Discovery of a Highly Selective CYP3A4 Inhibitor Suitable for Reaction Phenotyping Studies and Differentiation of CYP3A4 and CYP3A5^[S]

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ABSTRACT:

Current molecular tools lack the ability to differentiate the activity of CYP3A4 and CYP3A5 in biological samples such as human liver microsomes. Kinetic experiments and the CYP3A4 crystal structure indicate that the active sites of both enzymes are large and flexible, and have more than one binding subsite within the active site. 1-(4-Imidazopyridinyl-7-phenyl)-3-(4'-cyanobiphenyl) urea (SR-9186) was optimized through several rounds of structural refinement from an initial screening hit to obtain greater than 1000-fold selectivity for the inhibition of CYP3A4 versus CYP3A5. Characterization data demonstrate selectivity using midazolam and testosterone hydroxylation assays with recombinant cytochrome P450,

pooled human liver microsomes, and individually genotyped microsomes. Clear differences are seen between individuals with CYP3A5*1 and *3 genotypes. The antifungal drug ketoconazole is the most commonly used CYP3A inhibitor for in vitro and in vivo studies. A direct comparison of SR-9186 and ketoconazole under typical assay conditions used in reaction phenotyping studies demonstrated that SR-9186 had selectivity over CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A5 greater than or equal to that of ketoconazole. In addition, the long half-life (106 min) of SR-9186 in incubations containing 1 mg/ml human liver microsomes provided sustained CYP3A4 inhibition.

Introduction

The lack of proper chemical tools to differentiate the activity of CYP3A4 and CYP3A5 has led to the long-standing, yet erroneous, convention of treating the two enzymes as if they were one. Sometimes activity has been expressed as CYP3A to point out that the results are not specific for either CYP3A4 or CYP3A5. However, too often all activity has been attributed to CYP3A4. Furthermore, the practice of pooling tissue from multiple donors to generate an “average human” has given rise to the perception that CYP3A5 has less significance than CYP3A4. Although it is certainly true that the abundance of CYP3A4 exceeds that of CYP3A5 in a multidonor pool, this is not representative of actual patients. The concentrations of the two enzymes have been reported to be roughly equal in individuals who express CYP3A5 (Lin et al., 2002).

Enzyme-selective chemical inhibitors are commonly used in reaction phenotyping studies to determine the contribution of individual cytochrome P450 isoforms (Clarke, 1998). Knowledge of the metabolic pathway for a candidate compound allows more accurate pre-

dictions of potential drug-drug interactions. Quality chemical tools are available to determine the activity and inhibition of most of the major cytochromes P450 involved in xenobiotic metabolism. The commonly used CYP3A4 and CYP3A5 inhibitors inhibit both enzymes, precluding differentiation or activity in complex samples. Although no highly selective CYP3A4 or CYP3A5 inhibitors have been reported in the literature, several compounds have been shown to have modest 3- to 10-fold selectivity over CYP3A5 (Rendic, 2002; Williams et al., 2004). This degree of selectivity is not sufficient to inhibit 90+ % of CYP3A4 without significant CYP3A5 inhibition. Despite the inability of these mildly selective inhibitors to serve as in vitro tools for the isolation of CYP3A5 activity, they demonstrate that a degree of selectivity is achievable across numerous structural classes. In general, CYP3A4 appears to be more susceptible to irreversible inactivation in the presence of compounds that are metabolized to reactive metabolites, and many of the published selective inhibitors are time-dependent inhibitors (Khan et al., 2002; Stresser et al., 2004; Pearson et al., 2007). It should be stressed that many of the selective inactivators are not selective inhibitors because they display significant competitive inhibition of CYP3A5 despite their lack of time-dependent inactivation.

CYP3A5 has three common genetic alleles (Leskelä et al., 2007). CYP3A5*1 leads to the expression of active, full-length CYP3A5. The CYP3A5*3 (22893A→G) allele in intron 3 leads to a frameshift, resulting in the majority of the CYP3A5 mRNA yielding inactive protein and loss of CYP3A5 expression. Analysis by Western blot and RT-PCR demonstrates that individuals homozygous for CYP3A5*3

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ABBREVIATIONS: P450, cytochrome P450; LC, liquid chromatography; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; MS/MS, tandem mass spectrometry; SR-9186, 1-(4-imidazopyridinyl-7-phenyl)-3-(4'-cyanobiphenyl) urea.

SCHEME 1. Synthesis of SR-9186. Reaction conditions: a, CoCl_2 , toluene, triethylamine; b, ArCl , $\text{Pd}(\text{Ph}_3\text{P})_4$, K_2CO_3 , tetrahydrofuran; c, 2, 3-(4)-dimethylaminocinnamylidene.

have low levels of correctly spliced CYP3A5 mRNA, and this corresponds to sharply lower CYP3A5 protein levels (Leskelä et al., 2007). A second allele, *CYP3A5**6 (30597G→A), on exon 7 causes the deletion of exon 7 from the splice variant and is associated with lower CYP3A5 catalytic activity.

Selective inhibitors will refine the current prediction models for pharmacokinetic drug-drug interactions in which the catalytic efficiency of CYP3A4 and CYP3A5 can be accounted for and the influence of genetic polymorphisms can be incorporated in future models. A better understanding of both enzymes is important to make accurate clearance predictions before compounds are moved into human trials. The clinical relevance of the CYP3A5 genotype is seen with the immunosuppressant tacrolimus, which is metabolized by CYP3A5 3 times more efficiently than by CYP3A4 (Barry and Levine, 2010). To maintain the required tacrolimus trough concentrations of 5 to 15 ng/ml, patients who express active CYP3A5 (*1/*1 and *1/*3 genotypes) require approximately twice the dose as patients with the *3/*3 (inactive splice variant) genotype (Zhao et al., 2005). In addition, the CYP3A5 genotype has been implicated in vincristine metabolism, and CYP3A5 low expressers were found to have an increased risk of vincristine-induced neurotoxicity (Dennison et al., 2006, 2007, 2008a; Egbelakin et al., 2011).

The current article details a highly selective CYP3A4 inhibitor suitable for isolation of CYP3A5 activity in human liver microsomes. We have published details of the structure-activity relationship of SR-9186 and a number of related analogs for selective CYP3A4 inhibition (Song et al., 2012). In this study, we put special emphasis on the use of the inhibitor under conditions encountered in conducting reaction phenotyping studies, such as high concentrations of microsomal protein and long incubation times. Specific recommendations are made for the use of this new tool compound.

Materials and Methods

Chemicals Used. Midazolam, testosterone, phenacetin, coumarin, bupropion, paclitaxel, tolbutamide, (*S*)-mephenytoin, dextromethorphan, chlorzoxazone, vincristine, and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO). All solvents used for LC-mass spectrometry were of chromatography grade. HLM (pooled) were purchased from XenoTech, LLC (Lenexa, KS). CYP3A5 genotyped individual donor hepatic microsomes were purchased from both XenoTech, LLC, and BD Biosciences (San Diego, CA). Donor lots were as follows: *CYP3A5**1/*1 donors were HH739, HH47, HH867, HH785, HH860, 07100271, 0710272, and 0810554 (XenoTech, LLC, lots start with the HH prefix); *CYP3A5**1/*3 donors were HH757, HH868, HH54, 710239, 0710231, and 0710232; and *CYP3A5**3/*3 donors were HH61, HH792, HH189, HH837, 710233, 710253, and 710234. All solutions were prepared from Milli-Q-treated water with a specific resistance $\geq 17.8 \text{ M}\Omega$.

Synthesis of SR-9186. SR-9186 was synthesized in three steps starting from commercially available reagents as shown in Scheme 1. 4'-Amino-[1,1'-biphenyl]-4-carbonitrile (1) was converted to the corresponding isocyanate (2) by treatment with phosgene in toluene with essentially quantitative yield. The 4-pinacol borate ester of aniline (3) was coupled with 7-chloro-3*H*-imidazo[4,5-*b*]pyridine under typical Suzuki conditions to afford the biaryl aniline 4. Finally, coupling of this aniline with the isocyanate (2) yielded SR-9186 (5). Purification by reverse-phase preparative HPLC followed by lyophilization gave analytically pure material as a pale yellow solid. Purity was estimated at >99% based on HPLC-UV. The NMR spectrum is provided in Supplemental Fig. 1.

Enzyme Assays. Human liver microsomes. P450 activity was assayed using selective marker reactions. Details on substrates used and their corresponding metabolites plus concentrations and incubation times are given in Table 1. Substrate concentrations were approximately equal to their respective K_m values. All incubations were prepared in 0.1 M potassium phosphate buffer, pH 7.4, and incubated at 37°C with shaking. Testosterone and midazolam are referred to as CYP3A substrates because they are metabolized in HLM by both CYP3A4 and CYP3A5. Analysis was by LC-MS/MS using an API4000 mass spectrometer (Applied Biosystems, Foster City, CA) interfaced with an Agilent

TABLE 1
Incubation conditions

P450	Substrate	Metabolite	HLM mg/ml	Incubation Time min
1A2	50 μM phenacetin	Acetaminophen	0.05	30
2A6	1 μM coumarin	7-Hydroxycoumarin	0.05	10
2B6	75 μM bupropion	hydroxybupropion	0.1	15
2C8	10 μM paclitaxel	6 α -Hydroxypaclitaxel	0.1	15
2C9	100 μM tolbutamide	Hydroxytolbutamide	0.1	20
2C19	50 μM (<i>S</i>)-mephenytoin	4'-hydroxymephenytoin	0.2	30
2D6	5 μM dextromethorphan	Dextrorphan	0.05	10
2E1	100 μM chlorzoxazone	6-Hydroxychlorzoxazone	0.1	10
3A	5 μM midazolam	1'-Hydroxymidazolam	0.025	5
3A	75 μM testosterone	6 β -Hydroxytestosterone	0.05	10

FIG. 1. Selective inhibition of CYP3A4 versus CYP3A5 by SR-9186 in recombinant (r) P450s. Triplicate incubations with SR-9186 (4 nM–60 μ M) were coincubated with 1 mM NADPH and 1 nM recombinant P450 in 100 mM phosphate buffer, pH 7.4. Probe substrates were 2.5 μ M midazolam, 75 μ M testosterone, or 20 μ M vincristine.

1200 high-performance liquid chromatograph (Agilent Technologies, Palo Alto, CA). In most cases, chromatographic separation was achieved by using a Synergi Fusion RP C18 column (2.0 $\times$ 50 mm, 4 μ m; Phenomenex, Torrance, CA) with a mobile phase consisting of 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) run at a constant flow rate of 0.375 ml/min. A 2.5-min HPLC method was used with % B equal to 2% at $t = 0$ min, 80% at $t = 1.35$ to 1.6 min, and 2% at $t = 1.61$ to 2.5 min (all gradients were linear).

Because the selective inhibitor is likely to be used in phenotyping experiments, it was important to evaluate IC_{50} changes over a range of HLM concentrations. Incubation time was decreased to 1 min in these experiments to try to maintain linear kinetics. Specific changes from those conditions detailed in Table 1 are found under *Results* or in the figure legends.

Vincristine M1 formation in genotyped individual donor microsomes was evaluated with slight modification of established methods (Dennison et al., 2007, 2008b). Incubations containing 20 μ M vincristine, 0.1 mg of HLM protein/ml, and 1 mM NADPH prepared in 100 mM potassium phosphate buffer, pH 7.4, were stopped after 15 min by the addition of an equal volume of acetonitrile containing 1 μ M vinblastine as an internal standard. Analysis of vincristine M1 was by LC-MS/MS using a RP-amide column (Ascentis Express, 2.7 μ m, 2.1 $\times$ 100 mm; Supelco, Bellefonte, PA) at 300 μ l/min with a 9-min linear gradient elution from 85% A (water + 0.1% formic acid) to 80% B (acetonitrile + 0.1% formic acid).

Recombinantly expressed P450. Incubations using recombinantly expressed P450 (BD Supersomes; BD Biosciences) were conducted similarly to what is described for HLM. Enzyme concentrations for CYP3A4 and CYP3A5 incubations were 10 nM (2 pmol of enzyme in 0.2 ml). Substrate concentrations are the same as those listed in Table 1 unless otherwise indicated in the text. Incubations were initiated by the addition of 1 mM NADPH and terminated by the addition of an equal volume of acetonitrile.

Data Analysis. Data were curve-fit using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Unless otherwise noted, the built-in one-site competition model was used with the equation $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(X - \log IC_{50})})$, where X is $\log(\text{concentration})$, Y is percent inhibition, and bottom and top refer to the minimum and maximum for the curves. In most cases the values were constrained between 0 and 100%. Correlation analysis was done in GraphPad Prism with two-tailed P value determination using the Pearson method, which assumes that the data are from Gaussian populations.

Results

Initial screening efforts identified a compound with 30-fold selectivity for CYP3A4 over CYP3A5. This compound was optimized through successive rounds of medicinal chemistry as described in Song et al. (2012), and the synthesis of the optimized compound, SR-9186, is shown in Scheme 1.

In incubations using recombinantly expressed P450, SR-9186 was a potent CYP3A4 inhibitor with calculated IC_{50} values for inhibition

of midazolam $\rightarrow$ 1'-hydroxymidazolam, testosterone $\rightarrow$ 6 β -hydroxytestosterone, and vincristine $\rightarrow$ vincristine M1 of 9, 4, and 38 nM, respectively. Selectivity was demonstrated using recombinant CYP3A5, for which SR-9186 was a much weaker inhibitor. SR-9186 did not inhibit any of the reactions by 50% when tested at concentrations up to 60 μ M. When the CYP3A5 inhibition data were curve-fit to a sigmoidal one-site competition model, inhibition constants of 7.4, 0.36, and 1.5 μ M were calculated. The data are presented graphically in Fig. 1, showing a large concentration window in which CYP3A4 is 90+ % inhibited, but CYP3A5 is inhibited less than 20%. The reason for the larger CYP3A5 versus CYP3A4 S.D. values for SR-9186 inhibition using all three probe substrates is unknown, but similar results were obtained when repeated on three occasions.

To evaluate the potential utility of SR-9186 in phenotyping studies, several experiments were conducted. Phenotyping experiments are typically conducted using high concentrations of liver microsomes, whereas inhibition constants are usually determined at low microsomal concentration. The effect of HLM (pooled 150 donor) concentration was evaluated for SR-9186. Four HLM concentrations were used: 0.05, 0.25, 0.5, and 1 mg/ml. Testosterone hydroxylation was used as the measure of CYP3A activity, and the incubation time was reduced to 1 min to maintain linearity (testosterone levels were measured at the end of the reaction and did not decrease by more than 10% compared with $-NADPH$ controls). Increases in microsomal protein induced large shifts in the observed IC_{50} values for SR-9186 (Fig. 2). At the lowest HLM concentration, potency was similar to what was found using recombinant enzymes.

Selectivity of SR-9186 was compared with that of ketoconazole (Fig. 3) for 8 other hepatic P450 in incubations containing 1 mg/ml HLM, chosen to mimic conditions common in phenotyping experiments. Inhibitor concentrations were 2.5 μ M SR-9186, approximately 10-fold greater than the CYP3A4 IC_{50} in the presence of 1 mg/ml microsomal protein, and 1 μ M ketoconazole on the basis of historical precedence. Ketoconazole exhibited equivalent or greater inhibition than SR-9186 for each isoform tested. The lack of specific probe substrates precluded the direct evaluation of CYP3A4 and CYP3A5 activity in human liver microsomes. As a surrogate, four-donor CYP3A5 genotype specific pools were created by mixing equal amounts from genotyped individual hepatic microsomes. The pan-CYP3A inhibitor ketoconazole (1 μ M) inhibited midazolam hydroxylation by greater than 90% irrespective of the genotype (Fig. 3B). The remaining activity with SR-9186 (2.5 μ M) was 4-fold greater for microsomes from CYP3A5*1/*1 or *1/*3 donors compared with that for *3/*3 donors.

FIG. 2. Effect of human liver microsomal protein level on observed IC_{50} . The inhibition of testosterone 6β -hydroxylation by SR-9186 was evaluated in triplicate assays using four concentrations of human liver microsomal protein. The protein concentration ranged from 0.05 mg/ml, commonly used in enzyme inhibition assays, to 1.0 mg/ml, common in phenotyping experiments. Calculated IC_{50} values were 10, 51, 108, and 224 nM for 0.05, 0.25, 0.50, and 1.0 mg of HLM protein/ml, respectively.

SR-9186 and ketoconazole were both sufficiently stable in microsomal incubations to be used in phenotyping experiments. The half-life of 2.5 μ M SR-9186 or 1 μ M ketoconazole in incubations containing 0.2 or 1 mg/ml HLM is shown in Table 2. Under all conditions tested, the half-life of both compounds was greater than 1 h.

When a nonspecific substrate such as testosterone or midazolam is used, the majority of the CYP3A activity in incubations using a multiple-donor nonbiased pool of HLMs should come from CYP3A4. However, this does not translate to *CYP3A5**1 individual donors in whom both enzymes significantly contribute to CYP3A activity. To test whether or not the large CYP3A4 and CYP3A5 differential inhibition observed in incubations of recombinant enzyme would translate to physiological samples, hepatic microsomes from individually genotyped donors were evaluated. Six to eight donors with *1/*1, *1/*3, and *3/*3 *CYP3A5* polymorphisms were tested (Fig. 4A). Midazolam hydroxylation was used as a measure of total CYP3A activity because the catalytic efficiency of midazolam oxidation to 1'-hydroxymidazolam as described by V_{max}/K_m is similar for both CYP3A4 and CYP3A5 (Gibbs et al., 1999). The observed traces

for all three genotypes had similar IC_{50} values. The magnitude of the differences in calculated IC_{50} for *3/*3 and *1/*1 donors was small but statistically different ($p = 0.038$) (Fig. 4B). The maximal percent inhibition was highly significant ($p < 0.0002$) with individual donors lacking at least one *CYP3A5**1 gene exhibiting greater maximum inhibition (Fig. 4C).

To further evaluate the ability of SR-9186 to essentially isolate CYP3A5 activity, individually genotyped donors were profiled for their ability to catalyze the formation of vincristine M1. Vincristine M1 has previously been reported to be catalyzed more efficiently by CYP3A5 with approximately 10-fold selectivity over CYP3A4 (Dennison et al., 2006, 2007). Our results were consistent with this report as can be seen in Fig. 5, where vincristine M1 formation was much higher for *CYP3A5**1/*1 and *1/*3 individuals. Vincristine M1 formation slightly correlated with total CYP3A activity (estimated by following the formation of 1'-hydroxymidazolam) (Fig. 5A), but the correlation was greatly improved versus that with CYP3A5 (estimated by minimizing the CYP3A4 contribution with the addition of 2.5 μ M SR-9186) (Fig. 5B). The calculated two-tailed Pearson correlation coefficient increased from $r = 0.5524$ ($p = 0.0094$) to $r = 0.9798$ ($p = < 0.0001$).

It should be pointed out that when dissolved in aqueous solutions, SR-9186 extensively binds to plastic, leading to large errors at low concentrations (Fig. 6). This phenomenon is minimized with organic solvents or in the presence of proteins such as hepatic microsomes, and is of particular importance when dilutions are made. Dilutions should be made in organic solvents and directly transferred to the incubation. The use of an intermediate plate or spiking of SR-9186 into buffer and preparing a curve through serial dilutions will give poor results.

Discussion

Only a handful of compounds are known to have a significant (>5-fold) preference for CYP3A4 versus CYP3A5, either as a substrate or as an inhibitor. This is probably because few compounds have been examined for differences and the data that exist are largely unreported. With the recent trend in the pharmaceutical industry to minimize the potential for drug-drug interactions by moving away from CYP3A4 substrates and inhibitors, it is possible that we will inadvertently see an increased number of CYP3A5-biased inhibitors that would not have been recognized as potent inhibitors using tradi-

FIG. 3. Selectivity under phenotyping conditions. A, the effect of 1 μ M ketoconazole or 2.5 μ M SR-9186 was evaluated in incubations containing 1 mg/ml HLM protein (150 donor mixed gender) using the marker substrates and substrate concentrations indicated in Table 1. Incubation time was reduced to 1 min. B, inhibition of midazolam hydroxylation was similarly evaluated in *CYP3A5**1/*1, *1/*3, and *3/*3 pooled microsomes (four individual donors mixed 1:1:1:1).

TABLE 2

Stability in microsomal incubations

The half-lives of ketoconazole and SR-9186 were evaluated in microsomal incubations containing high levels of HLM protein and 1 mM NADPH in 100 mM phosphate buffer, pH 7.4.

	$t_{1/2}$ for HLM Protein	
	0.2 mg/ml	1.0 mg/ml
	min	
Ketoconazole (1 μ M)	130	68
SR-9186 (2.5 μ M)	380	110

tional methodologies of pooled HLM and pan-substrates such as midazolam or testosterone.

We believe that SR-9186 is an exciting new in vitro chemical tool for the differentiation of CYP3A4 and CYP3A5 activities. The massive costs required to demonstrate human safety for a compound without therapeutic benefit would make it naive to propose designing a CYP3A4-selective in vivo inhibitor. However, as appropriate chemical tools are developed to clearly differentiate CYP3A4 and CYP3A5 activity, approved drugs with sufficient selectivity to help elucidate the individual contributions of the two enzymes in vivo may be discovered.

SR-9186 is the first compound that can differentiate the contributions of CYP3A4 and CYP3A5. The data presented have been focused

on a reaction phenotyping application. Such experiments typically use high concentrations of hepatic microsomes and long incubation times. To have utility in phenotyping experiments, the inhibitor must be irreversible or have sufficient metabolic stability that the inhibition is maintained over the entire incubation. SR-9186 concentrations were minimally affected in HLM incubations, and it had a microsomal half-life similar to that of ketoconazole.

Data regarding time-dependent inhibition are presented in Supplemental Fig. 2. Preincubation of SR-9186 with or without NADPH in human liver microsomes did not alter the selectivity of SR-9186. There was a very small increase in inhibition potency against CYP3A4 after a 30-min preincubation, but the difference was negligible. Performance of SR-9186 as a selective inhibitor of CYP3A4 was not improved by preincubation. The small increase in potency appeared to be due to the formation of a metabolite-intermediate complex that could be displaced by potassium ferricyanide. The minimal importance of the time-dependent inhibition is hypothesized to occur because of the reversible nature of the metabolite-intermediate complex, the high inhibition potency of the parent (SR-9186) and the slow rate of SR-9186 metabolism.

The remaining CYP3A activity in the presence of SR-9186 was significantly higher in donors with at least one *CYP3A5*1* gene. This was in agreement with expectations and with earlier studies in which

FIG. 4. Inhibition of midazolam hydroxylation by SR-9186 in genotyped individual donor human liver microsomes. Triplicate incubations using individual donor microsomes were evaluated for formation of 1'-hydroxymidazolam. A, data corresponding to **1/*1* donors (blue with a narrow dashed line), **1/*3* donors (orange with wider dashed line), and **3/*3* donors (black solid lines) were analyzed for changes in the observed IC_{50} (B) and for maximal inhibition (C). Statistical analysis is a two-tailed unpaired *t* test assuming unequal variance.

FIG. 5. Isolation of CYP3A5 activity through selective inhibition of CYP3A4. Vincristine M1 formation in genotyped individual donor human liver microsomes was compared with total CYP3A activity, determined by following the hydroxylation of midazolam to 1'-hydroxymidazolam (A). B, vincristine M1 formation is compared with CYP3A5 through selective inhibition of CYP3A4 with the addition of 2.5 μ M SR-9186 to the incubation. Values reflect the ratio of the peak area detected by LC-MS/MS with an internal standard and are the average of $n = 3$ replicates.

Western blots were used to estimate CYP3A4 and CYP3A5 content in human intestinal samples from donors with different CYP3A5 genotypes (Lin et al., 2002). The Western blot data indicated that the ratio of CYP3A4 to CYP3A5 was similar in *1/*3 and in *1/*1 individuals. Our activity data support this observation in hepatic samples in which the remaining CYP3A activity was similar between homo- and heterozygous *1 individuals when CYP3A4 activity was inhibited by SR-9186. No differences were observed in the profiles from individual donor microsomes purchased from the separate vendors, Xeno-Tech, LLC, or BD Biosciences.

Catalysis of the conversion of vincristine to its major ring-opened metabolite, vincristine M1, has been shown to be more efficient for CYP3A5 than for CYP3A4 experimentally (Dennison et al., 2006, 2007) and clinically (Egbelakin et al., 2011). Under the conditions used in the present study, the rate of catalysis by CYP3A5 would have been expected to be approximately 10 times the rate by CYP3A4. This allowed vincristine M1 formation to be used as a marker for CYP3A5 activity in individual donor microsomes. The improved correlation between midazolam hydroxylation and vincristine M1 formation when SR-9186 was added to the incubation gives confidence to the conclusion that SR-9186 can selectively inhibit CYP3A4 and allow for the isolation of CYP3A5 activity.

Sufficient individual donors were not available to evaluate the selectivity of SR-9186 on the inhibition of CYP3A5*6. CYP3A7 is primarily considered to be a fetal version of CYP3A but has been

reported to be at higher levels in some adults and capable of contribution to total CYP3A activity in a meaningful way in a small percentage of patients (Sim et al., 2005; Daly, 2006). Inhibition of CYP3A7 activity was not observed by SR-9186 when tested in recombinantly expressed CYP3A7 (data not shown).

Although this article focuses on the use of SR-9186 as a probe molecule, it also demonstrates that high selectivity is achievable for the inhibition of CYP3A4 versus CYP3A5. Fluconazole, which has modestly greater potency for the inhibition of CYP3A4 versus CYP3A5, causes larger clinical pharmacokinetic drug-drug interactions with midazolam for patients with the CYP3A5*3/*3 genotype than for those that have at least one *1 allele (Isoherranen et al., 2008). The protection provided *1 individuals against the drug interaction would be expected to be even more pronounced with a more selective CYP3A4 inhibitor such as SR-9186.

The selectivity of SR-9186 was tested using three CYP3A substrates: testosterone, midazolam, and vincristine. CYP3A4 and CYP3A5 inhibition profiles using recombinant P450 had slightly different profiles for the three probe substrates. In all cases, SR-9186 was highly selective for the inhibition of CYP3A4.

In conclusion, for phenotyping experiments, a concentration of 2.5 μ M SR-9186 was shown to preferentially inhibit CYP3A4 in microsomal incubations. We have attempted to give guidance for use of the SR-9186 and anticipate possible problems that might be encountered by researchers such as nonspecific binding to plastic and the IC_{50} shift in the presence of high HLM protein concentrations. The need to account for nonspecific microsomal binding is well known and commonly corrected for when intrinsic hepatic clearance is calculated (Austin et al., 2002; Riley et al., 2005), but it is often neglected when inhibition is evaluated. To achieve maximal selectivity for CYP3A4 over CYP3A5, the concentration of SR-9186 may need to be adjusted, depending on the concentration of microsomal protein used in the incubation.

A better understanding of the effect of CYP3A5 $\pm$ genotype would be a positive step toward individualizing patient medication not only for efficacy but also for safety. Compounds for which in vitro assays indicate that CYP3A5 polymorphisms may be important in efficacy or susceptibility to drug-drug interactions can be evaluated in intelligently designed clinical drug-drug interaction studies similar to the way high-expressing and low-expressing CYP2D6 individuals are now evaluated for compounds that are cleared through the CYP2D6 pathway.

FIG. 6. Nonspecific binding to plastic. Triplicate standard curves were prepared in a 96-well polypropylene plate by adding 1 μ L of SR-9186 standards (prepared in dimethyl sulfoxide) to 199 μ L of water or acetonitrile. The plate was allowed to sit for 30 min and then directly analyzed by LC-MS/MS.

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Authorship Contributions

Participated in research design: Cameron and Li.

Conducted experiments: Li.

Contributed new reagents or analytic tools: Song and Kamenecka.

Performed data analysis: Cameron and Li.

Wrote or contributed to the writing of the manuscript: Cameron and Li.

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