Predicting the Drug Interaction Potential of AMG 853, a Dual Antagonist of the D-Prostanoid and Chemoattractant Receptor-Homologous Molecule Expressed on T Helper 2 Cells Receptors

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Abstract:

2-(4-(4-((tert-Butylcarbamoyl)-2-(2-chloro-4-cyclopropylphenylsulfonylamido)phenoxy)-5-chloro-2-fluorophenyl)acetamide (AMG 853) is an orally bioavailable and potent dual antagonist of the D-prostanoid and chemoattractant receptor-homologous molecule expressed on T helper 2 cells receptors. The drug interaction potential of AMG 853, both as a victim and a perpetrator, was investigated using in vitro, in silico, and in vivo methodologies. Experiments in human liver microsomes (HLM) and recombinant enzymes identified CYP2C8, CYP2J2, and CYP3A as well as multiple UDP-glucuronosyltransferase isoforms as being responsible for the metabolic clearance of AMG 853. With use of HLM and selective probe substrates, both AMG 853 and its acyl glucuronide metabolite (M1) were shown to be inhibitors of CYP2C8. AMG 853 and M1 did not inhibit any of the other cytochrome P450 isoforms tested, and AMG 853 exhibited minimal enzyme induction properties in human hepatocytes cultures. In light of the in vitro findings, modeling and simulation approaches were used to examine the potential for ketoconazole (a CYP3A inhibitor) to inhibit the metabolism of AMG 853 as well as for AMG 853 to inhibit the metabolism of paclitaxel, rosiglitazone, and montelukast, commonly used substrates of CYP2C8. A weak and clinically insignificant drug interaction (area under the drug concentration-time curve (AUC)/AUC <2) was predicted between ketoconazole and AMG 853. No drug interactions were predicted for AMG 853 and paclitaxel, rosiglitazone, or montelukast. Finally, administration of AMG 853 to healthy human subjects in clinical trials in the presence or absence of ketoconazole confirmed that AMG 853 is unlikely to be involved in clinically significant drug interactions.

Introduction

Prostaglandin D2 (PGD2) is involved in the endogenous control of allergic responses and exerts its activity through two G-protein-coupled receptors, D-prostanoid (DP) receptor and chemoattractant receptor-homologous molecule expressed on T helper cells (CRTH2) (Arimura et al., 2001; Hiri et al., 2001; Oguma et al., 2004). Both the DP and CRTH2 receptors are believed to regulate cell function in eosinophils and T helper 2 cells that are known to accumulate as a result of bronchial asthma and other inflammatory diseases (Sugimoto et al., 2003; Pettipher et al., 2007). Multiple studies using small molecule antagonists of DP and CRTH2 also support the critical role of these receptors in mediating inflammatory responses (Lai et al., 2007; Van Hecken et al., 2007; Norman, 2010; Barnes et al., 2011).

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ABBREVIATIONS: PGD2, prostaglandin D2; DP, D-prostanoid; CRTH2, chemoattractant receptor-homologous molecule expressed on T helper 2 cells; AMG 853, 2-(4-(4-((tert-Butylcarbamoyl)-2-(2-chloro-4-cyclopropylphenylsulfonylamido)phenoxy)-5-chloro-2-fluorophenyl)acetamide; EMA, European Medicines Agency; HLM, human liver microsomes; HLuM, human lung microsomes; UDPGA, UDP-glucuronic acid; P450, cytochrome P450; UGT, UDP-glucuronosyltransferase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; DMSO, dimethyl sulfoxide; ISEF, intersystem extrapolation factor; HPLC, high-performance liquid chromatography; AUC, area under the drug concentration-time curve; NME, new molecular entity.
drug interaction studies in vitro and in vivo (U.S. Food and Drug Administration, 2006; Zhang et al., 2009; European Medicines Agency, 2010). Drug interactions involve a test article serving as either victim (traditionally referred to as the probe substrate) or perpetrator (inhibitor of a drug-metabolizing enzyme) in a drug interaction and, thus, phenotyping and in vitro drug interaction studies are required to fully predict the potential for a drug to cause interactions in the clinic. Furthermore, because it has been shown that circulating metabolites can play a role in drug interactions and toxicity, it may be important to assess the drug interaction potential for circulating metabolites in addition to that of the parent drug (Isoherranen et al., 2009; Yeung et al., 2011).

The in vitro, in silico, and in vivo experiments described in this article were designed to examine the drug interaction potential of AMG 853. Upon elucidation of the enzymes responsible for the metabolism of AMG 853, experiments were performed to determine the effects of AMG 853 on selective probe substrates as well as the potential for AMG 853 to be inhibited by known inhibitors. Simcyp was used to predict the potential clinical relevance of the observed in vitro drug interactions of AMG 853. Finally, an in vivo drug interaction study in healthy human subjects was conducted to confirm the in vitro and in silico predictions.

Materials and Methods

Materials. AMG 853 and M2 were obtained from the Amgen Sample Bank. Materials. AMG 853 and M2 were obtained from the Amgen Sample Bank. M1 was prepared at J-Star Research Inc. (South Plainfield, NJ), and M3 was biosynthesized using human liver microsomes. Pooled human liver microsomes (nonsmoker) (HLuM) and cryopreserved human hepatocytes were obtained from Celsis (Chicago, IL). Phenacetin, acetaminophen, α-naphthoflavone, furafylline, bupropion, clotrimazole, diclofenac, dextromethorphan, midazolam, 1′-hydroxymidazolam, sulfaphenazole, quinidine, tolbutamide, hydroxybupropion, ketoconazole, diethyldithiocarbamate, 6-hydroxytestosterone, ticlopidine, tolbutamide, NADPH were purchased from Toronto Research Chemicals (Ann Arbor, MI). Montelukast, hydroxybupropion, and gemfibrozil 1-O-glucuronide were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada).

AMG 853 Metabolism by UDP-Glucuronosyltransferases and Cytochromes P450. Glucuronidation of AMG 853 was evaluated in vitro against recombinant expressed human UGT enzyme preparations (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17). UGT enzymes (0.05 mg) were activated by preincubation with alamethicin (25 μg/ml) in 50 mM Tris buffer on ice for 30 min. At the end of the preincubation period, incubation mixtures were diluted with purified water, and AMG 853 was added to achieve a final concentration of 10 μM. After a second preincubation period (5 min) at 37°C, reactions were initiated by addition of UDPGA cofactor (1 mM; final concentration) and incubated for 30 min at 37°C (100 μl final incubation volume). Control incubations with inactive microsomes (prepared from membranes not expressing UGT enzyme) were treated identically as described above. Reactions were terminated by addition of 200 μl of acetonitrile containing formic acid (0.1%, v/v) and 0.1 mM tolbutamide as an internal standard. After centrifugation (10 min at 14600g), the resulting supernatants were transferred to 96-well plates and analyzed for the presence of acetyl glucuronide (M1) by mass spectrometry (LC-MS/MS).

The oxidative metabolism of AMG 853 was previously evaluated in vitro against a subset of recombinant expressed P450s (J. A. Davis, M. P. Grillo, X. Han, J. T. Pearson, L. C. Wienkers, and B. M. Amore, manuscript in preparation). To expand on the previously reported data, experiments using a full complement of recombinant P450s were performed. Incubations consisted of 10 pmol of recombinant P450, 3 mM MgCl2, and 10 μM AMG 853 in 100 mM potassium phosphate buffer (pH 7.4). After a 5-min preincubulation period, reactions were initiated with the addition of 1 mM NADPH (final concentration). Reactions were terminated after 30 min with 2 volumes of ice-cold acetonitrile containing 0.1 mM tolbutamide as an internal standard. Samples were then centrifuged for 30 min at 11300g in a Beckman Allegra 6R tabletop centrifuge (Beckman Coulter, Fullerton, CA). Supernatants were transferred into sample vials and then were analyzed for the presence of M2 and M3 using LC-MS/MS.

To confirm the observations in recombinant P450s, AMG 853 was incubated in human liver microsomes in the presence of P450-selective inhibitors. In brief, human liver microsomes (0.1 mg/ml) were incubated with 2 μM AMG 853, 3 mM MgCl2, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) for 60 min in the presence of 100 nM α-naphthoflavone (CYP1A2), 600 nM montelukast (CYP2C8), 1000 nM sulfaphenazole (CYP2C9), 2000 nM (+)-N-3-benzyl-nirvanol (CYP2C19), 600 nM quindine (CYP2D6), or 800 nM ketoconazole (CYP3A). After the incubation time period, samples were prepared for LC-MS/MS analysis as described above using 0.1 μM tolbutamide as an internal standard. Formation of M2...
and M3 in the aforementioned incubations was compared with that in a DMSO solvent control (0.1%, v/v) to determine the percentage of inhibition by each of the isozyme-selective inhibitors.

**Enzyme Kinetics.** Experiments to determine the enzyme kinetics for the formation of M1, M2, and M3 in human microsomes and recombinant P450 or UGT enzymes were performed under conditions determined to be linear with respect to time and protein concentration (data not shown). For M2 and M3, human liver or lung microsomes (0.1 mg/ml, final protein concentration; pooled and individual donor genotyped for CYP3A5 status) or recombinant P450 enzymes (1 pmol, final concentration) were preincubated with 3 mM MgCl₂, 100 mM potassium phosphate buffer (pH 7.4), and varying concentrations of AMG 853 (0–100 μM), P450-dependent) for 3 min at 37°C. Reactions were initiated with the addition of NADPH (1 mM, final concentration) and quenched after 15 min with 2 volumes (v/v) of acetonitrile containing 0.1 μM tolbutamide as an internal standard. To characterize the kinetics of M1 formation, HLM (0.1 mg/ml) or recombinant UGT enzymes (10 pmol) were combined with alamethicin (25 μg/ml protein) and allowed to sit on ice for 30 min before the addition of 3 mM MgCl₂, 50 mM Tris buffer, and varying concentrations of AMG 853 (0–100 μM) for 3 min at 37°C. Reactions were initiated with the addition of NADPH (1 mM, final concentration) and quenched after 15 min with 2 volumes (v/v) of acetonitrile containing 0.1 μM tolbutamide as an internal standard. All reactions were vortex-mixed and centrifuged at 1460 g for 10 min before analysis by HPLC-MS/MS.

To qualitatively assess the contribution of each P450 to the formation of M2 and M3, intrinsic clearance values (V_{max}/K_{i}) were scaled using the previously reported intersystem extrapolation factor (ISEF) scaling approach (Proctor et al., 2004; Chen et al., 2011). Protein abundance and ISEF values used were as follows: CYP2C8, 24 pmol/mg, 1.41; CYP2J2, 2.52 pmol/mg, 0.0066; CYP3A4, 141 pmol/mg, 0.15; CYP3A5, 21 pmol/mg, 0.15; and CYP3A7, 2 pmol/mg 0.15.

**AMG 853 and M1 Drug Interaction Assessment.** The in vitro kinetics of each P450 probe substrate were characterized before initiation of inhibition studies. All incubations contained less than 1% (v/v) of organic solvent (acetonitrile-DMSO: 9:1). To evaluate the inhibitory potential of AMG 853 toward eight P450 isozymes for IC₅₀ studies, a single concentration (at the estimated K_{i} value) of the probe substrate was incubated in combination with varying concentrations of AMG 853 from 0 to 30 μM. To determine K_{i} values for AMG 853 inhibition in HLM, four concentrations of probe substrate were used, encompassing the estimated K_{i} value. Positive control experiments were also performed with varying concentrations of known P450 inhibitors (for CYP1A2, n-naphthoflavone, 0–1 μM; for CYP2B6, clotrimazole, 0–6 μM; for CYP2C8, montelukast, 0–0.5 μM; for CYP2C9, sulfaphenazole, 0–2 μM; for CYP2C19, (+)-N-3-benzyl-nirvanol, 0–2 μM; for CYP2D6, quinidine, 0–0.3 μM; for CYP2E1, diethyldithiocarbamate, 0–250 μM; and for CYP3A4, ketoconazole, 0–0.5 μM). All incubation mixtures (0.2 ml, final volume) contained microsomal protein (0.1 mg/ml) and 100 mM potassium phosphate buffer (pH 7.4). The microsomal protein content and incubation time were used, encompassing the estimated 10% of the initial substrate concentration was consumed over the course of the reaction, and product formation was linear with respect to time and protein concentration. Reactions were initiated with 1 mM NADPH and were allowed to proceed for 30 min, depending on the probe substrate used (37°C in an incubator shaker). Each reaction was terminated with the addition of 1 volume of acetonitrile (v/v) containing a stable-label internal standard. The samples were centrifuged (1460g for 10 min) and then analyzed by LC-MS/MS. For the CYP2C8 K_{i} determinations with p-acetylsalicylic acid, rosiglitazone, and montelukast as probe substrates, the incubation mixture (0.2 ml final volume) contained microsomal protein (0.1 mg/ml) and 100 mM potassium phosphate buffer (pH 7.4) containing 3 mM MgCl₂. Reactions were initiated with 1 mM NADPH and allowed to proceed for 20 min. The reaction was terminated with the addition of 2 volumes of acetonitrile (v/v) containing 0.1 μM tolbutamide as an internal standard. The samples were centrifuged (1460g for 10 min) and analyzed by LC-MS/MS.

To evaluate the potential of AMG 853 or M1 to inactivate P450 isozymes in a time-dependent manner, primary incubations (300 μl, final volume) containing AMG 853 or M1 (10 μM), microsomal protein (1.0 mg/ml), and 100 mM potassium phosphate buffer (pH 7.4) were initiated with 1 mM NADPH and allowed to proceed up to 30 min at 37°C in an incubator shaker.

**TABLE 1**

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Metabolite</th>
<th>In Vitro System</th>
<th>S_{0.5}</th>
<th>V_{max}</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td>μM</td>
<td>pmol·min⁻¹·mg⁻¹</td>
<td></td>
</tr>
<tr>
<td>AMG 853</td>
<td>M1</td>
<td>HLM</td>
<td>205.4 ± 18.7</td>
<td>43.0 ± 1.29</td>
<td>1.77 ± 0.13</td>
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<tr>
<td></td>
<td></td>
<td>rUGT1A1</td>
<td>218.4 ± 21.2</td>
<td>3.45 ± 0.06</td>
<td>2.53 ± 0.14</td>
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<td></td>
<td>rUGT1A3</td>
<td>379.2 ± 36.4</td>
<td>39.1 ± 2.24</td>
<td>1.73 ± 0.15</td>
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<td></td>
<td></td>
<td>rUGT2B7</td>
<td>183.4 ± 22.4</td>
<td>13.6 ± 0.27</td>
<td>1.55 ± 0.07</td>
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<tr>
<td></td>
<td></td>
<td>r, recombinant</td>
<td>N.A.</td>
<td>N.A.</td>
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**TABLE 2**

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Metabolite</th>
<th>In Vitro System</th>
<th>K_{i}</th>
<th>V_{max}</th>
<th>K_{i}</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
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<td>AMG 853</td>
<td>M2</td>
<td>HLM pooled</td>
<td>2.59 ± 0.30</td>
<td>0.027 ± 0.0007</td>
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<td></td>
<td>HLM_{CYP3A5*1/1}</td>
<td>2.75 ± 0.37</td>
<td>0.044 ± 0.001</td>
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<td>HLM_{CYP3A5*3/3}</td>
<td>4.11 ± 0.51</td>
<td>0.023 ± 0.0006</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLuM</td>
<td>31.4 ± 2.27</td>
<td>0.028 ± 0.0007</td>
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<td></td>
<td>rCYP2C8</td>
<td>1.21 ± 0.18</td>
<td>0.031 ± 0.001</td>
<td>31.7 ± 5.7</td>
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<tr>
<td></td>
<td></td>
<td>rCYP2J2</td>
<td>4.53 ± 0.32</td>
<td>0.384 ± 0.021</td>
<td>240.1 ± 32.3</td>
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<tr>
<td></td>
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<td>rCYP3A4</td>
<td>125.7 ± 11.8</td>
<td>0.960 ± 0.040</td>
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<td>rCYP3A5</td>
<td>5.07 ± 0.33</td>
<td>0.221 ± 0.004</td>
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<td>rCYP3A7</td>
<td>10.0 ± 0.77</td>
<td>0.260 ± 0.0005</td>
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<tr>
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<td>M3</td>
<td>HLM pooled</td>
<td>40.2 ± 3.28</td>
<td>0.292 ± 0.010</td>
<td>N.A.</td>
</tr>
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<td></td>
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<td>HLM_{CYP3A5*1/1}</td>
<td>38.5 ± 2.61</td>
<td>0.317 ± 0.008</td>
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</tr>
<tr>
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<td>HLM_{CYP3A5*3/3}</td>
<td>35.2 ± 2.67</td>
<td>0.296 ± 0.009</td>
<td>N.A.</td>
</tr>
<tr>
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<td></td>
<td>HLuM</td>
<td>30.2 ± 1.46</td>
<td>0.141 ± 0.003</td>
<td>N.A.</td>
</tr>
<tr>
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<td>rCYP2C8</td>
<td>49.1 ± 3.01</td>
<td>0.250 ± 0.007</td>
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<td>rCYP2J2</td>
<td>40.7 ± 3.86</td>
<td>0.299 ± 0.012</td>
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<td></td>
<td>rCYP3A4</td>
<td>111.1 ± 8.57</td>
<td>1.41 ± 0.031</td>
<td>N.A.</td>
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<td>rCYP3A5</td>
<td>59.4 ± 3.16</td>
<td>1.13 ± 0.020</td>
<td>N.A.</td>
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<td>rCYP3A7</td>
<td>37.4 ± 2.44</td>
<td>0.286 ± 0.008</td>
<td>N.A.</td>
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</tbody>
</table>

r, recombinant; N.A., not applicable.
Aliquots (10 μl; 20× dilution) of the primary incubation mixture were removed and added to a secondary incubation containing probe substrate, 1 mM NADPH, and phosphate buffer (pH 7.4) at select time points (0, 5, 10, 15, and 30 min). The microsomal protein content and incubation time in the secondary incubation wells were such that no more than 10% of the substrate was depleted, and product formation was linear with time. Each secondary probe substrate reaction was terminated with the addition of 1 volume of acetonitrile containing a stable label internal standard. The secondary probe substrates used were 200 μM phenacetin (CYP1A2), 200 μM bupropion (CYP2B6), 35 μM paclitaxel (CYP2C9), 20 μM diclofenac (CYP2C9), 100 μM (S)-mephenytoin (CYP2C19), 30 μM dextromethorphan (CYP2D6), 200 μM chloroxazone (CYP2E1), and 200 μM testosterone (CYP3A4). The samples were centrifuged (14,600 g for 10 min) and then analyzed by LC-MS/MS. Positive control experiments were also performed using a similar procedure with furafylline (CYP1A2), 1-aminobenzotriazole (CYP2B6 and CYP2E1), gemfibrozil 1-O-glucuronide (CYP2C8), tienilic acid (CYP2C9), ticlopidine (CYP2C19), paroxetine (CYP2D6), and troleandomycin (CYP3A4).

The ability for AMG 853 to induce CYP1A2, CYP2B6, CYP2C9, and CYP3A4 mRNA expression and enzyme activity in human hepatocytes was also assessed. In brief, human hepatocytes (n = 3 donors) were suspended in plating media and plated in 24-well collagen-coated plates at a cell density of 400,000 cells/well. After a 4-h attachment period under 95% ambient air/5% CO2, the plating media was removed, and the cells were incubated overnight in sandwich culture media. Upon removal of sandwich culture media, cells were acclimated for 24 h in incubation media before the addition of DMSO (0.1%, v/v), 3-methylcholanthrene, omeprazole, phenobarbital, rifampin, or AMG853 (0.3–30 μM). Test articles were added to hepatocyte cultures every 24 h for a total of 48 h. On day 5, hepatocytes were washed with Krebs-Henseleit buffer before the assessment of P450 activity using the same probe substrates as described for the inhibition experiments. Selective probe substrates (CYP1A2, 100 μM phenacetin, 45 min; CYP2B6, 100 μM bupropion, 45 min; CYP2C9, 100 μM diclofenac, 30 min; CYP3A4, and 50 μM midazolam, 20 min) were used to assess P450 activity. After the incubation time period, 10 μl of supernatant was removed and added to 200 μl of ice-cold acetonitrile for analysis by LC-MS/MS as described below.

P450 mRNA expression was also evaluated by washing the hepatocytes in phosphate-buffered saline followed by addition of a lysis buffer. mRNA levels were measured using an branched DNA amplification kit from Panomics (Santa Clara, CA) according to the manufacturer’s instructions. Expression levels were determined by luminescence on a Luminoskan Ascent multiplate reader (Thermo Labsystems, Helsinki, Finland). Probes for CYP1A2, CYP2B6, CYP2C9, and CYP3A4 were normalized to a glyceraldehyde-3-phosphate dehydrogenase probe control before statistical analysis.

In Vitro Sample Analysis. The analysis of all in vitro samples was conducted using LC-MS/MS technology. In brief, the LC-MS/MS platform used for all experiments comprised an 4000 Q TRAP system fitted with an electrospray ionization source (Applied Biosystems, Foster City, CA). Analyses were conducted using the Q TRAP through two LC-20AD pumps with an in-line CBM-20A controller and DGU-20A, solvent degasser (Shimadzu, Columbia, MD) coupled to a LEAP CTC HTS PAL autosampler equipped with a dual-solvent self-washing system (CTC Analytics, Carrboro, NC). An injection volume of 10 μl was used for all analyses.

HPLC separation of M1, M2, and M3 was achieved on a Kinetex C18 column (50 × 2.1 mm, 2.6-μm; Phenomenex, Torrance, CA). The 4000 Q TRAP system was operated in negative ionization mode for the detection of M1 and positive ionization mode for the detection of M2 and M3. Mass transitions for the three metabolites were 738.0/583.0 (M1), 625.0/536.1 (M2), and 625.1/552.1 (M3). Linear gradient elution (flow rate = 500 μl/min) was performed 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 to waste for the first 60 s to remove any nonvolatile salts. For probe substrates from the IC50, Ki, or time-dependent inhibition, HPLC separation was achieved using a Gemini C18 column (20 × 30 mm, 5-μm; Phenomenex). Gradient elution (flow rate = 400 μl/min) was performed with 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 to waste for the first 45 s to remove any nonvolatile salts. Generic Q TRAP source parameters included the following: curtain gas, 20 arbitrary units; collisionally activated dissociation gas, medium; ion spray voltage, 4500 V; source temperature, 550°C; and ion source gas 1 and 2, 40 arbitrary units each. Interface heaters were kept on for all analytes. Probe substrate mass transitions (Q1/Q3) were identical to those in previously published methods (Walsky and Obach, 2004).

Statistical Analysis of In Vitro Data. Analysis of IC50 and Ki data was performed using GraphPad Prism (version 5.04; GraphPad Software Inc., San Diego, CA). IC50 data were fit using a sigmoidal dose-response model (eq. 1), whereas Ki data were applied to either a competitive (eq. 2), linear-mixed (eq. 3), or biphasic (eq. 4) inhibition model based on the Akaike information criterion and visual inspection of the Dixon [I] versus 1/v and Lineweaver-Burk (1/[S] versus 1/v) plots. For eqs. 1 to 4, max and min refer to the activity of the probe substrate at the lowest and highest inhibitor concentrations, respectively, [I] is the concentration of inhibitor in the system, Ki is equal to half the substrate concentration at maximal reaction velocity, Ki is the dissociation constant for the enzyme-inhibitor complex, and Ki is the dissociation constant for the enzyme-substrate-inhibitor complex. Note that for eqs. 2 and 3, Ki, Ki, and Vmax, were treated as global parameters.

\[
\text{% activity} = \min\left(1 + \frac{(\max - \min)}{(1 + 10^{\text{IC50-LogIC50}})}\right)
\]

\[
v = \frac{V_{\max} \cdot [S]}{K_{\text{m}} + [S]} + \frac{[S]}{1 + [I]/K_{i}}
\]

\[
v = \frac{V_{\max} \cdot [S]}{K_{\text{m}} + [S]} + \frac{[S]}{1 + [I]/K_{i}}
\]

In Silico Predictions. Simcyp (version 11; Simcyp Ltd., Sheffield, UK) was used to predict potential drug interactions for AMG 853 and its acyl glucuronide metabolite (M1) with ketocazone, paclitaxel, rosiglitazone, and montelukast. For AMG 853, the following parameters were entered into Simcyp: molecular mass, 609.5 Da; logP, 5.1; blood/plasma ratio, 1; fraction unbound in plasma, 0.003; Papp, 11.1 (from an LLC-PK1 permeability assay with a propranolol reference value of 36); and volume of distribution at steady state, 2.7 l/kg; enzyme kinetic and inhibition parameters for M1, M2, and M3 were as noted in Tables 1, 2, and 3. For M1, the molecular weight was adjusted to 785.0, logP to 2.5, and fraction unbound to 0.01. First-order absorption and minimal physiology-based pharmacokinetic models were used for absorption and distribution predictions, respectively. Simulations designed to capture the effect of AMG 853 on CYP2C8 metabolism used a 200-mg oral dose of AMG 853 and either a 4-mg oral dose of rosiglitazone, a 3-mg oral dose of montelukast, or a 4.5-mg intravenous infusion of paclitaxel over a 24-h period (Sonnichsen and Relling, 1994; Karonen et al., 2012; Naik et al., 2012). For simulations of ketoconazole effects on AMG 853 metabolism, the in silico dosing regimen consisted of 200-mg b.i.d. oral doses of ketoconazole for 2 days before a 50-mg oral dose of AMG 853 coadministered with the first oral dose of ketoconazole on day 3. Simulations (n = 10 trials) were performed in healthy patient populations (100 subjects, 18–55 years of age) with female subjects accounting for half of the population.

In Vivo Drug Interaction Study. To further investigate the potential for AMG 853 to be involved in drug interactions in vivo, a drug interaction study was conducted in healthy human volunteers. The study was conducted according to the Declaration of Helsinki and the International Conference on Harmonisation Tripartite Guideline on Good Clinical Practice. Approvals from appropriate research ethics committees were obtained from each participating study center. All patients provided written informed consent before participating. An external data monitoring committee monitored patient safety throughout the study. In brief, the control arm consisted of human subjects (n = 6) who were orally administered 50 mg of AMG 853. The control arm was compared with a second set of human subjects (n = 6) who were pretreated with ketoconazole (200 mg b.i.d., oral administration) for 2 days before coadministration of AMG 853 (50 mg, oral administration) with the dose of
Sample Analysis for In Vivo Samples. Human plasma samples were prepared for quantitative analysis by precipitation and filtration of the plasma proteins with an acetonitrile solution containing a stable-label internal standard. After filtration, the supernatant was evaporated to dryness under a stream of nitrogen. The resulting residue was reconstituted with 1% formic acid in water) and 40% solvent B (acetonitrile-water, 9:1) at a flow rate of 0.5 ml/min. After injection of 10 μl of sample extract, solvent B was held at 40% for 1.60 min followed by a rapid increase to 90% in 0.15 min. AMG 853 and M1 was dependent on both the CYP3A5 genotype and the individual standard, respectively. Quantitation of AMG 853 and M1 over the concentration range of 0.500 to 1000 ng/ml was accomplished using a weighted quadratic regression.

Results

Recombinant expressed UGTs and P450s were incubated with AMG 853 (10 μM) to determine the enzymes responsible for the conjugative and oxidative metabolism of AMG 853. Results (n = 3) indicated that UGT1A1, UGT1A3, and UGT2B7 were capable of conjugating AMG 853, with UGT1A3 exhibiting the highest activity relative to those of the other UGTs. (Fig. 2A). There appeared to be minor contributions to glucuronide formation from UGT1A4 and UGT1A8. Acyl glucuronide formation of AMG 853 was not observed for the remainder of the recombinant UGTs investigated. Multiple P450 isoforms were responsible for the oxidative formation of M2 and M3. At 10 μM AMG 853, CYP2J2 and CYP3A5 produced the greatest amounts of M2, followed by CYP2C8, CYP3A4, and CYP3A7 (Fig. 2B). For M3, CYP2C8, CYP2J2, CYP3A4, CYP3A5, and CYP3A7 contributed approximately equally to the formation of the metabolite. A minor contribution from CYP2C19 was observed for M3 (Fig. 2C). Human liver microsomes with isofrom-selective inhibitors confirmed the observations in recombinant P450s. Upon coincubation, montelukast (CYP2C8) and ketoconazole (CYP3A4) inhibited the formation of M2 and M3 (Fig. 2D).

To further characterize the enzymes responsible for catalyzing the formation of M1, M2, and M3, enzyme kinetics were determined in various in vitro systems. The formation of M1 in HLM, UGT1A1, UGT1A3, and UGT2B7 displayed sigmoidal kinetics. Formation of M1 was not observed in HLuM fortified with UDPGA. The formation of M2 and M3 in HLM (pooled and individual donors genotyped for CYP3A5 status), HLuM, recombinant CYP2C8, CYP2J2, CYP3A4, CYP3A5, and CYP3A7 were fit to a Michaelis-Menten model, except for M2 with CYP2C8, which was fit to a substrate inhibition model. Kinetic parameters for all AMG 853 metabolites are listed in Tables 1 and 2. When enzyme kinetic parameters were scaled to account for relative activity and enzyme abundance using the well documented ISEF approach (Proctor et al., 2004; Chen et al., 2011), CYP2C8 is expected to account for approximately 67% of M2 formation, with the remainder being attributed to CYP3A4 and CYP3A5. In a similar fashion, CYP3A4 and CYP3A5 were responsible for approximately 65% of M3 formation with the rest being attributed to CYP2C8. Relatively low protein expression and ISEF values indicate that CYP2J2 and CYP3A7 will be expected to account for approximately 67% of M2 formation, with the remainder being attributed to CYP3A4 and CYP3A5. In a similar fashion, CYP3A4 and CYP3A5 were responsible for approximately 65% of M3 formation with the rest being attributed to CYP2C8. Relatively low protein expression and ISEF values indicate that CYP2J2 and CYP3A7 will be expected to play minor roles in the hepatic formation of M2 and M3.

To assess the impact of CYP3A5 genotype on the formation of M1, M2, and M3 in HLM, varying concentrations of AMG 853 were incubated in HLM genotyped to be homozygous for either the CYP3A5*1 allele (high CYP3A5 content) or the CYP3A5*3 allele (low CYP3A5 content). Under V<sub>max</sub> conditions, the formation rate of M1 was dependent on both the CYP3A5 genotype and the individual cofactors included in the incubation (Fig. 3). In the presence of NADPH and UDPGA, an overall decrease in the formation of M1 was observed. Higher formation rates of M1 were observed when only UDPGA was included in the incubation and were still dependent upon the CYP3A5 genotype. Likewise, M2 was formed to significantly greater amounts in HLM genotyped to be homozygous for CYP3A5*1.
In contrast to what was observed for M2, the oxidative metabolism of M3 was not dependent on the CYP3A5 genotype in HLM (Fig. 4B).

At substrate concentrations approximating the respective $K_m$ for each probe substrate, the reversible inhibitory potential of AMG 853 toward eight human liver microsomal P450 enzyme activities was evaluated (Table 3). Under the conditions used, less than 10% of AMG 853 was consumed through the course of the incubation. In comparison with the positive control inhibitors, AMG 853 exhibited no inhibitory potential ($IC_{50} = 30 \mu M$) toward CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. AMG 853 was found to be an inhibitor of CYP2C8-catalyzed paclitaxel 6-hydroxylation ($IC_{50} = 5.4 \pm 1.0 \mu M$, data not shown). For probe substrates for which the AMG 853-mediated $IC_{50}$ experiments to estimate the $K_i$ were conducted, AMG 853 inhibited CYP2C8-catalyzed paclitaxel 6-hydroxylation (competitive inhibition model, $K_i = 1.1 \pm 0.2 \mu M$), rosiglitazone demethylation (competitive inhibition model, $K_i = 6.0 \pm 0.8 \mu M$), and montelukast 36-hydroxylation (biphasic inhibition model, $K_i = 1.8 \pm 1.0 \mu M$). Likewise, the M1 acyl glucuronide metabolite of AMG 853 was also an inhibitor of CYP2C8 in vitro. $K_i$ values for M1 were $2.7 \pm 0.5 \mu M$ for paclitaxel 6-hydroxylation, $6.9 \pm 1.4 \mu M$ for rosiglitazone demethylation, and $7.3 \pm 3.3 \mu M$ for montelukast 36-hydroxylation. Data for paclitaxel and rosiglitazone with M1 were both fit to a linear-mixed inhibition model, whereas data for montelukast and M1 were fit to a biphasic inhibition model (Fig. 5).

At substrate concentrations approximately 5-fold higher than the estimated $K_m$ for each marker substrate and 10 $\mu M$ AMG 853 or M1, the potential of AMG 853 or M1 to inactivate CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 enzyme activity in a time-dependent manner was evaluated. In comparison with the positive control inhibitors, neither AMG 853 nor M1 exhibited time-dependent inactivation against any of the isoforms tested (data not shown).

Assessment of the induction potential of AMG 853 revealed no significant induction of CYP1A2, CYP2B6, CYP2C9, or CYP3A4 in plated human hepatocytes. Control inducers increased mRNA expression for CYP1A2 (omeprazole, 47.4-fold), CYP2B6 (phenobarbital, 22.1-fold), CYP2C9 (rifampin, 2.96-fold), and CYP3A4 (rifampin, 12.5-fold) as expected. In contrast, AMG 853 at concentrations up to 30 $\mu M$ increased mRNA and activity levels by less than 1.7- and 1.4-fold, respectively, indicating a low potential for induction at clinically relevant concentrations of AMG 853 (data not shown).

Simcyp was used to predict the potential effects of ketoconazole on the pharmacokinetic profile of AMG 853 and, conversely, of AMG 853 on the pharmacokinetic profiles of paclitaxel, rosiglitazone, and montelukast. In simulations in which ketoconazole was used as the perpetrator drug and AMG 853 as the victim drug, the AUC of AMG

(Fig. 4A). In contrast to what was observed for M2, the oxidative metabolism of M3 was not dependent on the CYP3A5 genotype in HLM (Fig. 4B).
The role of the DP and CRTH2 G-protein-coupled receptors in the regulation of inflammatory responses has been reported previously (Arimura et al., 2001; Hirai et al., 2001; Sugimoto et al., 2003; Oguma et al., 2004; Pettipher et al., 2007; Van Heeken et al., 2007; Norman, 2010; Barnes et al., 2011; Wang et al., 2012). AMG 853 is a novel inhibitor of PGD2 binding to each of the aforementioned receptors and has been evaluated for the attenuation of symptoms associated with various allergic or inflammatory responses (Liu et al., 2009, 2011). In support of the preclinical development of AMG 853, the aim of this article was to evaluate the enzymatic pathways involved in the clearance of AMG 853 and subsequently to examine the potential of AMG 853 to be involved in drug interactions, either as a victim or perpetrator.

The importance of evaluating the potential of new molecular entities (NMEs) to cause or be the victim of a drug interaction has been conveyed in recent guidance from both the U.S. Food and Drug Administration (2006) and the European Medicines Agency (Zhang et al., 2009; European Medicines Agency, 2010). Strategies for the assessment of potential drug interactions generally begin with gathering information on the in vitro metabolism of the NME and the enzyme responsible for catalyzing these routes of metabolism (Huang et al., 2007, 2008; Foti and Wahlstrom, 2008; Zhang et al., 2009, 2010; Foti et al., 2010; Lewis, 2010). At present, accepted methods for determining the enzymes involved in metabolism of an NME include the use of recombinantly expressed enzyme systems, pooled HLM with chemical inhibitors or inhibitory antibodies, and/or the use or correlation analysis using well characterized probe substrates in microsomes from individual donors (Wienkers and Stevens, 2003). In addition, it is also important to understand the potential of the NME to inhibit the metabolism of other drugs currently on the market. Generation of in vitro inhibition values (IC50 or KI) and comparing these values to predicted or observed in vivo drug concentrations ([I]) can provide a useful measure of the potential for an NME to cause in vivo drug interactions. In general, an [I]/K value >0.1 is considered to be an indication that there is the possibility of an in vivo drug interaction, and additional characterization of the potential drug interaction is recommended (Zhang et al., 2009).

Recent reports have also noted the importance of evaluating the contribution of metabolites to the inhibition potential of a NME. Similar to AMG 853, a primary metabolic pathway of the peroxisome proliferator-activated receptor α-activating drug gemfibrozil is glucuronidation of its carboxylic acid moiety, which results in formation of an acyl glucuronide metabolite (Okerholm et al., 1976; Randinitis et al., 1984, 1986). Subsequent studies have identified gemfibrozil 1-O-acyl glucuronide as both a competitive inhibitor and a hemmodifying ligand of CYP2C8 in vitro (Ogilvie et al., 2006; Baer et al., 2009; Jenkins et al., 2011). Furthermore, there has recently been increased focus on the assessment of circulating metabolites for both inhibition potential and other biochemical properties such as target potency. Although characterization of circulating metabolites is guided by strategies such as the Guidance for Industry: Safety Testing of Drug Metabolites (U.S. Food and Drug Administration, 2006), there are no recommendations on the assessment of the drug interaction potential of circulating metabolites. Recent efforts using the Metabolism and Transport Drug Interaction Database have noted that of 129 drug interactions (greater than 20% increase in AUC) involving the P450 family of enzymes, 106 had metabolites that circulated in plasma (Isoherranen et al., 2009). To this end, it becomes prudent to assess circulating metabolites not only for their potency against a biological target, but also for their potential to play a role in drug interactions.

The primary metabolism of AMG 853 in HLM and hepatocytes has been reported previously (J. A. Davis, M. P. Grillo, X. Han, J. T. Pearson, L. C. Wienkers, and B. M. Amore, manuscript in preparation). The major metabolites of AMG 853 in vitro include an acyl

![Enzyme kinetics for the formation of M2 and M3 in pooled HLM and donors genotyped to be CYP3A5*1/*1 (high expression) or CYP3A5*3/*3 (low expression). A, M2 exhibited a dependence on the CYP3A5 genotype, with the highest formation rates in donors genotyped to be CYP3A5*1/*1. B, no dependence on the CYP3A5 genotype status was observed for M3.](image-url)
glucuronide metabolite (M1), a t-butyl-hydroxylated metabolite (M2), and a chlorocyclopropylphenyl-hydroxylated metabolite (M3). Measured plasma concentrations of M1 in both humans and preclinical species suggest that glucuronidation is a major clearance pathway for AMG 853 (Banfield et al., 2010). Initial work in recombinantly expressed enzyme systems implicated UGT1A1, UGT1A3, and UGT2B7 in the conjugative formation of M1 and CYP2C8 and CYP3A in the oxidative formation of M2 and M3. The data described in this article support the previous assessment and also implicate CYP2J2 as potentially having a role in the formation of M2 and M3. CYP2J2 is primarily expressed in the small intestine and heart but is also found in the liver, skeletal muscles, lungs, and salivary glands (Wu et al., 1996; Gaedigk et al., 2006; Paine et al., 2006; Lee et al., 2010). Whereas CYP2J2 accounts for approximately 1 to 2% of total P450 content in both the liver and small intestine, expression of the isoform in lung tissue is of interest in light of the role of DP and CRTH2 in regulating bronchial asthma and other airway-related inflammatory diseases. CYP2J2 has been implicated in the metabolism of antihistamine drugs such as terfenadine, astemizole, and ebastine and, similar to what was observed in this study, has previously been shown to metabolize many of the same substrates as CYP3A4 (Hashizume et al., 2002; Matsumoto et al., 2002; Lee et al., 2010). Furthermore, as with CYP3A4, the enzyme is inhibited by ketoconazole (Stresser et al., 2004).

**FIG. 5.** Determination of $K_i$ values for AMG 853 and M1 against the CYP2C8 probes paclitaxel, rosiglitazone, and montelukast in HLM (activity expressed as relative response of analyte to internal standard). AMG 853 data were fit to a competitive inhibition model for paclitaxel ($K_i = 1.1 \mu M$) and rosiglitazone ($K_i = 6.0 \mu M$) and a biphasic inhibition model for montelukast ($K_i = 1.8 \mu M$). M1 data were fit to a linear-mixed inhibition model for paclitaxel ($K_i = 2.7 \mu M$) and rosiglitazone ($K_i = 6.9 \mu M$) and a biphasic inhibition model for montelukast ($K_i = 7.3 \mu M$).
The determination of enzyme kinetic parameters, a more mechanistic approach to identifying the relative contributions of each P450 or UGT to the clearance of an NME, supports the primary roles of CYP2C8, CYP2J2, CYP3A, and UGT1A3 in the metabolism of AMG 853. The highest observed $V_{\text{max}}$ value for the formation of M1 in recombinant UGT enzymes was obtained with UGT1A3 and is similar to the $V_{\text{max}}$ value observed in HLM. In light of recent evidence indicating that P450 enzymes may act as gatekeepers or functional modifiers to the UGT family of enzymes owing to their spatial relationship within the membrane of the endoplasmic reticulum (Ishii et al., 2010), the effect of NADPH and the CYP3A5 genotype on M1 formation was also evaluated. In all cases, formation of M1 was greater when only UDPGA was included in the incubation as opposed to UDPGA and NADPH, which indicates that NADPH-dependent metabolism may reduce the amount of aglycone (AMG 853) available to the chemical inhibitors or selective inhibitory antibodies (data not shown), a decrease in the clearance of AMG 853 and the formation of M1 in HLM was not observed.

A number of marketed drugs are currently recognized by the U.S. Food and Drug Administration as being probes for CYP2C8. Paclitaxel 6α-hydroxylation, amodiaquine N-deethylation and rosiglitazone para-hydroxylation are all recognized as being selectively catalyzed by CYP2C8 in vitro (VandenBrink et al., 2011). Both repaglinide and rosiglitazone are recommended as probe substrates for in vivo clinical studies (Huang et al., 2007). More recently, montelukast has been demonstrated to be primarily metabolized by CYP2C8 at clinically relevant concentrations (Karonen et al., 2010, 2012; Filippula et al., 2011; VandenBrink et al., 2011). In light of the observed in vitro inhibition parameters for AMG 853 and its acyl glucuronide metabolite M1, further exploration of the ability of AMG 853 to inhibit the metabolism of the previously mentioned drugs was warranted. When in silico predictions using Simcyp were performed to assess the potential for AMG 853 to be a perpetrator of CYP2C8-mediated drug interactions in vivo, no increase in the predicted $C_{\text{max}}$ or AUC values of paclitaxel, rosiglitazone, or montelukast was observed. Although AMG 853 had low micromolar $K_i$ values against CYP2C8 in vitro, one must also take into account the fact that the free fraction of AMG 853 in human plasma was previously determined to be 0.003. Therefore, one could expect the free in vivo concentrations of AMG 853 to be well below the observed in vitro $K_i$ values and would not anticipate a clinically significant drug interaction.

Although AMG 853 exhibits low potential to be the perpetrator of metabolism-dependent drug interactions, the observation that the oxidative metabolism of AMG 853 is primarily mediated by CYP2C8, CYP2J2, and CYP3A allows for the possibility of AMG 853 being a victim of a drug interaction by the quintessential nonselective CYP3A inhibitor, ketoconazole. In vitro phenotyping studies demonstrated that when either CYP2C8 or CYP3A was selectively inhibited with chemical inhibitors or selective inhibitory antibodies (data not shown), a decrease in the clearance of AMG 853 and the formation of M2 and M3 was noted. In silico modeling predicted a slight, albeit clinically insignificant (AUC/AUC < 2-fold), drug interaction potential for AMG 853 in the presence of ketoconazole. However, because

### TABLE 4

Summary of Simcyp parameters for the in silico estimation of the drug interaction potential of AMG 853 both as a victim (versus ketoconazole) or a perpetrator (versus paclitaxel, rosiglitazone, or montelukast)

<table>
<thead>
<tr>
<th>Victim Drug</th>
<th>Effector Drug</th>
<th>Proposed Interaction</th>
<th>Predicted Fold Change in AUC</th>
<th>Predicted Fold Change in $C_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMG 853 (200 mg)</td>
<td>Ketoconazole (200 mg)</td>
<td>Inhibition of CYP3A-mediated metabolism of AMG 853 by ketoconazole</td>
<td>1.91 ± 0.18 (1.63–2.10)</td>
<td>1.90 ± 0.39 (1.87–2.12)</td>
</tr>
<tr>
<td>Paclitaxel (4.5 mg)</td>
<td>AMG 853 (200 mg)</td>
<td>Inhibition of CYP2C8-mediated paclitaxel metabolism by AMG 853</td>
<td>1.01 ± 0.005 (1.00–1.02)</td>
<td>1.01 ± 0.003 (1.00–1.03)</td>
</tr>
<tr>
<td>Rosiglitazone (4 mg)</td>
<td>AMG 853 (200 mg)</td>
<td>Inhibition of CYP2C8-mediated rosiglitazone metabolism by AMG 853</td>
<td>1.00 ± 0.001 (0.99–1.02)</td>
<td>1.01 ± 0.004 (1.00–1.03)</td>
</tr>
<tr>
<td>Montelukast (4 mg)</td>
<td>AMG 853 (200 mg)</td>
<td>Inhibition of CYP2C8-mediated montelukast metabolism by AMG 853</td>
<td>1.01 ± 0.008 (1.00–1.02)</td>
<td>1.01 ± 0.007 (1.00–1.02)</td>
</tr>
</tbody>
</table>
the predicted change in AUC was near the point of clinical relevance and many CYP3A inhibitors may also affect the activity of the UGT family of enzymes, an in vivo drug interaction study with ketoca
zole was undertaken.

Plasma concentrations of AMG 853 obtained from the in vivo drug interaction study in the presence or absence of ketoca
zole indicated that the in vitro and in silico data accurately predicted the in vivo outcome (observed values within 2-fold of predicted values). The slight overprediction of C\text{max} and AUC ratios that were estimated can most likely be accounted for by the multiple clearance pathways of AMG 853. Whereas the drug interaction potential of AMG 853 in the presence of ketoca
zole is primarily centered on the interactions of ketoca
zole with CYP2C8, CYP2J2, and CYP3A from an in vitro and in silico standpoint, the importance of these metabolic pathways in vivo may be diminished when the contribution of glucuronidation and excretory pathways such as urinary or biliary elimination is accounted for. On the basis of the observed enzyme kinetic parameters for AMG 853 glucuronidation, the phase II pathway would have the capacity to metabolize any additional aglycone made available by the inhibition of oxidative metabolism at the clinically observed concentrations of AMG 853. In addition, the exact extent of AMG 853 metabolism that occurs in the intestine or the kidney is unknown and many CYP3A inhibitors may also affect the activity of the UGT isoforms and cytochrome P450 3A4. The data also suggest an important role for CYP3A5 with respect to the clearance of AMG 853. In vitro drug interaction studies identified AMG 853 as an inhibitor of CYP2C8-catalyzed metabolism. Incorporation of the in vitro data into in silico predictions using Simcyp predicted no potential drug interac
tions for CYP2C8 by AMG 853 and a weak and clinically insig
nificant (AUC/AUC < 2-fold) interaction for AMG 853 in the presence of ketoca
zole. A subsequent clinical drug interaction study in healthy human volunteers confirmed the lack of an effect of keto
ca
zole on the metabolism of AMG 853. On the basis of these observations, we do not expect that AMG 853 will serve as either a victim or perpetrator of clinically relevant drug interactions.

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Authorship Contributions

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EVALUATION OF AMG 853 DRUG INTERACTION POTENTIAL IN HUMANS

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