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-chloro-4-cyclopropylphenylsulfonylamido)phenoxy)-5-chloro-2-fluorophenyl)acetic acid (AMG 853) is a small molecular entity.

Pharmacokinetics and Drug Metabolism, Amgen Inc., Seattle, Washington

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; Hirai 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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Dosage and Administration

AMG 853 (2-(4-(4-(tert-Butylcarbamoyl)-2-chloro-4-cyclopropylphenylsulfonylamido)phenoxy)-5-chloro-2-fluorophenyl)acetic acid) 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 vivo, 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.
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 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. M1 was prepared at J-Star Research Inc. (South Plainfield, NJ), and M3 was biosynthesized using human liver microsomes. Pooled human liver microsomes (HLM) were purchased from CelZDirect (Durham, NC). Human lung microsomes (nonsmoker) (HLuM) and cryopreserved human hepatocytes were obtained from Celsis (Chicago, IL). Phenacetin, acetaminophen, α-naphthoflavone, furafylline, bupropion, clotrimazole, diclofenac, dextromethorphan, dextrorphan, midazolam, 1α-hydroxymidazolam, sulfaphenazole, quinidine, tolbutamide, nitrofurantoin, ketoconazole, diethyldithiocarbamate, 6β-hydroxytestosterone, ticlopidine, tolbutamide, 3-methylcholanthrene, omeprazole, phenobarbital, ritonavir, and phenytin, hepatocyte culture media, and UDP-glucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO). Chlorzoxazone, phenacetin, acetaminophen, α-naphthoflavone, furafylline, bupropion, clotrimazole, diclofenac, dextromethorphan, dextrorphan, midazolam, 1α-hydroxymidazolam, sulfaphenazole, quinidine, tolbutamide, nitrofurantoin, ketoconazole, diethyldithiocarbamate, 6β-hydroxytestosterone, ticlopidine, tolbutamide, 3-methylcholanthrene, omeprazole, phenobarbital, ritonavir, and phenytin, hepatocyte culture media, and UDP-glucuronic acid (UDPGA) 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 (1A, 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/mg) 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 1460g), the resulting supernatants were transferred to 96-well plates and analyzed for the presence of acetylglucuronide (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 preincubation 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 130g in a Beckman Allegra 6R tabletop centrifuge (Beckman Coulter, Fullerton, CA). Supernatants were transferred into sample vials and then 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. 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 preincubation 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 130g in a Beckman Allegra 6R tabletop centrifuge (Beckman Coulter, Fullerton, CA). Supernatants were transferred into sample vials and then 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 quinidine (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 mM 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 isoform-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). Reactions were initiated with the addition of NADPH and allowed to proceed for 10 min before analysis by LC-MS/MS. To qualitatively assess the contribution of each P450 to the formation of M2 and M3, intrinsic clearance values (V_max/K_m) were scaled using the previously reported intersystem extrapolation factor (IREF) scaling approach (Proctor et al., 2004; Chen et al., 2011). Protein abundance and IREF 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 IC_50 studies, a single concentration (at the estimated K_m 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_m value. Positive control experiments were also performed with varying concentrations of known P450 inhibitors (for CYP1A2, naphthoflavone, 0–1 μM; for CYP2B6, clotrimazole, 0–6 μM; for CYP2C8, montelukast, 0–0.5 μM; for CYP2C9, sulphanilazole, 0–2 μM; for CYP2C19, (+)-3-benzyl-nirvanol, 0–2 μM; for CYP2D6, quinidine, 0–0.3 μM; for CYP2E1, diethyldithiocarbamate, 0–250 μM; and for CYP3A, ketocazone, 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 K_m value. All incubation mixtures (0.2 ml, final volume) contained microsomal protein (0.1 mg/ml) and 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM tolbutamide as internal standard. The samples were centrifuged (1460g for 10 min) and then analyzed by LC-MS/MS.

**TABLE 1**

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Metabolite</th>
<th>In Vitro System</th>
<th>S_{0.5} (μM)</th>
<th>V_{max} (pmol/μg/min)</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<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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<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>
</tr>
<tr>
<td></td>
<td></td>
<td>rUGT1A3</td>
<td>379.2 ± 36.4</td>
<td>39.1 ± 2.24</td>
<td>1.73 ± 0.15</td>
</tr>
<tr>
<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>
</tr>
<tr>
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</tr>
</tbody>
</table>

r, recombinant. Table values are the mean of at least three experiments ± standard deviation.

**TABLE 2**

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Metabolite</th>
<th>In Vitro System</th>
<th>K_{i} (μM)</th>
<th>V_{max} (pmol/μg/min)</th>
<th>K_{M} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMG 853</td>
<td>M2</td>
<td>HLMpooled</td>
<td>2.59 ± 0.30</td>
<td>0.027 ± 0.0007</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLM/CYP3A5*5/5</td>
<td>2.75 ± 0.37</td>
<td>0.044 ± 0.001</td>
<td>N.A.</td>
</tr>
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<td></td>
<td></td>
<td>HLM/CYP3A5*5/5</td>
<td>4.11 ± 0.51</td>
<td>0.023 ± 0.0006</td>
<td>N.A.</td>
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<tr>
<td></td>
<td></td>
<td>HLM/CYP3A5*5/5</td>
<td>31.4 ± 2.27</td>
<td>0.028 ± 0.0007</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rCYP2C8</td>
<td>1.21 ± 0.18</td>
<td>0.031 ± 0.001</td>
<td>31.7 ± 5.7</td>
</tr>
<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>
<td></td>
<td>rCYP3A4</td>
<td>123.7 ± 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>
<td>N.A.</td>
</tr>
<tr>
<td>AMG 853</td>
<td>M3</td>
<td>HLMpooled</td>
<td>40.2 ± 3.28</td>
<td>0.292 ± 0.010</td>
<td>N.A.</td>
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<td>HLM/CYP3A5*5/5</td>
<td>38.5 ± 2.61</td>
<td>0.317 ± 0.008</td>
<td>N.A.</td>
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<td>HLM/CYP3A5*5/5</td>
<td>35.2 ± 2.67</td>
<td>0.296 ± 0.009</td>
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<td>HLM/CYP3A5*5/5</td>
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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>
<td>N.A.</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>
</tr>
<tr>
<td></td>
<td></td>
<td>rCYP3A5</td>
<td>59.4 ± 3.16</td>
<td>1.13 ± 0.020</td>
<td>N.A.</td>
</tr>
<tr>
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<td></td>
<td>rCYP3A7</td>
<td>37.4 ± 2.44</td>
<td>0.286 ± 0.008</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

r, recombinant; N.A., not applicable. TABLE values are the mean of at least three experiments ± standard deviation. V_max is expressed as pmol/mg 0.15.
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)-mephentoin (CYP2C19), 30 μM dextromethorphan (CYP2D6), 200 μM chlorozoxazone (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-oxo (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, 100 μl of supernatant was removed and added to 200 μl of ice-cold acetonitrile before 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 analyzed using a 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 introduced to the Q TRAP for the first 100 trials over a 24-h period (Sonnichsen and Relling, 1994; Karonen 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...
ketoconazole on the morning of day 3. Plasma concentrations of AMG 853 were monitored from 0 to 72 h postdose. Pharmacokinetic parameters were estimated using noncompartmental analysis (WinNonlin; Pharsight, Mountain View, CA). Drug interaction potential was assessed by comparing peak drug estimated using noncompartmental analysis (WinNonlin; Pharsight, Mountain View, CA). Drug interaction potential was assessed by comparing peak drug concentrations monitored from 0 to 72 h postdose. Pharmacokinetic parameters were estimated using noncompartmental analysis (WinNonlin; Pharsight, Mountain View, CA). Drug interaction potential was assessed by comparing peak drug concentrations monitored from 0 to 72 h postdose. Pharmacokinetic parameters were estimated using noncompartmental analysis (WinNonlin; Pharsight, Mountain View, CA). Drug interaction potential was assessed by comparing peak drug concentrations monitored from 0 to 72 h postdose. Pharmacokinetic parameters were estimated using noncompartmental analysis (WinNonlin; Pharsight, Mountain View, CA).

**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 were detected using an API4000 mass spectrometer (Applied Biosystems) set in the positive ion mode. The source temperature was set to 375°C, which minimized in source conversion of M1. The declustering potentials were set to 95.0 and 61.0 V for AMG 853 and M1, respectively. Multiple reaction monitoring scanning of transitions 609.1/338.0, 785.2/609.2, and 581.1/372.2 were used to monitor for AMG 853, M1, and the internal 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 isof orm-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 UGT1B7 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 play a minor role 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 Vmax 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 ($IC_{50} \approx 30 \mu M$) 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 853 was reduced by 1.5-fold.
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 (FDA) and the European Medicines Agency (EMEA). 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. In vitro data are then used to predict the potential for an NME to cause or be the victim of in vivo drug interactions. In general, an interaction is recommended if the predicted in vivo drug concentrations ([I]/K) can provide a useful measure of the potential for an NME to cause in vivo drug interactions. 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; Randinits et al., 1984, 1986). Subsequent studies have identified gemfibrozil 1-O-acyl glucuronide as both a competitive inhibitor and a heme-modifying 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...
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).

![Graphs showing the inhibition of AMG 853 and M1 against the CYP2C8 probes paclitaxel, rosiglitazone, and montelukast in HLM.](image-url)

**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 was also evaluated. In all cases, formation of M1 was greater when only UDPGA was included in the incubation as opposed to UDPGA was also evaluated. In all cases, formation of M1 was greater when only UDPGA was included in the incubation as opposed to UDPGA served in recombinant P450s for M2 were with CYP2J2, followed by CYP3A5*3/*3 allele (high expression) and lower in those homozgyous for the CYP3A5*3 allele (low expression). A plausible explanation may be that when either CYP450-UGT heterodimerization often observed in vitro (Fremont et al., 2005; Takeda et al., 2009), although it is important to note that it may also be related to a variation in the amount of UGT protein in each lot of liver microsomes.

With use of $V_{\text{max}}/K_m$ as a measure of the intrinsic contribution of an enzyme to a metabolic pathway, the highest intrinsic activities observed in recombinant P450s for M2 were with CYP2J2, followed by CYP3A5. An approximately 5.7-fold higher $V_{\text{max}}/K_m$ value was observed using recombinant CYP3A5 than with CYP3A4, which indicates a greater contribution from CYP3A5 for the formation of M2. This result is supported by the decreased formation of M2 in HLM genotyped to be CYP3A5*3/*3 compared with wild-type CYP3A5 or pooled microsomes. With respect to formation of M3, CYP3A5 exhibited the highest $V_{\text{max}}/K_m$ value of the recombinant P450s tested, although the relative difference between contributing isoforms was not as great as that noted for M2. Although CYP3A and CYP2J2 are known to be expressed in the lung (Zeldin et al., 1996; Hukkanen et al., 2002), the relatively small $V_{\text{max}}/K_m$ values observed in HLM indicate that metabolism of AMG 853 in the lungs should have only a minor impact on the overall clearance of AMG 853. 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 6a-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; Filipula 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**

<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</td>
<td>Ketoconazole</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</td>
<td>AMG 853</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</td>
<td>AMG 853</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</td>
<td>AMG 853</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>
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</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 ketoconazole was undertaken.

Plasma concentrations of AMG 853 obtained from the in vivo drug interaction study in the presence or absence of ketoconazole 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 ketoconazole is primarily centered on the interactions of ketoconazole 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 could also contribute to the overprediction of drug interaction values. Therefore, the overall percentage of AMG 853 clearance in vivo that is attributed to CYP2C8, CYP2J2, and CYP3A may actually be less than that predicted in vitro, as is the observed effect of ketoconazole on the clearance of AMG 853.

In summary, this article describes the reaction phenotyping and drug interaction studies undertaken to support the development of AMG 853, a novel inhibitor of the DP and CRTH2 G-protein-coupled receptors. In vitro studies implicated CYP2C8, CYP2J2, CYP3A, and multiple UGT isozymes as being responsible for the oxidative and conjugative metabolism of AMG 853. 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 interactions for CYP2C8 by AMG 853 and a weak and clinically insignificant (AUC/AUC <2-fold) interaction for AMG 853 in the presence of ketoconazole. A subsequent clinical drug interaction study in healthy human volunteers confirmed the lack of an effect of ketoconazole 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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Participated in research design: Foti, Pearson, Wong, Zalikowski, Banfield, Rock, Wahlstrom, and Amore.
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Performed data analysis: Foti, Pearson, Wong, Zalikowski, Boudreaux, Prokop, and Emery.
Wrote or contributed to the writing of the manuscript: Foti, Zalikowski, Banfield, Wickens, and Amore.

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