Selection of Alternative CYP3A4 Probe Substrates for Clinical Drug Interaction Studies Using In Vitro Data and In Vivo Simulation

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Alternative CYP3A4 DDI Probe Substrates

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Abbreviations:

DDIs, drug-drug interactions; HPLC, high-performance liquid chromatography,
LC-MS/MS, liquid chromatography/tandem mass spectrometry
Abstract

Understanding the potential for P450-mediated drug-drug interactions (DDI) is a critical step in the drug discovery process. DDI of CYP3A4 is of particular importance, due to the number of marketed drugs which are cleared by this enzyme. In response to studies suggesting the presence of several binding regions within the CYP3A4 active site, multiple probe substrates are often used for in vitro CYP3A4 DDI studies, including midazolam (the clinical standard), felodipine/nifedipine and testosterone. However, design of clinical CYP3A4 DDI studies may be confounded for cases such as AMG 458, where testosterone is predicted to exhibit a clinically relevant DDI (AUC/I/AUC ≥ 2), while midazolam and felodipine/nifedipine are not. In order to develop an appropriate path forward for such clinical DDI studies, the inhibition potency of twenty known inhibitors of CYP3A4 were measured in vitro using eight clinically relevant CYP3A4 probe substrates and testosterone. Hierarchical clustering suggested four probe substrate clusters: testosterone; felodipine; midazolam, buspirone, quinidine and sildenafil; and simvastatin, budesonide and fluticasone. The in vivo sensitivities of six clinically relevant CYP3A4 probe substrates (buspirone, cyclosporine, nifedipine, quinidine, sildenafil and simvastatin) were determined in relation to midazolam from literature DDI data. Buspirone, sildenafil and simvastatin exhibited similar or greater sensitivity than midazolam to CYP3A4 inhibition in vivo. Finally, SimCYP was used to predict the in vivo magnitude of CYP3A4 DDI caused by AMG 458 using midazolam, sildenafil, simvastatin and testosterone as probe substrates.
Introduction

The cytochrome P450 superfamily of drug metabolizing enzymes is involved in the metabolism of the majority of currently prescribed drugs and new chemical entities. Within the P450 superfamily, CYP3A4 is responsible for the metabolism of approximately 55% of marketed drugs (Wienkers and Heath, 2005). Due to its general importance in drug clearance, assessment and modeling of CYP3A4 inhibition is a critical part of the drug discovery and development process. Probe substrate-dependent inhibition profiles have been observed in vitro with CYP3A4, possibly due to the presence of multiple probe substrate binding regions within the CYP3A4 active site (Kenworthy et al., 1999). In response, a standard approach when testing for CYP3A4 inhibition has been to use multiple probe substrates such as midazolam (the clinical standard), felodipine/nifedipine or testosterone for in vitro experiments (Wienkers and Heath, 2005). Extrapolation of the in vitro data to the in vivo situation may be confounded in the multiple probe substrate scenario if a probe substrate that is not clinically relevant, such as testosterone, is markedly more susceptible to inhibition than midazolam (the clinical standard) and felodipine/nifedipine (Obach et al., 2005). Our first aim was to measure the in vitro inhibition profiles of eight clinically relevant CYP3A4 probe substrates (budesonide, buspirone, felodipine, fluticasone, midazolam, quinidine, sildenafil and simvastatin) and testosterone versus a panel of twenty known CYP3A4 inhibitors and to determine the similarity of the probe substrates based upon hierarchical clustering of the inhibition data.
Recent draft guidance from the U.S. Food and Drug Administration outlining the design of P450-mediated drug interaction experiments suggests that sensitive CYP3A4 probe substrates other than midazolam may be used for clinical DDI studies (Huang et al., 2007). However, few studies have compared the in vivo sensitivity of CYP3A4 probe substrates based upon clinical DDI data from the literature (Ragueneau-Majlessi et al., 2007). Our second aim was to mine the literature for clinical CYP3A4 DDI data and correlate the in vivo DDI sensitivity of clinically relevant probe substrates with midazolam. Our third aim was to integrate the in vitro correlation results and in vivo sensitivity analysis to develop a strategy for selecting alternate CYP3A4 probe substrates for the testosterone-selective inhibition situation, if needed.

Our fourth and final aim was to demonstrate a case study where evaluation of alternate CYP3A4 probe substrates was warranted based upon in vitro inhibition data. AMG 458 (Liu et al., 2008), a potent inhibitor of the receptor tyrosine kinase c-Met, exhibits markedly more potent inhibition of testosterone 6β-hydroxylation than midazolam 1'-hydroxylation and felodipine dehydrogenation in vitro. We used SimCYP, a physiologically-based modeling tool, to simulate the magnitude of effect AMG 458 would have on the alternate CYP3A4 probe substrates and to select an appropriate probe substrate for the clinical DDI study based upon the in silico predictions.
Materials and Methods

Materials. Pooled human liver microsomes (15 individual donors) were purchased from CellzDirect (Durham, NC). AMG 458 was obtained from the Amgen Sample Bank (Thousand Oaks, CA). Ammonium formate, HPLC-grade acetonitrile and HPLC-grade methanol were obtained from Alfa Aesar (Ward Hill, MA). Cyclosporine A was obtained from Biomol International (Plymouth Meeting, PA). Felodipine was obtained from Alltech Associates (Deerfield, IL). Dehydrofelodipine, fluticasone 17β-carboxylic acid, N-desmethylsildenafil, 6β-hydroxybudesonide, 6’-hydroxybuspirone and sildenafil were obtained from Toronto Research Chemicals, Inc (North York, ON, Canada). 3'-Hydroxysimvastatin was obtained from USBiological (Swampscott, MA). NADPH was purchased from EMD Biosciences (San Diego, CA). All other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available.

K_i Determination. Incubations were carried out using eight probe substrates of CYP3A4: budesonide, buspirone, felodipine, fluticasone, midazolam, quinidine, sildenafil, simvastatin and testosterone. Twenty known inhibitors of CYP3A4 exhibiting a wide range of inhibition potencies were selected for the in vitro studies. Stock solutions of all the inhibitors were made in dimethylsulfoxide (DMSO) and then diluted 10-fold with acetonitrile prior to addition to the incubation mixtures to minimize DMSO content. Four concentrations of each probe substrate [0.5*K_m, K_m, 2*K_m and 4*K_m: 0.5, 1, 2, and 4 µM for budesonide;
4, 8, 16, and 32 µM for buspirone; 1.0, 2.0, 4.0 and 8.0 µM for felodipine; 0.3, 0.6, 1.2 and 2.4 µM for fluticasone; 0.75, 1.5, 3.0, and 6.0 µM for midazolam; 15, 30, 60 and 120 µM for quinidine; 4.5, 9, 18, and 36 µM for sildenafil; 1, 2, 4, and 8 µM for simvastatin; and 25, 50, 100 and 200 µM for testosterone] and five concentrations of each inhibitor (spanning a ten-fold range of the expected $K_i$) were used for determination of $K_i$ in a 96-well plate format. Briefly, each reaction was carried out in duplicate containing 0.1 mg/mL human liver microsomal protein per incubation. Each incubation reaction mixture contained enzyme, probe substrate and inhibitor suspended in phosphate buffer (100 mM, pH 7.4) containing 3 mM MgCl₂ and was preincubated for three minutes in an incubator-shaker at 37 °C. The reactions were initiated by the addition of NADPH (1 mM final concentration). DMSO concentrations did not exceed 0.1% v/v and total organic solvent concentrations did not exceed 1% v/v. Solvent concentrations were the same for all experiments and turnover rates did not differ significantly from minimal solvent controls. The reactions were terminated with 100 µl of acetonitrile containing 0.1 µM of tolbutamide (internal standard). Length of the incubations were 20 min, except for midazolam, which was carried out for 5 min. The incubation time and protein concentrations used were within the linear range for each respective CYP probe reaction.

Liquid Chromatography/Tandem Mass Spectral Analysis. All analytical methods were conducted using HPLC-MS/MS technology. In brief, the LC-MS/MS system was comprised of an Applied Biosystems 4000 Q-Trap (operated in triple
quadrupole mode) equipped with an electrospray ionization source (Applied Biosystems, Foster City, CA). The MS/MS system was coupled to two LC-20AD pumps with an in-line CBM-20A controller and DGU-20A5 solvent degasser (Shimadzu, Columbia, MD) and a LEAP CTC HTS PAL autosampler equipped with a dual-solvent self-washing system (CTC Analytics, Carrboro, NC). The injection volume was 10 µL for each sample. For all assays except simvastatin hydroxylation, HPLC separation was achieved using a Gemini C18 2.0 x 30 mm 5 µm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 500 µL/min) was carried out using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. HPLC flow was diverted from the MS/MS system for the first 20 seconds to remove any non-volatile salts. For simvastatin hydroxylation, a Luna C18 (5 µm, 30 x 3.0 mm; Phenomenex, Torrance, CA) HPLC column was used. Gradient conditions utilized were similar to those used above, though the initial percentage of acetonitrile in the mobile phase was 50%. MS/MS conditions were optimized for individual analytes accordingly. Generic MS parameters included the curtain gas (10 arbitrary units), CAD gas (medium), ionspray voltage (4500 V), source temperature (450 °C) and ion source gas 1 and gas 2 (40 arbitrary units, each). Interface heaters were kept on for all analytes. Analysis masses were (positive ionization mode): dehydrofelodipine, m/z 382.1→354.2; N-desmethylsildenafil, m/z 461.1→283.3; 6β-hydroxybudesonide, m/z 447.0→339.2; fluticasone 17β-carboxylic acid, m/z 453.2→293.0; 6'-hydroxybuspirone, m/z 402.3→122.3; 1'-hydroxymidazolam, m/z 342.1→203.1; 3'-hydroxyquinidine, m/z 341.2→226.1; 3'
hydroxysimvastatin, \( m/z \ 435.1 \rightarrow 199.2 \); 6β-hydroxytestosterone, \( m/z \ 305.0 \rightarrow 269.0 \); tolbutamide, \( m/z \ 271.2 \rightarrow 91.1 \).

**Statistical Analysis.** Standard curve fitting was performed using Analyst (version 1.4; Applied Biosystems, Foster City, CA). In general, standard curves were weighted using \( 1/x \). Substrate saturation curves and inhibition data were plotted and analyzed using GraphPad Prism (version 4.01; GraphPad Software Inc., San Diego, CA). Data was then fitted to either a competitive (Equation 1), non-competitive (Equation 2), or linear-mixed inhibition model (Equation 3):

\[
(1) \quad v = \frac{V_{\text{max}} \bullet [S]}{K_{m} (1 + \frac{[I]}{K_{i}}) + [S]}
\]

\[
(2) \quad v = \frac{V_{\text{max}} \bullet [S]}{K_{m} (1 + \frac{[I]}{K_{i}}) + [S]((1 + \frac{[I]}{K_{i}})}
\]

\[
(3) \quad v = \frac{V_{\text{max}} \bullet [S]}{K_{m} (1 + \frac{[I]}{K_{i}}) + [S]((1 + \frac{[I]}{K_{i}})}
\]

In the preceding equations, \( K_{m} \) is equal to the substrate concentration at half maximal reaction velocity, \([I]\) is the concentration of inhibitor in the system, \( K_{i} \) is the dissociation constant for the enzyme-inhibitor complex and \( K_{i}' \) is the dissociation constant for the enzyme-substrate-inhibitor complex. Note that in the above equations, \( K_{m}, \ K_{i} \) and \( V_{\text{max}} \) were treated as global parameters. The mechanism of inhibition was determined by visual inspection of the data using
Hierarchical Clustering Analysis. Statistical and clustering analysis of the inhibition potency data was performed using Statistica 8.0 (StatSoft, Tulsa, OK). An UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering algorithm was used to determine similarity between the inhibition data sets and form successively larger clusters using a Euclidean distance similarity measure. Data were entered as inhibition potency ($K_I$) values. Compounds that exhibited activation or $K_I$ values above 50 µM were entered as a $K_I$ of 50 µM. For the purposes of hierarchical clustering, all probe substrate-effector pairings must have a numerical value; this poses an issue for instances where the probe substrate is also the effector (Kumar et al., 2006). For those instances, an average value of $K_I$ for that effector obtained with the other probe substrates was calculated and used.

Correlation Analysis of In Vivo Drug Interaction Potential. Literature data for AUC$_I$/AUC were obtained using the University of Washington Metabolism and Transport Drug Interaction Database™, where AUC$_I$ is defined as the area under the plasma concentration-time curve for a given probe substrate in the presence of an inhibitor and AUC is defined as the area under the plasma concentration-time curve for a given probe substrate in the absence of inhibitor. Studies were considered comparable if they had a similar dose regimen for both inhibitor and
probe substrate. For instances where multiple AUCI/AUC values were available in the literature, the AUCI/AUC values were averaged. A minimum of four shared AUCI/AUC values were deemed necessary to carry out the correlation analysis. Linear regression was carried out on untransformed data.

*Prediction of In Vivo Drug Interactions.* SimCYP (Version 8.01) was used to predict the *in vivo* drug interactions between AMG 458 and the probe substrates midazolam, sildenafil, simvastatin and testosterone. Drug interaction potentials were predicted for 500, 1000 and 2000 mg doses of AMG 458 based upon the anticipated therapeutic range (Liu et al., 2008); the following data for AMG 458 was entered into SimCYP: mw (molecular weight, 539.2 amu), logP (3.4), $f_a$ (fraction absorbed, 1.0), $f_{mCYP3A4}$ (fraction metabolized by CYP3A4, 0.99), $f_u$ (fraction unbound in plasma, 0.01), $f_{umic}$ (fraction unbound in microsomes, 0.9), *in vitro* microsomal clearance (18 µL/min/mg) and predicted $V_{dss}$ (0.88 L/kg), where $V_{dss}$ is defined as the volume of distribution at steady state. Physicochemical properties and dosing regimens for midazolam, sildenafil and simvastatin were taken directly from SimCYP default values. For testosterone, the following data was obtained from the literature and entered into SimCYP (White et al., 1998; Patki et al., 2003): mw (288.4 amu), logP (3.5), $f_{mCYP3A4}$ (0.99), $f_u$ (0.08, predicted), *in vitro* microsomal clearance (101 µL/min/mg) and $V_{dss}$ (1.0 L/kg). The remaining physiological and ADME parameters were predicted with SimCYP on the basis of the physicochemical data input using a one compartment distribution model. The pharmacokinetic simulations were designed to represent
100 healthy volunteers ranging in age from 18 to 65 and divided into 10 trials of 10 subjects each. Female subjects represented approximately 34% of the simulated population.
Results

The inhibition constants ($K_i$) for a set of twenty effectors were determined for the probe substrates budesonide, buspirone, felodipine, fluticasone, midazolam, quinidine, sildenafil, simvastatin and testosterone (Table 1). Competitive, noncompetitive and linear-mixed inhibition profiles were observed, depending on the probe substrate-inhibitor combination. Felodipine and fluoxetine exhibited linear-mixed inhibition using midazolam as a probe substrate; nifedipine exhibited linear-mixed inhibition using quinidine as a probe substrate. Eight effectors (cyclosporine, felodipine, fluoxetine, haloperidol, ketoconazole, nifedipine, sertraline and terfenadine) exhibited noncompetitive inhibition using midazolam as a probe substrate. Five effectors (fluoxetine, fluvoxamine, itraconazole, ketoconazole and sertraline) exhibited noncompetitive inhibition using buspirone as probe substrate. Four effectors (AMG 458, dextromethorphan, haloperidol and simvastatin) exhibited noncompetitive inhibition using felodipine as a probe substrate. Three effectors (felodipine, sertraline and simvastatin) exhibited noncompetitive inhibition using quinidine as a probe substrate. One effector exhibited noncompetitive inhibition using fluticasone (e.g. cyclosporine) and sildenafil (e.g. sertraline) as probe substrates, respectively.

Hierarchical clustering analysis was performed on the non-transformed inhibition potency data using an UPGMA clustering algorithm to obtain a Euclidean distance similarity measure. Results from the clustering analysis for the CYP3A4 data were visualized as a dendrogram (Figure 1), where the
horizontal axis of the dendrogram represents the Euclidean linkage distance between probe substrate clusters. Relative to midazolam, the average fold decrease in inhibition potency with standard error for each probe substrate was: buspirone (3 ± 1), quinidine (4 ± 6), sildenafil (5 ± 2), budesonide (8 ± 4), fluticasone (12 ± 7), felodipine (14 ± 5), simvastatin (22 ± 44) and testosterone (106 ± 45).

*In vivo* DDI data for CYP3A4 probe substrates was collected from the literature and compiled when similar study conditions were used relative to a midazolam comparator study (Table 2). For probe substrates with four or more DDI studies in common with midazolam, a linear correlation analysis was carried out (Figure 2). The line of unity of the correlation analysis is represented by a dashed line. Buspirone and simvastatin exhibited correlations that were greater than unity (2.7 and 1.8, respectively), sildenafil exhibited a correlation that was near unity (0.81), and cyclosporine, nifedipine and quinidine exhibited correlations that were markedly lower than unity (0.38, 0.01 and 0.25, respectively). Correlation analysis for budesonide, felodipine, fluticasone and erythromycin were not carried out as the literature contained less than four DDI studies in common with midazolam.

Prediction of the magnitude of *in vivo* DDI due to AMG 458 was obtained using SimCYP (Figure 3). Doses of 500, 1000, and 2000 mg of AMG 458 were chosen based upon coverage of the anticipated therapeutic range. Midazolam, simvastatin, sildenafil and testosterone were predicted to exhibit AUCr/AUC values of 1.1, 1.2, 2.0 and 2.1 at 500 mg doses of AMG 458; 1.2, 1.4, 2.6 and 3.0
at 1000 mg doses of AMG 458; and 1.5, 1.8, 3.8 and 4.9 at 2000 mg doses of AMG 458, respectively.
Discussion

Screening for and predicting the magnitude of P450-mediated DDIs is a crucial part of the drug discovery and development paradigm, potentially influencing both patient safety and product differentiation. Recent examples of drugs withdrawn from the market due to drug interactions include mibefradil (Po and Zhang, 1998) and cerivastatin (Davidson, 2002). Within the P450 superfamily, CYP3A4 is responsible for the metabolism of a majority of marketed drugs. As a result, assessment and modeling of CYP3A4 inhibition is a key component of reversible inhibition testing (Wahlstrom et al., 2006). While advancements have been made in the design of in vitro DDI experiments, the prediction of in vivo DDIs and database analysis of P450-mediated DDIs, a comprehensive understanding of probe substrate selection for CYP3A4 based upon both in vitro correlation data and in vivo sensitivity analysis has been lacking.

The selection of appropriate CYP3A4 probe substrates for in vitro studies has led to substantial debate. CYP2C9 (Kumar et al., 2006), CYP2C19 (Foti and Wahlstrom, 2008) and CYP3A4 (Kenworthy et al., 1999; Stresser et al., 2000) have exhibited probe substrate-dependent inhibition for in vitro studies. The correlation analysis of CYP3A4 DDI data from in vitro experiments has suggested that at least three probe substrate classes may exist for CYP3A4: benzodiazepine-like, dihydropyridine-like and testosterone-like, possibly due to the presence of multiple binding regions within the CYP3A4 active site (Kenworthy et al., 1999). While the use of midazolam, testosterone and
felodipine/nifedipine as probe substrates has been suggested based upon hierarchical clustering of in vitro data, implementation of a three probe substrate approach may be prohibitive depending on the number of candidates to be tested. Results from in vitro studies assaying the inhibition potential of forty-two marketed therapeutics against CYP3A4 using multiple probe substrates generally suggest the rank ordering of midazolam > testosterone > felodipine in terms of in vitro sensitivity to inhibition (Obach et al., 2006). Accuracy of the prediction of in vivo DDI magnitude from this data was dependent upon the statistical method used. None of the three probe substrates was clearly superior based upon performance of the in vivo DDI prediction. The use of quinidine as a CYP3A4 in vitro probe substrate has also been suggested based upon its kinetic properties and selectivity for CYP3A4 over CYP3A5 (Galetin et al., 2005).

Our selection of in vitro probe substrates was based upon the availability of clinical DDI data and structural characteristics of the probe substrate. Probe substrates with known correlation to midazolam and testosterone (e.g. cyclosporine, erythromycin and nifedipine) were excluded from the in vitro analysis. Although the correlation of quinidine to midazolam and testosterone has previously been determined usingazole-type inhibitors (Galetin et al., 2005), quinidine was included in the in vitro portion of this study in order to understand its correlation to the other probe substrates based upon a chemically diverse set of inhibitors. Due to a general industry paradigm using testosterone as a CYP3A4 probe substrate, we were particularly interested in identifying clinically relevant steroids for in vitro testing. Fluticasone and budesonide were selected.
as steroid probe substrates based upon the availability of clinical DDI data. Eplerenone, a steroid with clinical DDI data (Ragueneau-Majlessi et al., 2007), exhibited linear kinetics in our hands and was therefore unsuitable for use as an \textit{in vitro} probe substrate. Other probe substrates with a steroid chemotype, such as prednisolone, were excluded from consideration because a significant contribution to their clearance is mediated by enzymes other than P450s (Zurcher et al., 1989).

The correlation analysis of our \textit{in vitro} inhibition data suggested four clusters: felodipine-like, midazolam-like, simvastatin-like and testosterone-like. Testosterone was the least similar probe based upon both correlation analysis and average inhibition potency when compared to midazolam. A feature that differentiated testosterone from the other probe substrates was the number of effectors that caused activation rather than inhibition. While activation indicates interaction between the effector and probe substrate, the result is difficult to context within an \textit{in vivo} setting (Tracy, 2003). Based upon these results, we did not find a clinically relevant replacement for testosterone for use with \textit{in vitro} assays.

The \textit{in vivo} sensitivity of the CYP3A4 probe substrates is another criterion for probe substrate selection. The sensitivity of midazolam and simvastatin as CYP3A4 probe substrates has been directly compared in a clinical study using ketoconazole as the inhibitor (Chung et al., 2006). The authors concluded that simvastatin was a suboptimal \textit{in vivo} probe substrate due to its lack of CYP3A4 selectivity, as demonstrated by a marked increase in pharmacokinetic variability.
within the same patient population. Retrospective analysis of literature in vivo DDI studies, however, suggested that simvastin may generally exhibit increased sensitivity to inhibition in vivo based upon results from multiple CYP3A4 inhibitors (Ragueneau-Majlessi et al., 2007).

The availability of sufficient clinical CYP3A4 DDI data in the literature relative to midazolam (n = 4 studies) was our selection criteria for inclusion in the in vivo correlation analysis. Although previous comparisons to in vivo midazolam DDI data have been made for buspirone and simvastatin, they were included in this analysis because we averaged DDI data for instances where multiple clinical studies for the same inhibitor and probe substrate combination were available and carried out using similar conditions. Of the probe substrates tested in vitro, felodipine, fluticasone and budesonide were not included in the sensitivity analysis since they had less than four clinical DDI studies in common with midazolam. Cyclosporine, nifedipine and quinidine exhibited reduced sensitivity when compared to midazolam in vivo, sildenafil exhibited similar sensitivity and buspirone and simvastatin exhibited enhanced selectivity. The effect of this enhanced selectivity on the accuracy of in vivo DDI predictions is unclear. The estimation of inhibitor concentration used in the DDI prediction (total systemic $C_{\text{max}}$, free systemic $C_{\text{max}}$, total hepatic inlet $C_{\text{max}}$ or the free hepatic inlet $C_{\text{max}}$) has a profound impact on the prediction and has resulted in either underestimation or overestimation of DDI magnitude for these probe substrates depending on the methodology chosen (Galetin et al., 2005; Obach et al., 2006).
The reasons for the differences in probe substrate sensitivity may include competing clearance mechanisms that are not mediated by CYP3A4, experimental variability in the in vivo DDI studies, or differences in the susceptibility of each probe substrate to intestinal CYP3A4 inhibition. The f_mCYP value may have a marked impact on DDI predictions. Probe substrates with an f_mCYP value of 0.5 for a particular P450-mediated pathway may experience a maximal increase in AUC_i/AUC of 2-fold theoretically; as f_mCYP increases, the effect on the magnitude of AUC_i/AUC increases (Ito et al., 2005). The three probe substrates with the lowest in vivo sensitivity (cyclosporine, nifedipine and quinidine) have f_mCYP3A4 values lower than 0.8 (0.71, 0.71 and 0.76, respectively). Intestinal first pass metabolism may also have a marked impact on DDI prediction. For drugs with an intestinal extraction ratio less than 50%, a maximal increase in AUC_i/AUC of 2-fold is expected (Galetin et al., 2008). For drugs with a high extent of intestinal extraction, increases in AUC_i/AUC of 4-fold or more may be expected if maximal enzyme inhibition in the gut is achieved.

Midazolam is a clear CYP3A4 DDI probe substrate choice for most instances based upon its in vitro and in vivo characteristics, such as CYP3A selectivity and the availability of both intravenous and oral formulations. However, probe substrate selection for clinical CYP3A4 DDI studies may be confounded for cases such as AMG 458, where a probe substrate that is not clinically relevant (e.g. testosterone) has been tested in vitro, demonstrates markedly increased CYP3A4 inhibition potential relative to midazolam and felodipine/nifedipine, and is predicted to exhibit a clinically relevant DDI
(AUCi/AUC ≥ 2), while midazolam and felodipine/nifedipine are not. Due diligence suggests that a strategy is needed to evaluate whether additional or alternative clinical studies to a midazolam or felodipine/nifedipine DDI study may be necessary based upon in vitro results.

Ideal characteristics of a probe substrate for DDI studies include formation of a primary metabolite that is selectively mediated by the P450 of interest, the observation of Michaelis-Menten kinetics in vitro and a lack of confounding transporter activity in vivo. When identifying a potential alternative probe substrate to midazolam based upon in vitro inhibition potency, the most likely candidates would demonstrate inhibition profiles unique from midazolam. Based upon their in vitro inhibition profiles, we would select primary midazolam alternatives from the testosterone (cyclosporine and erythromycin), felodipine or simvastatin clusters. The low therapeutic index of cyclosporine makes it an undesirable probe substrate in vivo (Jorga et al., 2004). Erythromycin is often used in a single time point breath test (Frassetto et al., 2007), limiting the amount of clinical DDI data available for a full time course and may exhibit confounding transporter activity (Obach et al., 2005). These characteristics hindered our ability to create a direct correlation between the in vitro and in vivo data for cyclosporine and erythromycin, and were part of the rationale for excluding them from consideration as alternate probe substrates. Nifedipine is often cited as a probe substrate for in vitro CYP3A4 inhibition studies. However, it exhibits reduced sensitivity in vivo when compared to midazolam and has not been tested using a potent CYP3A4 inhibitor clinically to our knowledge. Felodipine has a
somewhat higher $f_{mCYP3A4}$ value (0.81) than nifedipine that should result in increased in vivo sensitivity and has been tested in vivo using a potent CYP3A4 inhibitor: $\text{AUC}_i/\text{AUC} = 6.3$ at 200 mg itraconazole (Jalava et al., 1997).

Simvastatin demonstrates unique inhibition profiles from midazolam in vitro, has been clinically tested with potent CYP3A4 inhibitors in vivo and demonstrates enhanced in vivo sensitivity when compared to midazolam. Based upon these characteristics, simvastatin is our primary choice as an alternative probe substrate when testosterone-selective inhibition of CYP3A4 is observed. Testing the inhibition potential of other probe substrates may be considered based upon in vitro results. However, since potent inhibitors are expected to be identified in vitro and testable in vivo using midazolam, alternative probe substrates should exhibit similar or better in vitro and in vivo sensitivity than midazolam for consideration. Although they are in the same in vitro inhibition cluster as midazolam, buspirone and sildenafil may be considered based upon acceptable in vitro characteristics and in vivo sensitivity.

The ability to predict in vivo exposure levels of a given drug (or inhibitor) using modeling and simulation programs such as SimCYP is a useful tool in the design of drug efficacy and safety studies (Rostami-Hodjegan and Tucker, 2007). Using the case study of AMG 458, the magnitude of in vivo DDI caused by AMG 458 was predicted for midazolam, sildenafil and simvastatin using SimCYP. Predictions based upon testosterone are shown for comparative purposes. Buspirone exhibited activation with AMG 458 and was therefore not included for the in silico predictions. Predictions for felodipine were not included since it was
inhibited less potently than midazolam by AMG 458. Sildenafil was predicted to exhibit clinically relevant DDI (AUC/I/AUC ≥ 2) across the anticipated dose range (500-2000 mg), while midazolam and simvastatin were not. At the lowest dose (500 mg), midazolam and simvastatin were predicted to demonstrate a < 1.2-fold increase in DDI magnitude; this magnitude of change may be difficult to detect based upon pharmacokinetic variability within an \textit{in vivo} DDI study. Since clinical DDI studies may be carried out at low doses of drug, often lower than anticipated efficacious doses, these predictions suggest that sildenafil would be an acceptable clinical CYP3A4 probe substrate for DDI studies using AMG 458. While simvastatin is our recommended probe substrate for testosterone-selective inhibition, the \textit{in vivo} predictions using AMG 458 demonstrate that other alternatives may be considered.

The selection of a CYP3A4 probe substrate for clinical DDI studies may be unclear for cases where a probe substrate, such as testosterone, is predicted to exhibit clinically significant DDI, while clinically relevant probe substrates, such as midazolam and felodipine/nifedipine, are not. Based upon hierarchical clustering of \textit{in vitro} data and correlation analysis of clinical DDI data, we recommend the use of simvastatin as a primary alternative CYP3A4 probe substrate for testosterone-selective inhibition. Buspirone or sildenafil may serve as useful secondary probe substrates for the testosterone-selective inhibition situation. The complexity of CYP3A4-mediated reactions suggests it is unlikely that a universal alternative to midazolam will be available in the near future.
References


Obach RS, Walsky RL, Venkatakrishnan K, Houston JB and Tremaine LM (2005) In vitro cytochrome P450 inhibition data and the prediction of drug-drug...


Figure Legends

Figure 1. CYP3A4 Probe Substrate Hierarchical Clustering Dendrogram

Figure 2. Correlation of Literature AUCi/AUC Values for CYP3A4 Probe
Substrates Relative to Midazolam: A) Buspirone, b) Cyclosporine, C) Nifedipine,
D) Quinidine, E) Sildenafil, F) Simvastatin

Figure 3. SimCYP Predicted AUCi/AUC Values for Midazolam, Simvastatin,
Sildenafil and Testosterone at AMG 458 Doses of 500, 1000 and 2000 mg
Table 1. \( K_i \) Values (µM) Obtained Using Pooled HLMs\(^a\)

<table>
<thead>
<tr>
<th>Probe Substrate(^b)</th>
<th>Effector</th>
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<tr>
<td></td>
<td>TST</td>
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<td>AMG 458</td>
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<td>Budenoside</td>
<td>ACT</td>
</tr>
<tr>
<td>Buspirone</td>
<td>50</td>
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<tr>
<td>Clozapine</td>
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<tr>
<td>Cyclosporine</td>
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<tr>
<td>Dextromethorphan</td>
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</tr>
<tr>
<td>Felodipine</td>
<td>ACT</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>8.7</td>
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<tr>
<td>Fluticasone</td>
<td>ACT</td>
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<tr>
<td>Fluvoxamine</td>
<td>20.4</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>27.4</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.013</td>
</tr>
<tr>
<td>Ketoconazole</td>
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<tr>
<td>Midazolam</td>
<td>6.4</td>
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<tr>
<td>Nifedipine</td>
<td>ACT</td>
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<tr>
<td>Sertraline</td>
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<tr>
<td>Sildenafil</td>
<td>50</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>35.0</td>
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<td>Terfenadine</td>
<td>11.2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>X</td>
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</tbody>
</table>

\(^a\)Global standard error for data fitting was less than 20% and \( r^2 \) > 0.80 for each effector

\(^b\)Abbreviations: Testosterone (TST); Midazolam (MDZ); Sildenafil (SIL); Fluticasone (FLU); Budesonide (BUD); Quinidine (QUI); Buspirone (BUS); Simvastatin (SIM); Felodipine (FEL)

\(^c\)Linear-mixed inhibition

\(^d\)Activation

\(^e\)Noncompetitive inhibition
### Table 2. AUC<sub>i</sub>/AUC Values Obtained from the Literature

<table>
<thead>
<tr>
<th>Inhibitor (mg/day)</th>
<th>AUC&lt;sub&gt;i&lt;/sub&gt;/AUC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BUD</td>
</tr>
<tr>
<td>Cimetidine (800-1200)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1</td>
</tr>
<tr>
<td>Clarithromycin (500)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Clarithromycin (1000)&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Diltiazem (60-120)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5</td>
</tr>
<tr>
<td>Erythromycin (800-1000)&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Erythromycin (1500)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0</td>
</tr>
<tr>
<td>Fluconazole (200)</td>
<td>1.8</td>
</tr>
<tr>
<td>Fluvoxamine (100-200)</td>
<td>2.4</td>
</tr>
<tr>
<td>Grapefruit juice (rs)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0</td>
</tr>
<tr>
<td>Grapefruit juice (ds)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.2</td>
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<td>Itraconazole (100)</td>
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<td>Itraconazole (200)</td>
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<tr>
<td>Ketoconazole (200)</td>
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<td>Ketoconazole (400)</td>
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<td>Mibefradil (50-100)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Ranitidine (300)</td>
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<tr>
<td>Ritonavir (500 bid)&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Saquinavir (600-1200 tid)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Verapamil (80)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: Budesonide (BUD); Buspirone (BUS); Cyclosporine (CYC); Felodipine (FEL); Midazolam (MDZ); Nifedipine (NIF); Quinidine (QUI); Sildenafil (SIL); Simvastatin (SIM);
<sup>b</sup>Time dependent inactivator of CYP3A4
<sup>c</sup>RS = regular strength grapefruit juice
<sup>d</sup>DS = double strength grapefruit juice
Figure 3

Predicted AUC/AUC vs. AMG 458 Dose (mg)

- MDZ
- SIM
- SIL
- TST