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

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ABSTRACT:
Understanding the potential for cytochrome P450-mediated drug-drug interactions (DDIs) is a critical step in the drug discovery and development process. DDIs of CYP3A4 are of particular importance because of the number of marketed drugs that are cleared by this enzyme. In response to studies that suggested 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, the design of clinical CYP3A4 DDI studies may be confounded for cases such as 1-(2-hydroxy-2-methylpropyl)-N-[5-(7-methoxyquinolin-4-yl)pyridin-2-yl]-5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazole-4-carboxamide (AMG 458), with which testosterone is predicted to exhibit a clinically relevant DDI whereas midazolam and felodipine/nifedipine are not. To develop an appropriate path forward for such clinical DDI studies, the inhibition potency of 20 known inhibitors of CYP3A4 were measured in vitro using 8 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 DDIs caused by AMG 458 using midazolam, sildenafil, simvastatin, and testosterone as probe substrates.

The cytochrome P450 (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). Because of its general importance in drug clearance, assessment and modeling of CYP3A4 inhibition are a critical part of the drug discovery and development process. Probe substrate-dependent inhibition profiles have been observed in vitro with CYP3A4, possibly owing 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 20 known CYP3A4 inhibitors and determine the similarity of the probe substrates based on hierarchical clustering of the inhibition data.

Recent draft guidance from the U.S. Food and Drug Administration outlining the designs 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 on 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 in which evaluation of alternate CYP3A4 probe substrates was warranted based on in vitro inhibition data. 1-(2-Hydroxy-2-methylpropyl)-N-[5-(7-methoxyquinolin-4-yl)pyridin-2-yl]-5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazole-4-carboxamide (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
the effect AMG 458 would have on the alternate CYP3A4 probe substrates and to select an appropriate probe substrate for the clinical DDI study based on the in silico predictions.

Materials and Methods

Materials. Pooled human liver microsomes (15 individual donors) were purchased from CelZDirect (Durham, NC). AMG 458 was obtained from the Argentum Sample Bank (Thousand Oaks, CA). Ammonium formate, HPLC-grade acetonitrile and HPLC-grade methanol were obtained from Alfa Aesar (Ward Hill, MA). Cyclosporine was obtained from BIOMOL International (Plymouth Meeting, PA). Fedelopine 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 US Biological (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 performed using eight probe substrates of CYP3A4: budesonide, buspirone, fedelopine, 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 dimethyl sulfoxide (DMSO) and then diluted 10-fold with acetonitrile before addition to the incubation mixtures to minimize DMSO content. Four concentrations of each probe substrate [0.5 × K_i, 2 × K_i, 5 × K_i, and 4 × K_i: 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 fedelopine; 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 10-fold range of the expected K_i) were used for determination of K_i in a 96-well plate format. In brief, each reaction was carried out in duplicate, containing 0.1 mg/ml human liver microsomal protein/incubation. Each incubation reaction mixture contained enzyme, probe substrate, and inhibitor. Incubations and turnover rates did not differ significantly from minimal incubations and turnover rates did not exceed 1% v/v. Solvent concentrations were the same for all experiments and incubation reaction mixture contained enzyme, probe substrate, and inhibitor. Studies were considered comparable if they had a similar dose regimen for both inhibitor and probe substrate. For instances when multiple inhibitor-effector pairings must have a numerical value; this poses an issue for instances for which 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.

Liquid Chromatography/Tandem Mass Spectral Analysis. All analytical methods were conducted using HPLC-MS/MS technology. In brief, the liquid chromatography-MS/MS system consisted of an Applied Biosystems 4000 Q-Trap spectrometer (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 C18-200A controller and DGU-20A solid 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 × 30 mm 5-μm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 500 μl/min) was performed using a mobile phase consisting of 5 mM ammonium formate with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). HPLC flow was diverted from the MS/MS system for the first 20 s to remove any nonvolatile salts. For simvastatin hydroxylation, a Luna C18 (5 μm, 30 × 3.0 mm; Phenomenex) HPLC column was used. Gradient conditions used were similar to those used above, although 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 382.2 → 354.2; N-desmethylsildenafil, m/z 461.1 → 283.3; 6β-hydroxybudesonide, m/z 447.0 → 339.2; fluticasone 17β-carboxylic acid, N-desmethylsildenafil, 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'-hydroxyimivastatin, m/z 435.1 → 199.2; 6β-hydroxytestosterone, m/z 305.0 → 269.0; and tolbutamide, m/z 271.2 → 91.1.

Statistical Analysis. Standard curve fitting was performed using Analyst (version 1.4; Applied Biosystems). 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 were then fitted to either a competitive (eq. 1), noncompetitive (eq. 2), or linear-mixed inhibition model (eq. 3):

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

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 and V_max were treated as global parameters. The mechanism of inhibition was determined by visual inspection of the data using Dixon ([I] versus 1/v) and Lineweaver-Burk ([1/S] versus 1/v) plots and comparative model assessment using the Akaike information criterion.

Hierarchical Clustering Analysis. Statistical and clustering analysis of the inhibition potency data were performed using Statistica 8.0 (StatSoft, Tulsa, OK). An unweighted pair group method with arithmetic mean clustering algorithm was used to determine similarity between the inhibition data sets and to 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 greater than 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 for which 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 an inhibitor. Studies were considered comparable if they had a similar dose regimen for both inhibitor and probe substrate. For instances when multiple AUC_i/AUC values were available in the literature, the AUC_i/AUC values were averaged. A minimum of four shared AUC_i/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, imatinib, and testosterone. Drug interaction potentials were predicted for 500-, 1000-, and 2000-mg doses of AMG 458 based on the anticipated therapeutic range (Liu et al., 2008); the following data for AMG 458 were entered into Simcyp: molecular weight (539.2 amu), logP (3.4), I_f (fraction absorbed, 1.0), I_f,max (fraction metabolized by CYP3A4, 0.99), f_u (fraction unbound in plasma, 0.01), f_m (fraction unbound in microsomes, 0.9), in vitro microsomal clearance (18 μl/min/mg), and

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predicted $V_{\text{ss}}$ (0.88 l/kg), where $V_{\text{ss}}$ 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 were obtained from the literature and entered into Simcyp (White et al., 1998; Patki et al., 2003): molecular weight (288.4 amu), logP (3.5), $f_{\text{relCYP3A4}}$ (0.99), $f_{\text{d}}$ (0.08, predicted), in vitro microsomal clearance (101 μM/Min), and $V_{\text{ss}}$ (1.0 l/kg). The remaining physiological and absorption, distribution, metabolism, and excretion 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 years 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 20 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 fluticasone 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, fluticasone, 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 nontransformed inhibition potency data using an unweighted pair group method with arithmetic mean clustering algorithm to obtain a Euclidean distance similarity measure. Results from the clustering analysis for the CYP3A4 data were visualized as a dendrogram (Fig. 1), for which the horizontal axis of the dendrogram represents the Euclidean linkage distance between probe substrate clusters. Relative to midazolam, for each probe substrate the average fold decrease in inhibition potency with S.E. was as follows: 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 were 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 performed (Fig. 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 performed because the literature contained fewer than four DDI studies in common with midazolam.

Prediction of the magnitude of in vivo DDIs due to AMG 458 was obtained using Simcyp (Fig. 3). Doses of 500, 1000, and 2000 mg of AMG 458 were chosen based on coverage of the anticipated therapeutic range. Midazolam, simvastatin, sildenafil, and testosterone were predicted to exhibit AUC/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 process. Predicting the magnitude of in vivo DDIs due to AMG 458 was obtained using Simcyp (Fig. 3). Doses of 500, 1000, and 2000 mg of AMG 458 were chosen based on coverage of the anticipated therapeutic range. Midazolam, simvastatin, sildenafil, and testosterone were predicted to exhibit AUC/AUC ratios 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.

### Table 1

<table>
<thead>
<tr>
<th>Effector</th>
<th>Values with Probe Substrate</th>
<th>$K_i$ Values with Probe Substrate</th>
<th>$\mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMG 458</td>
<td>TST</td>
<td>MDZ</td>
<td>SIL</td>
</tr>
<tr>
<td>Budesonide</td>
<td>ACT 0.10</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>Buspirone</td>
<td>50 0.54</td>
<td>2.1</td>
<td>9.3</td>
</tr>
<tr>
<td>Clozapine</td>
<td>7.4 0.42</td>
<td>17.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>24.2 3.1</td>
<td>9</td>
<td>6.9</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>50 8.6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Felodipine</td>
<td>ACT 0.25</td>
<td>0.65</td>
<td>0.44</td>
</tr>
<tr>
<td>Fluticasone</td>
<td>8.7 4.2</td>
<td>3.3</td>
<td>50</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>20.4 2.6</td>
<td>2.7</td>
<td>50</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>27.4 2.3</td>
<td>2.4</td>
<td>3.1</td>
</tr>
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<td>Itraconazole</td>
<td>0.013 0.013</td>
<td>0.016</td>
<td>0.012</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.023 0.014</td>
<td>0.017</td>
<td>0.044</td>
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<td>Midazolam</td>
<td>6.4 X</td>
<td>1.8</td>
<td>0.74</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>ACT 1.8</td>
<td>8.7</td>
<td>0.46</td>
</tr>
<tr>
<td>Sertraline</td>
<td>10.4 3.1</td>
<td>2.1</td>
<td>12.6</td>
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<tr>
<td>Sildefinil</td>
<td>50 0.71</td>
<td>X</td>
<td>3.9</td>
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<td>Simvastatin</td>
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<td>0.38</td>
</tr>
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<td>Terfenadine</td>
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<td>0.21</td>
<td>0.31</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.7</td>
<td>9.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

TST, testosterone; MDZ, midazolam; SIL, sildenafil; FLU, fluticasone; BUC, budesonide; QUI, quinidine; BUS, buspirone; SIM, simvastatin; FEL, felodipine.

* Linear-mixed inhibition.
* Activation.
* Noncompetitive inhibition.
paradigm, potentially influencing both patient safety and product differentiation. Recent examples of drugs withdrawn from the market because of 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). Although 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 on 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). Although the use of midazolam, testosterone, and felodipine/nifedipine as probe substrates has been suggested based on 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 42 marketed therapeutic agents 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 these data was dependent on the statistical method used. None of the three probe

![Fig. 1. CYP3A4 probe substrate hierarchical clustering dendrogram.](image-url)
substrates was clearly superior based on performance of the in vivo DDI prediction. The use of quinidine as a CYP3A4 in vitro probe substrate has also been suggested based on its kinetic properties and selectivity for CYP3A over CYP3A5 (Galetin et al., 2005).

Our selection of in vitro probe substrates was based on 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 was determined previously using azole-type inhibitors (Galetin et al., 2005), quinidine was included in the in vitro portion of this study to understand its correlation to the other probe substrates based on a chemically diverse set of inhibitors. Because of 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 on 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 in vitro probe substrate. Other probe substrates with a steroid chemotype, such as pred-
The correlation analysis of our in vitro inhibition data suggested four clusters: felodipine-like, midazolam-like, simvastatin-like, and testosterone-like. Testosterone was the least similar probe based on both correlation analysis and average inhibition potency compared with midazolam. A feature that differentiated testosterone from the other probe substrates was the number of effectors that caused activation rather than inhibition. Whereas activation indicates interaction between the effector and probe substrate, the result is difficult to extrapolate to an in vivo setting (Tracy, 2003). Based on these results, we did not find a clinically relevant replacement for testosterone for use with in vitro assays.

The 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 in vivo probe substrate because of its lack of CYP3A4 selectivity, as demonstrated by a marked increase in pharmacokinetic variability within the same patient population. However, retrospective analysis of literature in vivo DDI studies suggested that simvastatin may generally exhibit increased sensitivity to inhibition in vivo based on 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 with 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 in which multiple clinical studies for the same inhibitor and probe substrate combination were available and performed using similar conditions. Of the probe substrates tested in vitro, felodipine, fluticasone, and budesonide were not included in the sensitivity analysis because they had fewer than four clinical DDI studies in common with midazolam. Cyclosporine, nifedipine, and quinidine exhibited reduced sensitivity compared with 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 \( C_{\text{max}} \), or the free hepatic \( C_{\text{max}} \)) 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 (Gaalten 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 \( f_{\text{in CYP}} \) value may have a marked impact on DDI predictions. Probe substrates with an \( f_{\text{in CYP}} \) value of 0.5 for a particular P450-mediated pathway may experience a maximal increase in \( \text{AUC}_p/\text{AUC} \) of 2-fold theoretically; as \( f_{\text{in CYP}} \) increases, the effect on the magnitude of \( \text{AUC}_p/\text{AUC} \) increases (Ito et al., 2005). The three probe substrates with the lowest in vivo sensitivity (cyclosporine, nifedipine, and quinidine) have \( f_{\text{in CYP}_{3A4}} \) values lower than 0.81 (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 \( \text{AUC}_p/\text{AUC} \) of 2-fold is expected (Gaalten et al., 2008). For drugs with a high extent of intestinal extraction, increases in \( \text{AUC}_p/\text{AUC} \) 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 on 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, in which a probe substrate that is not clinically relevant (e.g., testosterone) has been tested in vitro, demonstrates markedly increased CYP3A4 inhibition potential relative to that of midazolam and felodipine/nifedipine, and is predicted to exhibit a clinically relevant DDI (\( \text{AUC}_p/\text{AUC} \geq 2 \)), whereas midazolam and felodipine/nifedipine are not. Due diligence suggests that a strategy is needed to evaluate whether clinical studies additional or alternative to a midazolam or felodipine/nifedipine DDI study may be necessary based on in vivo 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 a potential alternative probe substrate to midazolam is identified based on in vitro inhibition potency, the most likely candidates would demonstrate inhibition profiles unique from that of midazolam. Based on 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 compared with midazolam and has not been tested using a potent CYP3A4 inhibitor clinically to our knowledge. Felodipine has a somewhat higher \( f_{\text{in CYP}_{3A4}} \) value (0.81) than nifedipine that should result in increased in vivo sensitivity and has been tested in vivo using a potent CYP3A4 inhibitor: \( \text{AUC}_p/\text{AUC} = 6.3 \) at 200 mg of itraconazole (Jalava et al., 1997).

Simvastatin demonstrates inhibition profiles unique from those of midazolam in vitro, has been clinically tested with potent CYP3A4 inhibitors in vivo, and demonstrates enhanced in vivo sensitivity compared with that of midazolam. Based on 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 on in vivo results. However, because potent inhibitors are expected to be identified in vitro and are 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 on 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). By 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 on 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 because it was inhibited less potently than midazolam by AMG 458. Sildenafil was predicted to exhibit clinically relevant DDls (AUC/AUC = 2) across the anticipated dose range (500–2000 mg), whereas midazolam and simvastatin were not predicted to demonstrate a <1.2-fold increase in DDI magnitude; this magnitude of change may be difficult to detect based on pharmacokinetic variability within an in vivo DDI study. Because clinical DDI studies may be performed 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. Although simvastatin is our recommended probe substrate for testosterone-selective inhibition, the 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 in which a probe substrate, such as testosterone, is predicted to exhibit clinically significant DDls, whereas clinically relevant probe substrates, such as midazolam and felodipine/riedpine, are not. Based on hierarchical clustering of 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 that it is unlikely that a universal alternative to midazolam will be available in the near future.

References

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