenz, bupropion, 4-hydroxybupropion, ritonavir, midazolam, 1'-hydroxymidazolam, and desethylamodiaquine were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Coumarin, glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase, 8-methoxypsolaren, dextromethorphan, dextrophan, chloroquine, desmethyldiazepam, phenacetin, and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO). Amodiaquine and levallorphan were purchased from the United States Pharmacopeia (Rockville, MD).
S-Mephenytoin was purchased from Biomol (Plymouth, PA). All other chemicals and solvents were of high-performance liquid chromatography (HPLC) grade.

HLMs. Human liver microsomal preparations (lot no. SD101, SD109) were purchased from Celldirect (Pittsburg, NC). HLMs (HL 09/14/99) were prepared from liver tissues medically unsuitable for transplantation by ultracentrifugation by standard protocols (8), and protein concentrations were determined by the method of Bradford (5) with bovine serum albumin as a standard. The microsomal pellets were suspended in a reaction buffer to a protein concentration of 10 mg/ml (stock). Insect cells in which human P450s were expressed by baculovirus (with oxidoreductase) were purchased from BD Biosciences (San Jose, CA). All microsomal preparations were stored at -80°C until they were used.

Experiments. (i) General incubation conditions. The inhibitory effects of voriconazole on the activities of eight different CYP isoforms were studied with HLMs (and expressed CYPs, when required). The isoform-specific probe reactions were performed with phenacetin O-deethylation (CYP1A2) (58), coumarin 7-hydroxyl- 3.3 mM MgCl2, and 0.4 U/ml glucose-6-phosphate dehydrogenase) (final incubation volume of an NADPH-generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 150 mM NaCl, 20 mM sodium acetate buffer, pH 5.3), 10 U/ml bupropion for CYP2B6, 10 to 500 μM tolbutamide, 50 μM S-mephenytoin, 25 μM dextromethorphan, and 10 μM midazolam was incubated at 37°C in duplicate with HLMs and the NADPH-generating system in the absence or the presence of a range of voriconazole concentrations (up to 100 μM). Processing of the incubation mixture and HPLC analysis of the metabolites formed were performed as described above.

(ii) Kinetic analysis. Kinetic analysis was performed for each isoform to determine the potency of inhibition and to select isoforms for further detailed study of their inhibition. A single isoform-specific substrate concentration at about the respective Kc values was used in the IC50 determination for each isoform. The IC50 determination was performed with UV detection at 245 nm. The rate of coumarin 7-hydroxylation was assayed by methods described below (see “Specific enzyme assays”) and the presence and concentration of voriconazole (IC50 determination) was compared with that in controls in which the inhibitor was replaced with vehicle.

(iii) Kinetic analysis. Kinetic analysis was performed for each substrate probe reaction before initiation of the inhibition experiments with voriconazole, and the data generated were used as a guide for selection of the appropriate concentrations of the substrate probes in the subsequent inhibition experiments. Thus, the kinetic parameters for the metabolism of each probe substrate were determined by incubating increasing concentrations of the substrate (without the inhibitor) at 37°C in duplicate with HLMs and the NADPH-generating system. Phenacetin (5 to 500 μM), coumarin (0.1 to 50 μM), efavirenz (0.1 to 500 μM), bupropion (1 to 500 μM), amiodarquine (0.1 to 100 μM), tolbutamide (5 to 500 μM), S-mephenytoin (5 to 500 μM), dextromethorphan (1 to 200 μM), and midazolam (1 to 300 μM) were used. The rates of formation of metabolites versus the substrate concentrations were fit to appropriate enzyme kinetic models to estimate the apparent kinetic parameters. Kc values and the maximum rate of metabolism (Vmax) were 43.8 μM and 84.9 pmol/min/mg protein, respectively, for CYP1A2 (high affinity component); 10.5 μM and 1.525 pmol/min/mg protein, respectively, for CYP2A6; 29.7 μM and 2.311 pmol/min/mg protein, respectively, for CYP2B6; 3.3 μM and 2.197 pmol/min/mg protein, respectively, for CYP2C8; 154.1 μM and 200 pmol/min/mg protein, respectively, for CYP2C9; 77.9 μM and 640 pmol/min/mg protein, respectively, for CYP2C19; 28.6 μM and 57 pmol/min/mg protein, respectively, for CYP2D6; 2.1 μM and 1.369 pmol/min/mg protein, respectively, for CYP3A-catalyzed midazolam 1'-hydroxylation; and 30.6 μM and 789 pmol/min/mg protein, respectively, for CYP3A-catalyzed midazolam 4'-hydroxylation. These Kc values were determined by incubating the substrate probe at multiple concentrations for the determination of the IC50 values for each isoform. The isoform-specific probe substrate concentrations used were 10 to 75 μM efavirenz and bupropion for CYP2B6, 10 to 500 μM tolbutamide for CYP2C9, 10 to 75 μM S-mephenytoin for CYP2C19, and 5 to 25 μM midazolam for CYP3A. The voriconazole concentrations used were 0 to 25 μM.

Specific enzyme assays. (i) CYP1A2 activity (phenacetin O-deethylation) using recombinant CYP1A2 was performed with UV detection at 245 nm. The rate of coumarin 7-hydroxylation was assayed by methods described below (see “Specific enzyme assays”) and the presence and concentration of voriconazole (IC50 determination) was compared with that in controls in which the inhibitor was replaced with vehicle.

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for 15 min. The reaction was terminated by adding 100 μl of acetonitrile. After the internal standard, 8-methoxypsoralen (50 μl of 20 μg/ml), was added, the mixture was vortex mixed and centrifuged and an aliquot (150 μl) of the supernatant was transferred to HPLC vials; 100 μl of this aliquot was injected onto the HPLC system. The internal standard and 7-hydroxycoymarin were separated by using a Zorbax SB-C18 column (150 by 4.6 mm; particle size, 3.5 μm; Phenomenex), a Luna C18 guard column (30 by 4.6 mm; particle size, 5 μm; Phenomenex), and a mobile phase composed of 70% 10 mM KH₂PO₄ (pH 3.0) and 30% (vol/vol) acetonitrile (flow rate, 0.8 ml/min). The column eluate was monitored by UV detection at 280 nm (internal standard) or with a fluorescence detector at an excitation wavelength of 370 nm and an emission wavelength of 450 nm (7-hydroxycoumarin).

(iii) CYP2B6 activity (efavirenz 8-hydroxylation to 8-hydroxyefavirenz) (66). The assay of efavirenz 8-hydroxylation was performed as described in our earlier publication (66), with a slight modification. A mixture of efavirenz (with or without inhibitors), HLMs (0.5 mg/ml) or expressed CYP2B6 (52 pmol/ml), and the NADPH-generating system was incubated at 37°C for 10 min. The reaction was terminated by addition of 0.5 ml acetonitrile. After an internal standard (ritonavir, 10 μg/ml) was added and the mixture was centrifuged at 14,000 rpm for 5 min, the supernatant was extracted with ethyl acetate under alkaline pH. The organic layer was removed, evaporated to dryness with a speed vacuum. The residue was reconstituted in mobile phase (75% 50 mM KH₂PO₄ (pH 2.4) and 25% (vol/vol) acetonitrile) (flow rate, 0.8 ml/min). Detection was performed with UV detection at 245 nm.

Evidence exists that drug interactions involving CYP2B6 might be substrate dependent (7). The effects of substrate specificity differences in inhibition by voriconazole on CYP2B6 activity (efavirenz 8-hydroxylation to 8-hydroxyefavirenz) were measured with two additional CYP2B6-catalyzed substrate reactions: bupropion 4-hydroxylation to 4-hydroxybupropion (15) and 8-hydroxyefavirenz 14-hydroxylation to 8,14-dihydroxyefavirenz (66). To determine the IC₅₀, bupropion (50 μM) or 8-hydroxyefavirenz (5 μM) was incubated with HLMs and the NADPH-generating system in the absence or the presence of voriconazole (0 to 100 μM). Dixon plots were determined by using efavirenz and bupropion, as described above, as the probes used for the 8-hydroxyefavirenz 14-hydroxylation. The incubation conditions and sample processing for the bupropion assay were similar to those described above for the efavirenz metabolism assay, except that the incubation period was 15 min and the internal standard was acetonitrile (25 μl of 10 μg/ml). A symmetry C₁₈ column (4.6 by 150 mm; particle size, 3.5 μm) with a guard column, a mobile phase that consisted of 85% 10 mM KH₂PO₄ (pH 2.4) and 15% (vol/vol) acetonitrile, and a mobile phase composed of 70% 10 mM KH₂PO₄ (pH 3.0) and 30% (vol/vol) acetonitrile (flow rate, 0.8 ml/min). Detection was performed with UV detection at 245 nm.

The assay of efavirenz 8-hydroxylation was performed as described in our earlier publication (66), with a slight modification. A mixture of amodiaquine (with or without voriconazole), HLMs (0.1 mg/ml) or expressed CYP2B6 (52 pmol/ml), and the NADPH-generating system were incubated for 60 min at 37°C. Incubation and HPLC assay methods described by Ko et al. (32) were used (32). The reaction was terminated by addition of 20 μl 60% CCl₄. Controls were incubated for 0 min without the test inhibitor and without the NADPH-generating system. The total volume of the preincubation mixture was 225 μl. After 0 and 15 min of preincubation, 25 μl of probe substrate was added at a final concentration corresponding to the Fmax, and the mixture was further incubated for the time specific for each assay. The reaction was stopped and processed as described above for the coinduction experiments.

Data analysis and prediction of in vivo data from in vitro data. The rates of formation of metabolite versus the substrate concentrations were fit to appropriate enzyme kinetic models by using the WinNonlin (version 5.0) program (Pharsight, Mountain View, CA) to estimate the apparent kinetic parameters (Km and Vmax). IC₅₀ was determined by analysis of the plot of the logarithm of the inhibitor concentration versus the percentage of activity remaining after inhibition. For this purpose, the relationship described by the Michaelis-Menten model was fitted to the data by assuming that voriconazole has a level of plasma protein binding of 58% (51).

The cooperativity parameter (Kc), which is a measure of the degree of cooperativity, was calculated by assuming that voriconazole has a level of plasma protein binding of 58% (51). On the basis of the predicted in vivo changes, voriconazole’s ability to alter (inhibit) the pharmacokinetics of the coadministered drugs was classified as
RESULTS

Estimation of IC_{50}s. The inhibitory effects of multiple concentrations of voriconazole (0 to 100 μM) on the activity of each CYP isoform determined by the metabolism of a single concentration of isoform-specific probe were tested with HLMs (or expressed CYPs, when needed). Voriconazole showed potent inhibition of CYP2B6 (efavirenz 8-hydroxylation), CYP2C9 (tolbutamide 4'-hydroxylation), CYP2C19 (S-mephénytoin 4'-hydroxylation), and CYP3A (midazolam 1'-hydroxylation), with IC_{50}s of 1.71 μM, 3.62 μM, 5.25 μM, and 2.90 μM, respectively. Relatively higher IC_{50}s were previously reported: for CYP2C9, 8.4 μM when tolbutamide was used as a probe (42); for CYP2C19, 8.7 μM (42) or 32 μM (16) when S-mephénytoin was used as a probe; and for CYP3A, 50 μM when felodipine was used as a probe (16), 10.5 μM when nifedipine was used as a probe (42), 54 μM when testosterone was used as a probe (16), and 10.5 μM when midazolam was used as a probe (42). There is evidence that drug interactions involving CYP3A might be substrate dependent (29). An inhibitor might show inhibition when only one substrate is used, while it may not inhibit (or may have a lower level of inhibition) when another substrate is used. Thus, it is often recommended that two structurally unrelated CYP3A4/CYP3A5 substrate probes be used to assess the potential for drug interactions involving this enzyme system (17). Using midazolam (the preferred substrate probe), we have shown potent inhibition at therapeutically relevant concentrations of voriconazole (see also below). Since these data are consistent with in vivo data showing that voriconazole is a potent inhibitor of chemically diverse CYP3A substrates, we do not believe that the differences in IC_{50}s are due to the substrate probes used. Differences in experimental conditions most likely explain the differences in IC_{50}s observed, particularly given that the estimation of IC_{50}s is highly dependent on the substrate concentration used.

The inhibitory effect of voriconazole on the activity of CYP1A2, CYP2A6, CYP2C8, and CYP2D6 was negligible (by less than 25% at 100 μM voriconazole) (Fig. 1). The lack of inhibition of these isoforms by voriconazole is unlikely a result of the substrate probes used. For example, we used dextromethorphan as a probe for CYP2D6 in this study and found no inhibition, consistent with in vitro data obtained with debrisoquin (41) and bufuralol (16) as probes or in a clinical study of the voriconazole-venlafaxine interaction (25). We believe that both probes are good as in vitro probes, but dextromethorphan might be preferred because of its availability for in vivo studies, while special permission for the use of debrisoquin is needed.

The extent of inhibition of CYPs by voriconazole was not modified during the preincubation experiments (data not shown). As expected, the activity of each isoform was substantially inhibited by the isoform-specific inhibitor, and the isoform-specific inhibitors served as positive controls (data not shown).

The initial goal of this study was to characterize the ability of voriconazole to inhibit CYP2B6 with the view toward finding an inhibitor probe that can be used in vivo to dissect the role of this enzyme in human drug metabolism. Thus, the interaction of voriconazole with CYP2B6 compared to its interaction with other CYPs was examined in more detail. In particular, since there is evidence that drug interactions involving CYP2B6 might be substrate dependent (7), IC_{50}s were determined by using two additional CYP2B6-catalyzed reactions. Consistent with data obtained with efavirenz as a substrate in HLMs (IC_{50} = 1.71 μM), voriconazole showed potent inhibition of CYP2B6-mediated bupropion 4-hydroxylation (IC_{50} = 1.19 μM) and 8-hydroxyefavirenz 14-hydroxylation (IC_{50} = 0.79 μM) in HLMs (Fig. 2A). In expressed CYP2B6, this inhibitory effect of voriconazole was less pronounced when efavirenz, bupropion, and 8-hydroxyefavirenz were used as probe substrates (IC_{50}s = 17.5 μM, 3.7 μM, and 1.9 μM, respectively) (Fig. 2B).

Further experiments were performed to determine the causes for the differential inhibitory potency of voriconazole in HLMs and by CYP2B6, particularly when efavirenz was used as a probe. One possibility is that this difference is due to the conversion of voriconazole in HLMs by CYPs other than CYP2B6 to a metabolite(s) that is a more potent inhibitor of CYP2B6. The main human metabolite of voriconazole is voriconazole N-oxide, which is mainly formed by CYP2C19 (and to some extent by CYP2C9 and CYP3A) (24), while a minor metabolite is hydroxyvoriconazole (40). Since we had no access to these metabolites to directly test their ability to inhibit CYP2B6, we conducted a series of experiments to better understand the difference in susceptibility to voriconazole inhibition between CYP2B6 and HLMs. First, voriconazole was preincubated with HLMs or expressed enzyme for 5, 15, 20, 25, and 30 min in the pres-
The amount of voriconazole formed increased with the incubation time (Fig. 3C). Third, we incubated expressed CYP2B6 in the absence and the presence of expressed CYP2C19. While the presence of expressed CYP2C19 increased the formation of voriconazole N-oxide, the inhibitory potency of voriconazole on CYP2B6 was not modified (IC_{50} = 12.2 μM). None of the data obtained from these experiments suggest that the more potent inhibition of CYP2B6 in HLMs is because of the voriconazole metabolites that are formed during incubation. Efavirenz 8-hydroxylation is predominantly catalyzed by CYP2B6 (66). However, although it is unlikely, the potent inhibition of efavirenz 8-hydroxylation in HLMs (but less so in expressed CYP2B6) by voriconazole may suggest the inhibition of CYPs in addition to CYP2B6 in efavirenz 8-hydroxylation in vitro.

Estimation of K_i values. While IC_{50} values are qualitatively informative and help to address whether inhibition has occurred, their values are of limited use because they can be influenced by the substrate concentration selected, and it may not be accurate to use these parameters for the quantitative prediction drug interactions in vivo. Therefore, we performed additional experiments designed to estimate K_i values. The preliminary inhibition data generated from a single probe substrate reaction (Fig. 1) were used to simulate the appropriate range of substrate and inhibitor concentrations for use in the construction of Dixon plots for the inhibition of CYP isoforms by voriconazole in HLMs, from which precise K_i values were estimated.

For CYP2B6, K_i values were determined by using efavirenz and bupropion as the probe substrates. Of all the CYPs tested, CYP2B6 was the most sensitive to voriconazole inhibition (Table 1). Representative Dixon plots for the inhibition of CYP2B6 in HLMs are shown in Fig. 4. Visual inspection of the Dixon plots and further analysis of the parameters of the enzyme inhibition models suggested that the inhibition data fit well to a competitive type of inhibition. The K_i values estimated by using a nonlinear regression model for competitive enzyme inhibition of CYP2B6-catalyzed efavirenz 8-hydroxylation and bupropion 4-hydroxylation in HLMs were less than 0.5 μM (Table 1).

Figure 5 shows Dixon plots for the inhibition of CYP2C9 (Fig. 5A) and CYP2C19 (Fig. 5B) by voriconazole in HLMs. Voriconazole inhibited CYP2C9 and CYP2C19 competitively, with estimated K_i values of 2.79 μM and 5.07 μM, respectively (Table 1).

As shown in Fig. 6, the inhibition of CYP3A-catalyzed midazolam 1'-hydroxylation (Fig. 6A) and 4'-hydroxylation (Fig. 6B) by voriconazole fit well to both noncompetitive and competitive enzyme inhibition models. The K_i values estimated from a noncompetitive inhibition equation and a competitive inhibition equation were less than 3 μM and 1 μM, respectively (Table 1), while the K_i value derived for inhibition of midazolam 4-hydroxylation by noncompetitive inhibition model was 2.9 ± 1.3 μM. To further understand the mechanism by which voriconazole inhibits the activity of CYP3A, voriconazole was preincubated in the presence of an NADPH-generating system and HLMs before initiation of the reaction by the addition of midazolam. Preincubation with voriconazole did not enhance its potency toward the inhibition of CYP3A activity (data not shown), suggesting that the noncompetitive type of inhibition...
observed during coinubcation is unlikely due to a time-depen-
dent inactivation of CYP3A by the inhibitor.

**DISCUSSION**

In the present study, (i) we have shown for the first time that voriconazole is a highly potent inhibitor of CYP2B6 and that its effect on CYP2A6 and CYP2C8 activity is marginal; and (ii) we have confirmed previous findings (16, 42) that voriconazole is a potent inhibitor of CYP2C9, CYP2C19, and CYP3A and also reported $K_i$ values that can be used to predict the magnitude of drug interactions in vivo. On the basis of our in vitro data, voriconazole would be predicted to slow the elimination of drugs predominantly cleared by CYP2B6, CYP2C9, CYP2C19, and CYP3A or by a combination of these CYPs (Table 1). These data help to explain the multiple voriconazole drug interactions that have been reported in the literature and can be used to predict and to identify new drug-drug interactions. Our data and findings from other in vitro studies (16, 41) and in vivo studies (25, 27) suggest that it is unlikely that voriconazole alters the pharmacokinetics of drugs metabolized by CYP1A2, CYP2A6, CYP2C8, CYP2D6, and CYP2E1.

Of the eight CYPs studied, CYP2B6 was most sensitive to inhibition by voriconazole ($K_i < 0.5 \mu M$) (Table 1). Although findings from in vivo studies are generally limited, our in vitro data are consistent with the findings of in vivo studies that showed that voriconazole interacts with certain CYP2B6 substrates. Voriconazole has recently been reported to significantly slow the elimination of efavirenz in healthy volunteers (37). Although the authors of that study (37) suggested that CYP3A was the mechanism for this interaction, the available in vitro evidence (9, 66) and in vivo evidence (21, 38, 69; Bristol-Myers Squibb Company, efavirenz [Sustiva] package insert, updated March 2008) strongly support the conclusion that CYP2B6 (but not CYP3A) is important in the clearance of

![FIG. 3. Time-dependent inhibition of CYP2B6-catalyzed efavirenz 8-hydroxylation by voriconazole in HLMs. (A) Voriconazole (10 \mu M) was preincubated in duplicate with HLM and a phosphate reaction buffer (pH 7.4) (without or with the NADPH-generating system) in the absence or the presence of a substrate probe for 15 min at 37°C. The preincubation reaction was started by adding the NADPH-generating system. The controls were preincubated for 0 min without the test inhibitor and without the NADPH-generating system. The total volume of the preincubation mixture was 225 \mu l. After 0 and 15 min of preincubation, 25 \mu l of efavirenz (final concentration, 100 \mu M) was added and the mixture was further incubated for 10 min with cofactors. The reaction was stopped and processed as described for the coincubation experiments (see Materials and Methods). (B and C) Effects of duration of incubation on the degree of inhibition of efavirenz 8-hydroxylation by voriconazole in HLMs (B) and metabolism of voriconazole, as shown by parent compound depletion and metabolite formation (C). Efavirenz (10 \mu M) was incubated with HLMs (0.5 mg/ml) and cofactors in the absence or the presence of voriconazole (1 \mu M) for up to 60 min. Each point represents the average of duplicate incubations.](http://aac.asm.org/)

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efavirenz and that inhibition of CYP2B6 is the main mechanism by which voriconazole increases efavirenz exposure in humans (37). Voriconazole has also been shown to increase the level of exposure to methadone, particularly that to racemic methadone (36), an enantiomer whose clearance appears to be independent of the dose or the plasma concentrations (51). The change in the AUC was calculated by using the formula described in the text (see “Data analysis and prediction of in vivo data from in vitro data”), assuming competitive and noncompetitive modes of inhibition.

The ability of voriconazole to slow the elimination of CYP3A substrates in vivo, e.g., immunosuppressant drugs (cyclosporine, sirolimus, and tacrolimus) (46, 47), fentanyl and alfentanil (55), oxycodone (22), etoricoxib (28), and midazolam (53), has been well documented. Using the most reliable

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**Table 1.** $K_i$ values of voriconazole for the inhibition of CYPs in HLMs and prediction of changes in AUC in vivo

<table>
<thead>
<tr>
<th>CYP</th>
<th>$K_i$ value (µM)</th>
<th>Predicted fold change in AUC at $C_{\text{min}}$</th>
<th>Experimental conditions and observed fold change in AUC</th>
<th>Fold change</th>
<th>Reference</th>
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<tr>
<td></td>
<td></td>
<td>Total $[I]$ ($C_{\text{min}}$-$C_{\text{max}}$)</td>
<td>Free ($I_u$) ($C_{\text{min}}$-$C_{\text{max}}$)</td>
<td>Voriconazole dose</td>
<td>Substrate (dose)</td>
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<tr>
<td>CYP2B6</td>
<td>0.40 ± 0.1$^a$</td>
<td>16–32</td>
<td>7.5–14</td>
<td>400 mg BID$^e$ on day 1, 200 mg BID on days 2–3</td>
<td>Efavirenz (400 mg/day)</td>
</tr>
<tr>
<td></td>
<td>0.34 ± 0.1$^f$</td>
<td>16–32</td>
<td>7.5–14</td>
<td>400 mg BID on day 1, 200 mg BID on days 2–3</td>
<td>S-Methadone (30–100 mg/day racemic methadone orally)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>2.8 ± 0.2</td>
<td>3.0–5.1</td>
<td>1.9–2.71</td>
<td>400 mg/day for 10 days</td>
<td>Phenytoin (300 mg/day orally)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>400 mg BID on day 1, 200 mg BID on day 2</td>
<td>Diclofenac (50 mg, single dose)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>300 mg BID on day 2</td>
<td>S-Ibuprofen (400 mg, single oral dose)</td>
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<td></td>
<td></td>
<td>400 mg BID on day 1, 200 mg BID</td>
<td>Warfarin (30 mg, single dose)</td>
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<td>CYP2C19</td>
<td>5.1 ± 0.3</td>
<td>2.1–3.3</td>
<td>1.5–1.9</td>
<td>400 mg BID on day 1, 200 mg BID on days 2–6</td>
<td>Omeprazole (40 mg/day for 7 days)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>200 mg BID</td>
<td>Omeprazole (40 mg/day)</td>
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<tr>
<td>CYP3A</td>
<td>2.97 ± 0.2$^g$</td>
<td>2.9–4.8</td>
<td>1.8–2.6</td>
<td>400 mg BID on day 1, 200 mg BID on day 2</td>
<td>Midazolam (7.5 mg orally)</td>
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<tr>
<td></td>
<td>0.66 ± 0.2$^h$</td>
<td>9.6–18.4</td>
<td>4.6–8.3</td>
<td>400 mg BID on day 1, 200 mg BID on day 2</td>
<td>Midazolam (0.05 mg/kg of body weight intravenously)</td>
</tr>
</tbody>
</table>

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$^a$ The average plasma voriconazole concentrations after administration of the usual therapeutic dose (400 mg every 12 h on day 1 and then 200 mg every 12 h for days 2 and 4) were approximately 4 µg/ml (11.5 µM) at $C_{\text{max}}$ and 2 µg/ml (5.7 µM) at $C_{\text{min}}$ (12), and these concentrations were used. $C_{\text{min}}$ and $C_{\text{max}}$ values are in micromolar.

$^b$ The fraction unbound ($I_u$) (2.4 to 4.8 µM for $C_{\text{min}}$ and $C_{\text{max}}$, respectively) was calculated on the basis of 58% plasma protein binding of voriconazole, which was independent of the dose or the plasma concentrations (51). The change in the AUC was calculated by using the formula described in the text (see “Data analysis and prediction of in vivo data from in vitro data”), assuming competitive and noncompetitive modes of inhibition.

$^c$ BID, twice a day.

$^d$ Indicates the fold change in pharmacodynamics.

$^e$ Efavirenz as a substrate.

$^f$ Bupropion as a substrate.

$^g$ Voriconazole dose, Substrate (dose), Fold change.

$^h$ $K_i$ for inhibition of midazolam 1'-hydroxylation derived from noncompetitive model.

$^i$ $K_i$ for inhibition of midazolam 1'-hydroxylation derived from competitive model.
standard substrate for evaluation of the in vivo inhibition of CYP3A, midazolam (19), we found that voriconazole is a strong inhibitor of CYP3A (IC$_{50}$ = 2.90 μM; $K_i$ = 0.66 μM for the competitive enzyme inhibition model and $K_i$ = 2.97 μM for the noncompetitive enzyme inhibition model) (Table 1). In other studies, IC$_{50}$S for the inhibition of the CYP3A-catalyzed metabolism of nifedipine, felodipine, and testosterone by voriconazole were reported to be relatively high (10.5, 50, and 54 μM, respectively) (16, 42). Although the difference in inhibition constants may reflect in part the substrate-dependent interaction of CYP3A substrates with voriconazole, as this possibility has been reported previously (29), this seems unlikely given that voriconazole alters the in vivo elimination of several structurally diverse CYP3A substrates. The discrepancy could simply reflect differences in experimental conditions. In clinical studies, voriconazole increases the levels of exposure to midazolam administered orally and intravenously by 10.3-fold and 3.7-fold, respectively (53). When the $K_i$ values derived by using the noncompetitive model were used to predict the change in AUC in vivo, the changes in AUC observed after the oral administration of midazolam were much higher than those predicted from our in vitro data (2.9- to 4.9-fold changes in the midazolam AUC when the total $C_{min}$ and $C_{max}$ of voriconazole were used and 1.8- to 2.6-fold changes in the midazolam AUC when the fraction unbound is considered); the values observed after the intravenous administration of midazolam (53) were consistent with our predicted values (Table 1). As no differences in the potency of inhibition between the preincubation and the coinubation experiments were observed, this observation is not due to the contribution of time-dependent inhibition. The possibility that circulating oxidative metabolites of voriconazole (24, 40) might contribute to the in vivo inhibition of CYP3A cannot be excluded. Also, as CYP3A is the main enzyme catalyzing midazolam not only in the liver but also in the gut wall (19, 53, 60), it is likely that the greater interaction observed in vivo after the oral administration of midazolam (53) can be attributed to the inhibition of CYP3A by voriconazole at both of these sites (53). Alternatively, in vivo inhibition might occur predominantly through competitive inhibition. Using the competitive inhibition model, we predicted 9.6- to 18.4-fold changes in the AUC when total $C_{min}$ and $C_{max}$ were considered and 4.6- to 8.3-fold when the $C_{min}$ and the $C_{max}$ for the fraction unbound were used. These values are broadly consistent with the AUC changes that have been observed previously (53). Together, these data indicate that voriconazole can be considered a strong inhibitor of CYP3A, an enzyme system that is abundant in the gut wall and the liver.
and that is involved in the metabolism of more than 50% of those clinically used drugs cleared by oxidation (67).

The $K_i$ value for the inhibition of CYP2C9 by voriconazole in HLMs was 2.79 $\mu$M. On the basis of these data, we would expect changes in the AUC of 3- to 5-fold for the CYP2C9 substrate during voriconazole administration when the total $C_{\text{min}}$ and the $C_{\text{max}}$ of voriconazole are considered and 1.9- to 2.7-fold when the $C_{\text{min}}$ and the $C_{\text{max}}$ of the fraction unbound are considered. Consistent with our in vitro data and the predicted values, voriconazole has been shown to increase (1.8- to 2-fold) the AUCs of known CYP2C9 substrates (30) (Table 1).

Approximately 100 clinically used drugs are metabolized by CYP2C9 (30). Although voriconazole can be categorized as a weak inhibitor of CYP2C9, it may have clinically important consequences for some substrates with narrow therapeutic ranges (e.g., warfarin, phenytoin, and sulfonylureas).

CYP2C19 is responsible for the metabolic detoxification or activation of a number of clinically important drugs (11). Our data show that voriconazole is a moderate inhibitor of CYP2C19 ($IC_{50} = 5.25 \mu$M, $K_i = 5.07 \mu$M), consistent with the findings of a previous in vitro study, which reported an $IC_{50}$ of 8.7 $\mu$M (42); in contrast, another study (16) reported a higher $IC_{50}$ (32 $\mu$M) when the same substrate probe was used. We predicted changes in the AUC of 2.1- to 3.3-fold for CYP2C19 substrates predominantly metabolized by the enzyme when the total $C_{\text{min}}$ and $C_{\text{max}}$ of voriconazole were used and 1.5- to 2-fold when the $C_{\text{min}}$ and the $C_{\text{max}}$ of the fraction unbound were considered (Table 1). According to information provided in the package insert, the level of omeprazole exposure was increased fourfold by voriconazole. Since omeprazole is predominantly cleared by CYP2C19 and to some extent by CYP3A (1, 2), the fact that the observed effect of voriconazole was relatively higher than our predicted effect probably reflects a net effect of voriconazole on both of these enzymes. While voriconazole is expected to moderately alter the exposure of drugs primarily cleared by CYP2C19 (11), the magnitude of the interaction may be greater if alternative metabolic pathways are inhibited simultaneously. In addition, the clinical impact of voriconazole might be greater when it is coprescribed with prodrugs requiring CYP2C19-mediated metabolic activation, e.g., clopidogrel (23), cyclophosphamide (57), and thalidomide (35).

In summary, we have shown that voriconazole is a potent inhibitor of CYP2B6, CYP2C9, CYP2C19, and CYP3A at clinically relevant concentrations. Our in vitro data broadly predict clinically important drug interactions of voriconazole with substrates of these enzymes, and there is convincing evidence that this is the case in clinical studies. Together, these four enzymes are the clearance mechanisms for ~67% of the drugs currently marketed (70). Given that the number of patients requiring voriconazole is on the increase and that these patients are often seriously ill, the likelihood that voriconazole will be coprescribed with drugs that interact and thus elicit frequent and severe adverse drug interactions in the population is very high. However, it is important to note that the extent of drug interactions with this drug varies greatly among individuals, because the level of voriconazole exposure after the administration of therapeutic doses exhibits large intersubject variability due to factors that include CYP2C19 genetic polymorphisms, nonlinear pharmacokinetics at different doses, the duration of treatment, the routes of administration, the level of hepatic impairment, the presence of inflammatory conditions, age, and the concurrent administration of substrate drugs that reduce or enhance the clearance of voriconazole (e.g., efavirenz, carbamazepine, ritonavir) (37, 47). In addition, the simultaneous inhibition of alternative routes of metabolism of substrates (e.g., omeprazole) (47) or the simultaneous inhibition of gut wall and hepatic metabolism (e.g., CYP3A substrates) versus the inhibition of hepatic metabolism after drug administration by the intravenous route (inhibition only at the hepatic site) (e.g., midazolam) (53) could influence the extent of the interaction with voriconazole.

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