0090-9556/08/3607-1261–1266$20.00
DRUG METABOLISM AND DISPOSITION
DMD 36:1261–1266, 2008

Vol. 36, No. 7
19000/3347843
Printed in U.S.A.

Prediction of Pharmacokinetic Drug-Drug Interactions Using Human Hepatocyte Suspension in Plasma and Cytochrome P450 Phenotypic Data. III. In Vitro-in Vivo Correlation with Fluconazole

Chuang Lu, Cicely Berg, Shimoga R. Prakash, Frank W. Lee, and Suresh K. Balani

Drug Metabolism and Pharmacokinetics, Drug Safety and Disposition, Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts

Received September 24, 2007; accepted March 31, 2008

ABSTRACT:

Whereas ketoconazole is often used to study the worst-case scenario for clinical pharmacokinetic drug-drug interactions (DDIs) for drugs that are primarily metabolized by CYP3A4, fluconazole is considered to be a moderate inhibitor of CYP3A4, providing assessment of the moderate-case scenario of CYP3A-based DDIs. Fluconazole is also a moderate inhibitor of CYP2C9 and CYP2C19. For predicting clinical DDIs using conventional approaches, determining the in vivo inhibitor concentration at the enzymatic site [I], a critical parameter, is still not practical. In our previous study, a novel method involving hepatocyte suspension in plasma was used to circumvent the need to determine the elusive [I] value. In this study, the CYP1A2, 2C9, 2C19, 2D6, and 3A4 activities remaining in the presence of fluconazole were determined in human hepatocytes suspended in human plasma, covering a range of fluconazole clinical plasma concentrations (C_{avg} and C_{max}). Because the protein-binding effect of fluconazole is expected to be close to that in vivo, the inhibition observed in vitro will be similar to that in vivo. This inhibition information was then applied to the cytochrome P450 (P450) phenotypic data to predict DDIs. Using the available P450 phenotypic information on theophylline, tolbutamide, omeprazole, *S*-warfarin, phenytoin, cyclosporine, and midazolam and that determined in this study for sirolimus and tacrolimus, we found that the predictions for area under the curve increases for most of these drugs in the presence of fluconazole were remarkably similar (within 35%) to the observed clinical values. This study proves the general applicability of our approach using human hepatocyte incubation in human plasma to predict DDIs.

Fluconazole is an antifungal agent being widely used for oropharyngeal candidiasis and coccidioidal meningitis (Hardman et al., 2001). Fluconazole is also a moderate inhibitor of multiple cytochromes P450 (P450s) in humans, such as CYP2C9, CYP2C19, and CYP3A4 (Niwa et al., 2005a). Fluconazole shows linear pharmacokinetics (Balant, 1981; Balani et al., 2006), high gastrointestinal absorption, low plasma protein binding, and low metabolic clearance. It is cleared largely via renal excretion (>90%) (Debruyne, 1997). In addition, fluconazole is found to inhibit UDP glucuronosyltransferases (Trapnell et al., 1998; Uchaipichat et al., 2006), as well as transporters such as Pgp (Kodawara et al., 2001). One advantage of fluconazole over other antifungal drugs such as ketoconazole and itraconazole is that it can "freely" penetrate into tissues including cerebrospinal fluid (Lazar and Wilner, 1990). In the clinic, for the purpose of overcoming possible drug resistance, the usage of fluconazole is often very high. At a 200 mg b.i.d. dose, the maximum plasma concentration (C_{max}) could reach as high as 34.6 μM (Hardman et al., 2001). Whereas ketoconazole is often used to study the worst-case scenario of CYP3A4-mediated drug-drug interaction (DDI) potential for a drug candidate, fluconazole can be considered to study the moderate-case scenario of CYP3A4-mediated DDI. In addition, the contribution of other P450 isoforms affected by fluconazole also has to be taken into consideration.

P450 reactive phenotyping is a quantitative measurement of the relative contribution of each P450 to the overall metabolism of a drug, if the drug is primarily metabolized by several P450s. Among the limited literature reports available, Soars et al. (2003) determined quantitative P450 phenotyping of 14 market compounds using average percent content and relative activity factor approaches. Methods commonly used in the determination of reactive phenotyping include the relative activity factor (Crespi, 1995; Venkatakrishnan et al., 2001), chemical inhibitors (Newton et al., 1995; Bourri é et al., 1996; Lu et al., 2003), and monoclonal antibodies (Gelboin et al., 1999; Shou et al., 2000; Soars et al., 2003). In a comparison, Uttamsingh et al. (2005) applied all three methods to determine the relative P450 contributions to the metabolism of a proteasome inhibitor, bortezomib (Velcade). With the introduction of the potent and selective CYP2C19 and CYP3A4 inhibitors benzylnirvanol and azamulin, respectively (Walsky and Obach, 2003; Stresser et al., 2004), using chemical inhibitors to determine the reactive phenotyping has become an easy and cost-effective choice.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.
doi:10.1124/dmd.107.019000.

ABBREVIATIONS: CYP, cytochrome P450; CYP3A4, CYP3A4/5; Pgp, P-glycoprotein; DDI, drug-drug interaction; LC/MS/MS, liquid chromatography coupled to tandem mass spectrometry; f_m, fraction of metabolism by a given enzyme; f_A, fraction of activity remaining of a given enzyme in the presence of inhibitor.

Predicting the clinical DDI from in vitro data is one of the major and important efforts in the pharmaceutical industry. It is especially important for drugs that are primarily metabolized by CYP3A4, which is the case for the majority of small molecule drugs. The current DDI draft guidance calls for a clinical DDI study if more than 25% of the drug clearance is from a specific pathway, e.g., CYP3A4 (Food and Drug Administration Drug-Drug Interaction Draft Guidance, 2006, http://www.fda.gov/cder/guidance/6695dft.htm). Besides the pharmacokinetic properties of the substrate drug, two factors that dictate the DDI potential are the inhibitor's inhibition constant (K_i) and the enzyme site free concentration of that inhibitor ([I]). The K_i usually can be determined from in vitro microsomal incubation assays with the consideration of protein binding. But the enzyme site inhibitor concentration, [I], at this point cannot be determined. For reliable prediction of DDIs, vast efforts have been focused on defining the enzyme site inhibitor concentration. As pointed out in our previous report, several attempts have been made to estimate [I], but no one way with wide applicability has been discovered thus far. In part I of this article, we developed a method for DDI predictions that circumvented the need to assess the [I] value and to use the [I]/K_i ratio (Lu et al., 2007). In that method, various concentrations of the potent CYP3A inhibitor ketoconazole were incubated in human hepatocytes suspended in human plasma to construct an inhibition titration curve. After equilibrium, the P450 activities remaining were measured using prototypical substrates. In this model the extracellular (plasma) concentration of the inhibitor is considered to mimic that in vivo, and hence the intracellular concentration of that inhibitor at the enzyme site in vitro is considered to be similar to that in vivo. Furthermore, if the intracellular concentrations are comparable, the enzyme activity remaining measured in vitro can represent that in vivo. Therefore, without the need to know the [I] and K_i of that inhibitor, information on the enzyme activity remaining could be used to aid the DDI prediction based on the fraction of enzyme contribution to the total drug clearance (Rostami-Hodjegan and Tucker, 2004; Ito et al., 2005; Obach et al., 2006; Lu et al., 2007).

In the present study, human hepatocytes were incubated in human plasma in the presence of various concentrations of fluconazole to determine the enzyme activity remaining and inhibition of five major P450s: 1A2, 2C9, 2C19, 2D6, and 3A4. Reactive phenotyping information for nine marketed drugs was either extracted from the literature or determined in-house. Application of the P450 activity remaining and the reactive phenotyping data in our model (Lu et al., 2007) showed a decent correlation between the predicted and observed clinical DDI. Thus, this report further strengthens the general applicability of our approach to predict DDIs.

Materials and Methods

Reagents. Pooled human liver microsomes from 50 donors were purchased from XenoTech, LLC (Kansas City, KS). Cryopreserved human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD) and AP Sciences Inc. (Baltimore, MD). 4-Hydroxytolbutamide, 4-hydroxymephenytoin, 1′-hydroxymidazolam, (*S*)-(+)-(*N*)-(3)-benzylnirvanol (benzylnirvanol), and azamulin were purchased from BD Gentest (Woburn, MA). Phenacetin, acetaminophen, tolbutamide, dextromethorphan, dextrorphan, furafylline, sulfaphenazole, quinidine, fluconazole, midazolam, NADPH, and $MgCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO). Human plasma was purchased from Bioreclamation Inc. (Hicksville, NY). *S*-Mephenytoin was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Fluconazole P450 Inhibition Determination in Human Hepatocytes. This study was performed in human plasma fortified with 2 mM salicylamide. The salicylamide was used to reduce possible phase II conjugation of the phase I metabolites, which are the analytes in this study (Lu and Li, 2001). Serially diluted fluconazole solutions were prepared in 0.4% dimethylsulfoxide-2% acetonitrile-plasma (v/v/v) at final concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 μM. Human hepatocytes (pooled from two female and two male donors) were thawed and prepared in plasma as described previously (Li et al., 1999). The hepatocytes (25 μl in plasma, final concentration of 1.0×10^6 hepatocytes/ml, viability >80%) were mixed with 25 μl of fluconazole solutions at various concentrations at room temperature for 20 min followed with a prewarm period of 10 min at 37°C to allow equilibration. The P450 isozyme-specific substrates prepared in 1% acetonitrile-plasma (v/v, 50 μl) were added to start the P450 activity assays. The final concentrations for the substrates were 30 μM phenacetin, 150 μM tolbutamide, 100 μM *S*-mephenytoin, 8 μM dextromethorphan, or 5 μM midazolam. To increase compound solubility, with minimal effect on P450 activity, 0.1% dimethylsulfoxide and 1% acetonitrile were added. The incubations were carried out in a 37°C CO_2 (5%) incubator for 45 min for phenacetin, dextromethorphan, and midazolam. The incubations for tolbutamide and *S*-mephenytoin were extended to 90 min because of low turnover rates of the substrates. All incubation conditions were in a predetermined linear range. The reactions were stopped by adding 2 volumes of acetonitrile containing 1 μM of carbutamide (internal standard). The samples were kept in a refrigerator for 30 min and then centrifuged at 3000*g* for 10 min. The supernatants were analyzed by LC-MS/MS for the amount of metabolite formed. The percentage of metabolic activity remaining was calculated by comparing the P450 activities in samples with various concentrations of fluconazole to their relative vehicle controls.

Determination of Extracellular Fluconazole Concentrations. After the equilibrium period of fluconazole in human hepatocytes of 20 min at room temperature and 10 min at 37°C, the hepatocytes were separated from the plasma via a centrifugation method modified from a previous report (Shitara et al., 2003). To separate hepatocytes from a Krebs-Henseleit buffer incubation (the commonly used buffer for hepatocyte incubation) the hepatocytes are generally centrifuged through an oil mix consisting of 46.5 g of silicon oil (d = 1.050 g/ml; Aldrich Chemical Co., Milwaukee, WI) and 8.6 g of mineral oil (d = 0.84 g/ml; Sigma-Aldrich) that has a final density of ~1.011 g/ml. However, in the hepatocyte-plasma incubation, the density of the human plasma is close to this range and sometimes plasma passes through the oil layer along with the hepatocytes upon centrifugation. Thus, a new oil mixture was used in our studies [1 g of silicon oil (d = 1.102 g/ml; Aldrich) mixed with 0.15 g of mineral oil (d = 0.84 g/ml; Sigma-Aldrich)], which generated a final density of ~1.12 g/ml. Fluconazole concentrations in these extracellular media were analyzed using LC-MS/MS with standard curves prepared in the same matrices. To determine whether accountable metabolism occurred during this equilibrium period, two sets of fluconazole-hepatocyte samples were prepared. The reaction-terminating solution, acetonitrile containing 1 μM of carbutamide, was added to one set at 0 min and to the second set after the equilibrium period. The percentage of fluconazole remaining was determined using LC-MS/MS. The LC-MS/MS system used to determine fluconazole and P450 substrate metabolites consisted of an Agilent 1100 high-performance liquid chromatograph, a CTC PAL autosampler (LEAP Technologies, Carrboro, NC), and a SCIEX API 4000 detector (Applied Biosystems, Framingham MA). Metabolite separation was achieved on a Phenomenex Synergi C18 column (75 × 4.6 mm) with a gradient consisting of 0.1% formic acid-water (mobile phase A) and 0.1% formic acid-acetonitrile (mobile phase B) at a flow rate of 1.0 ml/min. Specifically, 5% of mobile phase B was applied for 0.5 min after injection and increased linearly to 95% B from 0.5 to 3.5 min. Mobile phase B was held at 95% from 3.5 to 3.6 min, and the column was reequilibrated to 5% B from 3.6 to 5.0 min. A positive ion spray in the multiple-reaction monitoring mode was applied with a predetermined parent/product mass transition ion pairs for fluconazole and P450 probe substrate metabolites.

Calculation of AUC Changes from the in Vitro Data. The P450 contents in the gut are a small fraction of that in the liver (Obach et al., 2006), and the contribution of metabolism by gut to the overall metabolism in humans is compound and dose regimen-dependent. For oral drugs that are subject to gut metabolism, the effect becomes significant if those drugs are highly subject to CYP3A clearance and have low permeability or are substrates of efflux pumps. However, the information on Fg′/Fg factor is not available for the compounds of interest (Rostami-Hodjegan and Tucker, 2004; Ito et al., 2005; Galetin et al., 2006). Thus, this factor was not included in our calculation. Equation 1, which was described in our previous report (Lu et al., 2007), was used to calculate

TABLE 1

Effect of fluconazole on P450 activity in human hepatocytes suspended in human plasma

Fluconazole was equilibrated with human hepatocytes in human plasma to allow non-specific binding. After equilibration, the extracellular concentration of fluconazole was measured. The remaining P450 activities in hepatocytes (f_A) were also determined using the probe substrates.

Total Fluconazole in Incubation	Fluconazole Extracellular	f_A				
		CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
	μM			%		
100 μM	98.4	91.9	21.2	10.6	74.2	15.4
50 μM	47.2	112	36.8	14.9	97.6	23.3
25 μM	26.5	89.5	55.2	27.8	102	34.9
12.5 μM	13.1	97.5	66.3	45.2	87.4	39.8
6.25 μM	6.10	90.8	78.9	64.7	115	50.6
3.13 μM	3.13	85.1	86.7	75.7	110	59.9
1.56 μM	1.46	90.0	89.3	89.2	89.0	66.7
0 μM	0	100	100	100	100	100

AUC changes, assuming linear pharmacokinetics and a C_{max} of 34.6 μM at 200 mg b.i.d. or q.d. dose of fluconazole (Hardman et al., 2001).

Results

The inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 by fluconazole in human hepatocytes in human plasma is presented in Table 1. Figure 1 shows the plot of fluconazole concentration versus the P450 activity remaining from the data in Table 1. The data for CYP2D6 showed some variation, which is considered to be within experimental error. In Table 1, column 1 represents the total concentrations in the incubation: the initial concentration of fluconazole. "Fluconazole extracellular" (column 2) represents the extracellular concentration of fluconazole remaining in the plasma after the equilibration period. Comparing the values in columns 1 and 2, the difference was less than 10%, which is within the experimental error range. Thus, fluconazole seems to be evenly distributed between hepatocytes and plasma. This is attributed to the low protein binding and high permeability of fluconazole (Debruyne, 1997; Gibbs et al., 1999). It is also corroborated by the clinical observation showing that fluconazole is readily distributing to all body tissues (Lazar and Wilner, 1990; Hardman et al., 2001). Fluconazole, upon incubation with human hepatocytes in human plasma, showed a dose-dependent inhibition of CYP2C9, CYP2C19, and CYP3A4. However, within the concentration range tested in this study and considering the variability in the data and the low degree of change in the data, dose-dependent inhibition of fluconazole on CYP1A2 and CYP2D6 was not inferred. These results agree with earlier studies in human liver microsomes (Niwa et al., 2005a,b). In this study, the substrates to measure the P450 activity were added into the incubation after the 30-min equilibration period. To confirm that the majority of fluconazole was still available for inhibitions at that time, the total fluconazole concentrations before and after the equilibrium were determined. As expected, fluconazole is a low metabolism compound, and it was found to be stable in the hepatocyte during the 30-min equilibration. Table 2 lists the f_m values, the fluconazole clinical doses, and the estimated C_{max} used for DDI prediction. Table 3 presents DDI predictions for nine marketed compounds. Also included is a comparison between the values predicted from this study and the values observed in clinical trials. A correlation coefficient (r^2) of 0.71 (Fig. 2) was derived from the data in Table 3. The omeprazole data point was omitted from correlation calculations for the reasons described under *Discussion*. A slope close to unity (1.16) suggested a fairly good prediction of clinical DDI from the in vitro data. Relative prediction errors were calculated by dividing the difference between the predicted value and the observed value by the observed value. For most compounds, the relative errors were less than 35%, except for omeprazole being 58%.

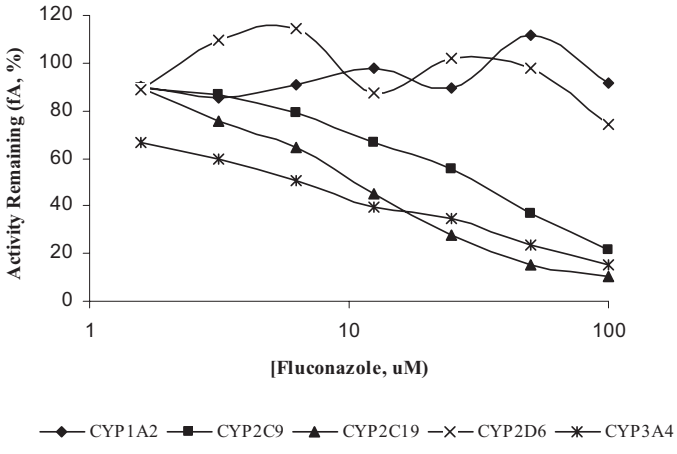

FIG. 1. Relationship of the extracellular plasma concentration of fluconazole and P450 activity remaining (f_A) in incubation of human hepatocytes suspended in human plasma.

Discussion

In continuation of our previous study (Lu et al., 2007), we have extended our model of hepatocyte suspension in plasma for the prediction of DDIs to studies using the moderate CYP3A inhibitor fluconazole. This process will help predict the fold change in AUC for CYP3A substrates with fluconazole, positive results from which may help justify the concomitant use of similar moderate CYP3A inhibitor drugs, depending also on the therapeutic range of the drug in question. In this study, the P450 inhibition in hepatocytes was measured over a wide range of concentrations of fluconazole to cover all possible in vivo plasma C_{max} values. Cryopreserved hepatocytes are known to preserve the P450 activities as well as most of the phase II enzymes (Li et al., 1999; Madan et al., 1999). Uptake transporters, such as organic anion transporting polypeptide and sodium-taurocholate cotransporting polypeptide, are also mostly preserved in the cryopreserved human hepatocytes (Shitara et al., 2003), but some efflux transporters, such as Pgp, multidrug resistance-associated protein 2, and breast cancer resistance protein, are internalized in hepatocyte suspension (Liu et al., 1999; Xia et al., 2005). Nevertheless, fluconazole inhibits these efflux transporters (Kodawara et al., 2001); thus, they are rendered nonfunctional in both in vitro and in vivo situations. Therefore, it is reasonable to assume that when the fluconazole concentration in the extracellular plasma in the in vitro model is comparable with the plasma concentration in vivo, the concentration of the inhibitor at the enzyme site in the in vitro model is also comparable with that in vivo. In this way, estimating the actual enzyme site inhibitor concentration becomes unnecessary. This ap-

TABLE 2

Relative P450 contribution to the hepatic metabolism, fluconazole dose, and estimated C_{max}

Compound	f_m					Fluconazole Dose[a]	C_{max}[b]
	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4/5		
							μM
Theophylline	1	NA	NA	NA	NA	200 mg b.i.d.	34.6
Tolbutamide	NA	0.72	0.28	NA	NA	200 mg q.d.	34.6
Omeprazole	NA	NA	0.60	NA	0.40	100 mg q.d.	17.3
S-Warfarin	NA	1	NA	NA	NA	300 mg q.d.	51.9
Phenytoin	NA	0.90	0.10	NA	NA	200 mg q.d.	34.6
Midazolam	NA	NA	NA	NA	0.94	200 mg q.d.	34.6
Sirolimus	0	0.15	0	0	0.85	200 mg q.d.	34.6
Cyclosporine	NA	NA	NA	NA	0.71	200 mg q.d.	34.6
Tacrolimus	0	0.08	0	0.01	0.47	200 mg q.d.	34.6

NA, no information available.
[a] For reference, see Table 3.
[b] Assume linear pharmacokinetics, i.e., double the dose, double the C_{max}.

TABLE 3

Drug-fluconazole interaction prediction using the hepatocytes in plasma model

Compound	-Fold of AUC Change				Reference
	Predicted[a]	Observed	Prediction Error	f_{renal}[b]	
			%		
Theophylline	1.00	1.19	−16.0	0.18	Obach et al., 2006[c,d]
Tolbutamide	2.44	2.09	16.5	0.001	Ito et al., 2004[d] Soars et al., 2003[e]
Omeprazole	2.61	6.29	−58.5	<0.01	Obach et al., 2006[d] Soars et al., 2003[e]
S-Warfarin	2.76	2.84–4.31	−22.9	<0.02	Ito et al., 2004[c,d] Obach et al., 2006[c,d]
Phenytoin	2.19	1.75	25.1	0.02	Ito et al., 2004[d] Rosemary et al., 2006[e]
Midazolam	2.89	3.60	−19.7	<0.01	Obach et al., 2005[d] Galetin et al., 2006[e]
Sirolimus	3.02	4.70	−35.7	Negligible	Ito et al., 2004[d] Lu et al., 2008[e]
Cyclosporine	1.98	1.84	7.6	<0.01	Ito et al., 2005[d] Galetin et al., 2006[e]
Tacrolimus	1.57	1.19	31.8	<0.01	Osowski et al., 1996[d] Lu et al., 2008[e]

[a] Predictions are based on our model assuming 200 mg b.i.d. dose of fluconazole have plasma C_{max} of 34.6 μM and linear pharmacokinetics of drugs. Prediction error is expressed as 100 × (predicted value − observed value)/observed value.
[b] Information on renal clearance was from Hardman et al. (2001).
[c] Assume theophylline and S-warfarin are selective substrates for CYP1A2 and CYP2C9, respectively.
[d] References for observed AUC changes.
[e] References for f_m.

proach should apply to most reversible P450 inhibitors. The effect of inhibitors and test compounds that are substrates and inhibitors of transporters is yet to be fully evaluated. The current approach has now been demonstrated to work with two CYP3A inhibitors, ketoconazole and fluconazole, which are not substrates but are inhibitors of Pgp. However, any inhibitor with properties similar to these inhibitors should work equally well. Of note, if a test compound is primarily metabolized by phase II enzymes, the salicylamide should not be used to block phase II conjugations.

The fold increase in AUC was predicted using the model [eq. 1 as described previously (Lu et al., 2007)]. In this model, the AUC ratio for a compound primarily cleared by metabolism can be expressed as a function of the remaining activity of each enzyme toward the metabolism of this compound or the remaining clearance routes:

$$\frac{AUC_I}{AUC} = \frac{CL}{CL_I} = \frac{1}{f_{m,\,hep}\,(f_{m\,3A4} f_{A\,3A4} + f_{m\,2C9} f_{A\,2C9} + \ldots) + f_{other} f_{A\,other}} \quad (1)$$

where $f_{m,\,hep}$ is the fraction of clearance by hepatic metabolism, $f_{m,\,P450}$ is the relative contribution of an isoenzyme to the total

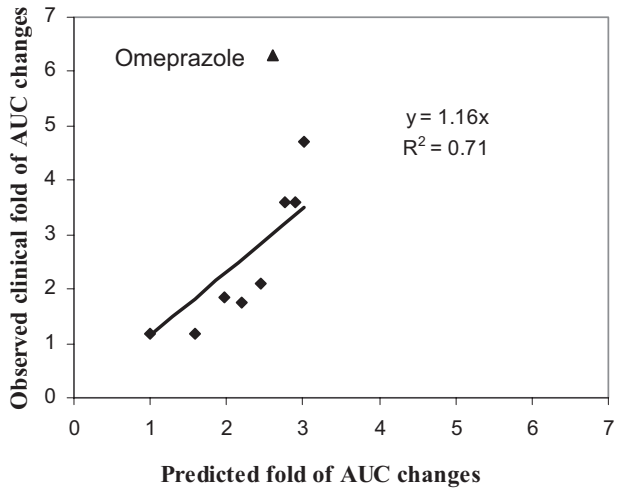

FIG. 2. Correlation of the observed clinical and the predicted DDI (fold of AUC change) using eq. 1. The data are from Table 3. Average value of *S*-warfarin was used.

metabolism of a compound (without inhibitor), f_A is the fraction of enzyme activity remaining in the presence of an inhibitor (Table 1), and f_{other} represents the clearance routes other than hepatic metabolism, such as renal or biliary.

Although $f_{m,\,P450}$ could be determined in several ways (Uttamsingh et al., 2005) including using the selective chemical inhibitors (Table 1), f_A in vivo can be estimated from f_A in vitro when the extracellular (plasma) concentrations are comparable in both systems. In the in vitro system, what is being measured is the velocity of the enzyme to metabolize a substrate. Under a competitive inhibition scenario (Segel, 1974):

$$f_A = \frac{v_1}{v} = \frac{\dfrac{V_{max}[S]}{K_m\left(1 + \dfrac{I}{K_i}\right) + [S]}}{\dfrac{V_{max}[S]}{K_m + [S]}} = \frac{K_m + [S]}{K_m\left(1 + \dfrac{I}{K_i}\right) + [S]} \quad (2)$$

When $[S] \ll K_m$,

$$f_A = \frac{v_1}{v} = \frac{1}{1 + \dfrac{I}{K_i}} \quad (3)$$

On the other hand, under a noncompetitive inhibition scenario (Segel, 1974):

$$f_A = \frac{v_1}{v} = \frac{\dfrac{V_{max}[S]}{K_m\left(1 + \dfrac{I}{K_i}\right) + [S]\left(1 + \dfrac{I}{K_i}\right)}}{\dfrac{V_{max}[S]}{K_m + [S]}} = \frac{1}{1 + \dfrac{I}{K_i}} \quad (4)$$

thus proving that the inverse of f_A represents the term $(1 + I/K_i)$, which has been used previously to calculate the exposure increases in the presence of inhibitors, encompassing the inhibitor concentration at the enzyme site. As we discussed previously (Lu et al., 2007), the plasma C_{max} at the steady state can be used to assess the inhibitory effect, keeping in mind, though, that C_{max} may vary from person to person because of interindividual variability in pharmacokinetics or from study to study owing to different dosing regimens. For a given C_{max} or a comparable in vitro concentration of an inhibitor, the fraction of enzyme activity remaining (f_A) can be calculated from the titration curves (Fig. 1; Table 1). For concentrations in between the range of two values listed in the table the corresponding f_A values can be determined by linear extrapolation of data. Equation 1 assumes linear pharmacokinetics and a plasma C_{max} of 34.6 μM at a 200 mg b.i.d. or q.d. dose of fluconazole (Hardman et al., 2001). For example, in an omeprazole study, fluconazole was dosed at 100 mg q.d. (Obach et al., 2006); thus, the C_{max} for our studies was adjusted to 17.3 μM. The $f_{A,\,3A4}$ and $f_{A,\,2C19}$ were calculated to be 0.379 and 0.620, respectively (Table 1 and eq. 6). Together with $f_{m,\,3A4}$ of 0.40 and $f_{m,\,2C19}$ of 0.60 from the literature (Soars et al., 2003) and assuming clearance only due to metabolism (i.e., $f_{m,\,hep} = 1$), a 2.61-fold increase in AUC is predicted.

The literature available on compounds with information on both clinical DDI with fluconazole and quantitative reactive phenotyping (f_m) is limited. Table 3 lists several compounds found in the recent literature (Osowski et al., 1996; Soars et al., 2003; Ito et al., 2004; Galetin et al., 2006; Obach et al., 2006; Rosemary et al., 2006) with the observed clinical DDI. The f_m values of sirolimus and tacrolimus were determined in house (Lu et al., 2008). Although no reactive phenotyping information is available for theophylline and *S*-warfarin, these two compounds were treated as prototypical substrates for CYP1A2 and CYP2C9, respectively, for retroactive prediction based on our study. Midazolam is widely considered to be exclusively a CYP3A4 substrate. Omeprazole was found to be metabolized by CYP2C19 (60%) and CYP3A4 (40%), whereas tolbutamide was found to be metabolized by CYP2C9 (72%) and CYP2C19 (28%) (Soars et al., 2003). Phenytoin is 90% cleared by CYP2C9, and the rest is cleared by CYP2C19 (Rosemary et al., 2006). CYP3A4 was reported to contribute 71% to the metabolism of cyclosporine (Galetin et al., 2006), but the rest of the clearance routes have not been documented. In this study, the prediction for cyclosporine was based on the assumption that fluconazole does not affect the nonhepatic clearance routes. The dosing regimen of fluconazole was not available for the interaction studies with tolbutamide and phenytoin; therefore, it was assumed that the normal 200 mg b.i.d. dose was applied. In vivo, human plasma fluconazole concentration values vary in the literature; a C_{max} of 34.6 μM at 200 mg b.i.d. was adapted to calculate the f_A values (Hardman et al., 2001). The observed clinical AUC increase values were adapted from Osowski et al. (1996), Ito et al. (2005), and Obach et al. (2006). For most of the compounds, renal clearance plays a minor role ($\leq$1%) (Table 3), and thus the renal clearance contribution was not included in the calculation. For theophylline, the effect of fluconazole on renal clearance is unknown. Thus, the effect was not taken into consideration. Likewise, no biliary clearance was available for any compounds, and hence this contribution was not considered. For *S*-warfarin clinically observed AUC change ranges from 2.84 (Ito et al., 2005) to 4.31 (Obach et al., 2006). An average of these values was used for Fig. 2. With the use of this information and the fluconazole cross-inhibition data in Table 1, the prediction of AUC changes are presented in Table 3. Notably, these predictions compare fairly closely to the observed clinical values (r^2 = 0.71 and slope of 1.16) (Fig. 2), except for omeprazole. Omeprazole is known to be taken up by active transport and accumulates in cytosol of hepatocytes (Sewell et al., 1994). It is assumed that the cryopreserved hepatocytes used in our study may have only partially preserved the uptake capacity (Shitara et al., 2003). Therefore, the in vitro results underpredicted the in vivo outcome. Also, there was only a single clinical DDI study reported in the literature. Thus, omeprazole was treated as an outlier and was not included for correlation analysis.

In summary, under linear kinetics, the DDI predictions were made using reactive phenotyping (the relative contributions of enzymes to the overall metabolism of a drug, an intrinsic property of the drug) and the P450 cross-inhibition by the inhibitor fluconazole at close to the physiological conditions (hepatocyte incubation in human plasma). In contrast to our previous work on ketoconazole, which provides information for the worst-case DDI scenario, fluconazole as used in this study provides a moderate-case DDI scenario, also taking into account inhibition of P450s other than CYP3A by fluconazole. Considering that the correlation shown in Fig. 2 was built on data from many different studies by different investigators, with some information missing in the literature, and certain assumptions were made, the observation of r^2 of 0.71 is considered remarkable. Thus, this model can be considered to have general applicability to P450 inhibitors for predicting the DDI quantitatively early in the preclinical development stage, with the main advantage being not having to estimate the elusive inhibitor concentration at the enzyme site.

Acknowledgments. The authors thank Drs. Gerald T. Miwa and Liang-Shang Gan for valuable discussions.

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Address correspondence to: Dr. Chuang Lu, Millennium Pharmaceuticals, Inc., 40 Landsdowne St., Cambridge, MA 02139. E-mail: chuang.lu@mpi.com