Predicting the Drug Interaction Potential of AMG 853, a Dual Antagonist of the D-Prostanoid (DP) and Chemoattractant Receptor-Homologous Molecule Expressed on TH2 Cells (CRTH2) Receptors

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Evaluation of AMG 853 Drug Interaction Potential in Humans

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Abbreviations:  DP, D-prostanoid;  CRTH2, chemoattractant receptor-homologous molecule expressed on TH2 cells;  PGD2, prostaglandin D2;  UGT, UDP-glucuronosyltransferase;  CYP, cytochrome P450;  UDPGA, UDP-glucuronic acid;  HLM, human liver microsomes;  HLuM, human lung microsomes;  LC-MS/MS, liquid chromatography / tandem-mass spectrometry;  NME, new molecular entity;  AUC, area under the drug concentration-time curve;  C_{max}, peak drug concentration observed in plasma
Abstract

AMG 853 is an orally bioavailable and potent dual antagonist of the D-prostanoid (DP) and chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) receptors. The drug interaction potential of AMG 853, both as a victim and 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 UGT isoforms as being responsible for the metabolic clearance of AMG 853. Using 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 CYP 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 utilized 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 (AUC_i/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; Hirai et al., 2001; Oguma et al., 2004). Both the DP and CRTH2 receptors are believed to regulate cell function in eosinophils and TH2 cells that are known to accumulate as a result of bronchial asthma and other inflammatory diseases (Pettipher et al., 2007; Sugimoto et al., 2003). Multiple studies utilizing small molecule antagonists of DP and CRTH2 also support the critical role of these receptors in mediating inflammatory responses (Barnes et al., 2011; Lai et al., 2007; Norman, 2010; Van Hecken et al., 2007).

AMG 853, 2-(4-(4-(tert-butylcarbamoyl)-2-(2-chloro-4-cyclopropylphenylsulfonamido)phenoxy)-5-chloro-2-fluorophenyl)acetic acid (Figure 1), a novel inhibitor of PGD2 binding to both DP and CRTH2, has been evaluated for treatment of allergic diseases (Liu et al., 2009; Liu et al., 2011). In vitro, IC50 values for AMG 853 against DP and CRTH2 ranged from 3 to 35 nM. AMG 853 has also shown efficacy in a guinea pig model of PGD2-induced airway constriction. The preclinical pharmacokinetics of AMG 853 were characterized by extensive oral absorption and moderate clearance across species. The compound was highly bound to plasma proteins (fu = 0.003) and its major metabolites include hydroxylation on the both the t-butyl and cyclopropyl moieties (M2 and M3), as well as glucuronidation on the carboxylic acid moiety (M1) (Davis et al., 2012).

The prediction of drug interactions for a potential drug candidate is a key aspect of the drug development process. Recent guidelines by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have provided useful recommendations for the
conduct of drug interaction studies *in vitro* and *in vivo* (EMA, 2010; Zhang et al., 2009). 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 as such, phenotyping and *in vitro* drug interaction studies are required to fully predict the potential for a drug to cause interactions in the clinic. Furthermore, as 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 parent drug (Isoherranen et al., 2009; Yeung et al., 2011).

The *in vitro*, *in silico* and *in vivo* experiments described in this manuscript 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 carried out 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. M1 was prepared at J-Star Research Inc. (South Plainfield, NJ) and M3 was bio-synthesized using human liver microsomes. Pooled human liver microsomes (HLM) were purchased from CellzDirect (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, dextorphan, paroxetine, midazolam, 1'-hydroxymidazolam, sulfaphenazole, quinidine, troleandomycin, ketoconazole, diethyldithiocarbamate, 6β-hydroxytestosterone, ticlopidine, tolbutamide, 3-methylcholanthrene, omeprazole, phenobarbital, rifampin, hepatocytes culture media and UDP-glucuronic acid (UDPGA) were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorzoxazone and paclitaxel were purchased from MP Biomedicals (Solon, OH). (S)-mephenytoin was purchased from Biomol International (Plymouth Meeting, PA). 6-Hydroxypaclitaxel and NADPH were purchased from Calbiochem (San Diego, CA). Recombinant cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) Supersomes, CYP3A5 genotyped HLM, 4'-hydroxy-(S)-mephenytoin, 4'-hydroxydiclofenac, 6-hydroxychlorzoxazone, (+)-N-3-benzyl-nirvanol, hepatocytes sandwich media and stable-label internal standards were purchased from BD Biosciences (Bedford, MA). Testosterone was purchased from Steraloids (Newport, RI). Tienilic acid was ordered from Cayman Chemical Co. (Ann Arbor, MI). Montelukast, hydroxybupropion and gemfibrozil 1-O-glucuronide were purchased from Toronto Research Chemicals (North York, Ontario, Canada).
**AMG 853 Metabolism by UDP-Glucuronosyltransferases and Cytochrome P450s.**

Glucuronidation of AMG 853 was evaluated *in vitro* against recombinant expressed human UGT enzymes preparations (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17). UGT enzymes (0.05 mg) were activated by pre-incubating with alamethicin (25 µg/mg) in 50 mM Tris buffer on ice for 30 min. At the end of the pre-incubation period, incubation mixtures were diluted with purified water and AMG 853 was added to achieve a final concentration of 10 μM. Following a second pre-incubation period (5 min) at 37 ºC, reactions were initiated by addition of UDPGA cofactor (1 mM, final concentration) and incubated for 30 min at 37 ºC (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 acetonitrile containing formic acid (0.1%, v/v) and 0.1 μM tolbutamide as an internal standard. Following centrifugation (10 min x 1460 g) the resulting supernatants were transferred to 96-well plates and analyzed for the presence of acyl 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 CYPs (Davis et al., 2012). To expand upon the previously reported data, experiments utilizing a full complement of recombinant CYPs were carried out. Incubations consisted of 10 pmol recombinant CYP, 3 mM MgCl₂, and 10 μM AMG 853 in 100 mM potassium phosphate buffer (pH 7.4). Following a 5 min pre-incubation 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 μM tolbutamide as an internal standard. Samples were then centrifuged for 30 min x 1130 g in a Beckman Allegra 6R table top 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 CYPs, AMG 853 was incubated in human liver microsomes in the presence of CYP 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 minutes 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). Following 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 to a DMSO solvent control (0.1%, v/v) to determine the percent 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 CYP or UGT enzymes were carried out 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 CYP enzymes (1 pmol, final concentration) were pre-incubated with 3 mM MgCl₂, 100 mM potassium phosphate buffer (pH 7.4) and varying concentrations of AMG 853 (0 – 1000 µM, CYP dependent) for 3 min at 37 ºC. 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/mg protein) and allowed to sit on ice for 30 min prior to the addition of 3 mM MgCl₂, 50 mM Tris buffer and varying concentrations of AMG 853 (0 – 100 µM). Reactions were initiated with the
addition of UDPGA and quenched after 45 min with two volumes of acetonitrile containing 0.1 µM tolbutamide as an internal standard. All reactions were vortex mixed and centrifuged at 1460 g for 10 min prior to analysis by LC-MS/MS.

In order to qualitatively assess the contribution of each CYP to the formation of M2 and M3, intrinsic clearance values (Vmax / Km) were scaled using the previously reported ISEF scaling approach (Chen et al., 2011; Proctor et al., 2004). 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; CYP3A7, 2 pmol/mg 0.15.

**AMG 853 and M1 Drug Interaction Assessment**

The *in vitro* kinetics of each CYP probe substrate were characterized prior to initiating 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 towards eight CYP isozymes for IC50 studies, a single concentration (at the estimated Km value) of the probe substrate was incubated in combination with varying concentrations of AMG 853 from 0 to 30 µM (Table 1). To determine Ki values for AMG 853 inhibition in HLM, four concentrations of probe substrate were used, encompassing the estimated Km value. Positive control experiments were also performed with varying concentrations of known CYP inhibitors (CYP1A2 – α-naphthoflavone, 0 – 1 µM; CYP2B6 – clotrimazole, 0 – 6 µM; CYP2C8 – montelukast, 0 – 0.5 µM; CYP2C9 – sulfaphenazole, 0 – 2 µM; CYP2C19 – (+)-N-3-benzyl-nirvanol, 0 – 2 µM; CYP2D6 – quinidine, 0 – 0.3 µM; CYP2E1, diethylthiocarbamate, 0 – 250 µM; CYP3A – 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 such that no more than 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 5 to 30 min, depending on the probe substrate used (37 °C in an incubator shaker). Each reaction was terminated with the addition of 1 volume acetonitrile (v/v) containing a stable label internal standard. The samples were centrifuged (1460 g for 10 min) and then analyzed by LC-MS/MS. For the CYP2C8 $K_i$ determinations with paclitaxel, 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 MgCl$_2$. 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 internal standard. The samples were centrifuged (1460 g for 10 min) and analyzed by LC-MS/MS.

To evaluate the potential of AMG 853 or M1 to inactivate CYP 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. Aliquots (10 μL; 20x 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 (CYP2C8), 20 μM diclofenac
(CYP2C9), 100 µM (s)-mephenytoin (CYP2C19), 30 µM dextromethorphan (CYP2D6), 200 µM chlorzoxazone (CYP2E1) and 200 µM testosterone (CYP3A4). The samples were centrifuged (1460 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. Following a 4 hour 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 hours in incubation media prior to 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 hours for a total of 48 hours. On Day 5, hepatocytes were washed with Krebs-Henseleit buffer prior to the assessment of CYP activity using the same probe substrates as described for the inhibition experiments. Selective probe substrates (CYP1A2, 100 µM phenacetin, 45 minutes; CYP2B6, 100 µM bupropion, 45 minutes; CYP2C9, 100 µM diclofenac, 30 minutes; CYP3A4, 50 µM midazolam, 20 minutes) were used to assess CYP activity. Following the incubation time period, 100 µL supernatant was removed and added to 200 µL ice-cold acetonitrile prior to analysis by LC-MS/MS as described below.

CYP mRNA expression was also evaluated by washing the hepatocytes in PBS followed by addition of a lysis buffer. mRNA levels were analyzed using a branched deoxyribonucleic acid amplification kit from Panomics and according to 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 GAPDH probe control prior to statistical analysis.

**In Vitro Sample Analysis.** The analysis of all *in vitro* samples was conducted using LC-MS/MS technology. Briefly, the LC-MS/MS platform utilized for all experiments was comprised of an Applied Biosystems 4000 Q-Trap fitted with an electrospray ionization source (Applied Biosystems, Foster City, CA). Analytes were introduced to the Q-Trap through two LC-20AD pumps with an in-line CBM-20A controller and DGU-20A5 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 2.6 μm Kinetex C18 50 x 2.1 mm column (Phenomenex, Torrance, CA). The 4000 Q-Trap 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 783.0/583.0 (M1), 625.0/536.1 (M2) and 625.1/552.1 (M3). Linear gradient elution (flow rate = 500 μL/min) was carried out using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. HPLC flow was diverted to waste for the first 60 seconds to remove any non-volatile salts.

For probe substrates from the IC$_{50}$, K$_i$ or time-dependent inhibition, HPLC separation was achieved using a Gemini C18 2.0 x 30 mm 5 μm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 400 μL/min) was carried out using a mobile phase system...
consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. HPLC flow was diverted to waste for the first 45 seconds to remove any non-volatile salts. Generic Q-Trap source parameters included the curtain gas (20 arbitrary units), CAD gas (medium), ion spray voltage (4500 V), source temperature (550 °C) and ion source gas 1 and gas 2 (40 arbitrary units, each). Interface heaters were kept on for all analytes. Probe substrate mass transitions (Q1/Q3) were identical to previously published methods (Walsky and Obach, 2004).

**Statistical Analysis of In Vitro Data.** Analysis of IC\textsubscript{50} and K\textsubscript{i} data was performed using Graphpad Prism (version 5.04; Graphpad Software Inc., San Diego, CA). IC\textsubscript{50} data was fit using a sigmoidal dose-response model (Equation 1), while K\textsubscript{i} data was applied to either a competitive (Equation 2), linear-mixed (Equation 3) or biphasic (Equation 4) inhibition model based upon the Akaike Information Criteria and visual inspection of the Dixon ([I] vs 1/v) and Lineweaver-Burke (1/[S] vs 1/v) plots. For the equations below, 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, K\textsubscript{m} is equal to half the substrate concentration at maximal reaction velocity, K\textsubscript{i} is the dissociation constant for the enzyme-inhibitor complex and K\textsubscript{i} is the dissociation constant for the enzyme-substrate-inhibitor complex. Note that for equation 2 and 3, K\textsubscript{m}, K\textsubscript{i} and V\textsubscript{max} were treated as global parameters.

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\text{%activity} = \text{min} + \frac{(\text{max} - \text{min})}{(1 + 10^{\text{log}[I] - \text{log IC}_{50}})}
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(1)
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 ketoconazole, paclitaxel, rosiglitazone and montelukast. For AMG 853, the following parameters were entered into Simcyp: molecular weight, 609.5 Da; logP, 5.1; blood:plasma ratio, 1; fraction unbound in plasma, 0.003; $P_{\text{eff}}$, 11.1 (from LLC-PK1 permeability assay with a propranolol reference value of 36); volume of distribution at steady-state, 2.7 L/kg; enzyme kinetic and inhibition parameters for M1, M2 and M3 as noted in Tables 1 and 2. For M1, the molecular weight was adjusted to 785.0, logP to 2.5, and the 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 utilized 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 hour period (Karonen et al., 2011; Naik et al., 2012; Sonnichsen and Relling, 1994). For simulations of ketoconazole effects on AMG 853 metabolism, the in silico dosing regimen consisted of 200 mg BID oral doses of ketoconazole for two days prior to a 50
mg oral dose of AMG 853 co-administered with the first oral dose of ketoconazole on Day 3. Simulations (n = 10 trials) were performed in healthy patient populations (100 subjects, 18 to 55 years of age) with female subjects accounting for half of the population.

In Vivo Drug Interaction Study. In order 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 to a second set of human subjects (n = 6) who were pretreated with ketoconazole (200 mg BID, oral administration) for 2 days prior to co-administration 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 hours post-dose. Pharmacokinetic parameters were estimated using noncompartmental analysis (WinNonlin, Pharsight, CA). Assessment of drug interaction potential was determined by comparing \( C_{\text{max}} \) and \( \text{AUC}_{0-\text{inf}} \) values for each arm of the study.

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 50/50 (v/v) methanol/water.

AMG 853 and the acyl glucuronide metabolite M1 were chromatographically separated on a Phenomenex (Torrance, CA) Synergi Polar RP column (4.0µm, 30 x 2.0 mm) that had been equilibrated with 60% solvent A (0.1% formic acid in water) and 40% solvent B (9:1 acetonitrile:water) 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 Applied Biosystems (Applied Biosystems, Foster City, CA) API4000 mass spectrometer 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 61.0V 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 CYPs were incubated with AMG 853 (10 μM) in order to determine the enzymes responsible for the conjugative and oxidative metabolism of AMG 853. Results (n=3) indicated that UGT 1A1, 1A3 and 2B7 were capable of conjugating AMG 853 with UGT1A3 exhibiting the highest activity relative to the other UGTs. (Figure 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 CYP 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 (Figure 2b). For M3, CYP 2C8, CYP2J2, CYP3A4, CYP3A5 and CYP3A7 contributed approximately equally to the formation of the metabolite. A minor contribution from CYP2C19 was observed for M3 (Figure 2c). Human liver microsomes with isoform-selective inhibitors confirmed the observations in recombinant CYPs. Upon co-incubation, montelukast (CYP2C8) and ketoconazole (CYP3A) inhibited the formation of M2 and M3 (Figure 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 Table 1. When enzyme kinetic parameters were scaled to account for relative activity and enzyme abundance using the well documented ISEF approach (Chen et al.,
2011; Proctor et al., 2004), 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 minor roles in the hepatic formation of M2 and M3.

In order 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\textsubscript{max} conditions, the formation rate of M1 was dependent upon both the CYP3A5 genotype as well as the individual cofactors included in the incubation (Figure 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. Similarly, M2 was formed to significantly greater amounts in HLM genotyped to be homozygous for CYP3A5*1 (Figure 4a). In contrast to what was observed for M2, the oxidative metabolism of M3 was not dependent on the CYP3A5 genotype in HLM (Figure 4b).

At substrate concentrations approximating the respective K\textsubscript{m} for each probe substrate, the reversible inhibitory potential of AMG 853 towards eight human liver microsomal CYP enzyme activities was evaluated (Table 2; Figure 5). Under the conditions used, less than 10% of AMG 853 was consumed through the course of the incubation. In comparison to the positive control inhibitors, AMG 853 exhibited no inhibitory potential (IC\textsubscript{50} ≥ 30 μM) towards CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. AMG 853 was found to be an inhibitor of CYP2C8-catalyzed paclitaxel 6-hydroxylation (IC\textsubscript{50} = 5.4 ± 1.0 μM, data not shown).
For probe substrates where the AMG 853-mediated IC$_{50}$ < 30 μM, experiments to estimate the $K_i$ were conducted. AMG 853 inhibited CYP2C8-catalyzed paclitaxel 6-hydroxylation (competitive inhibition model, $K_i = 1.1 \pm 0.2$ μM), rosiglitazone demethylation (competitive inhibition model, $K_i = 6.0 \pm 0.8$ μM) and montelukast 36-hydroxylation (biphasic inhibition model, $K_i = 1.8 \pm 1.0$ μM). Similarly, the M1 acyl glucuronide metabolite of AMG 853 was also an inhibitor of CYP2C8 in vitro. $K_i$ values for M1 were 2.7 ± 0.5 μM for paclitaxel 6-hydroxylation, 6.9 ± 1.4 μM for rosiglitazone demethylation and 7.3 ± 3.3 μM for montelukast 36-hydroxylation. Data for paclitaxel and rosiglitazone with M1 were both fit to a linear-mixed inhibition model while data for montelukast and M1 was fit to a biphasic inhibition model.

At substrate concentrations approximately 5-fold higher than the estimated $K_m$ for each marker substrate and 10 μM 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 to 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 μM increased mRNA and activity levels by less than 1.7-fold 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 where ketoconazole was utilized as the perpetrator drug and AMG 853 as the victim drug, the AUC of AMG 853 was predicted to increase approximately 1.91-fold and the $C_{\text{max}}$ AMG 853 to increase by approximately 1.90-fold in the presence of ketoconazole (Figure 6). Conversely, simulations were conducted to assess the effects of AMG 853 as the perpetrator drug on the victim drugs paclitaxel, rosiglitazone and montelukast. No changes in either the AUC or $C_{\text{max}}$ values of the victim drugs were predicted following co-administration with 200 mg AMG 853 (Table 3).

In light of the $in vitro$ and $in silico$ data for AMG 853 with ketoconazole, a clinical drug interaction study was conducted using a parallel study design in two groups of healthy human volunteers. Following a 50 mg oral administration of AMG 853, a $C_{\text{max}}$ of 227 ng/mL was observed (Figure 7). In volunteers who were treated with ketoconozole (200 mg BID), the $C_{\text{max}}$ of AMG 853 increased to 270 ng/mL, an increase of approximately 1.18-fold. A similar observation was made when $\text{AUC}_{0-\text{inf}}$ values were compared between the two study cohorts. An $\text{AUC}_{0-\text{inf}}$ of 671 ng*hr/mL was observed in the control group as compared to an $\text{AUC}_{0-\text{inf}}$ of 800 ng*hr/mL in the ketoconazole arm, which indicates an increase of 1.19-fold upon concurrent dosing of ketoconazole.
Discussion

The role of the DP and CRTH2 G-protein coupled receptors in the regulation of inflammatory responses has been previously reported (Arimura et al., 2001; Barnes et al., 2011; Hirai et al., 2001; Norman, 2010; Oguma et al., 2004; Pettipher et al., 2007; Sugimoto et al., 2003; Van Hecken et al., 2007; Wang et al., 2011). 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; Liu et al., 2011). In support of the preclinical development of AMG 853, the aim of this manuscript 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 (NME) 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 as well as the European Medicines Agency (EMA, 2010; Zhang et al., 2009). 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 (Foti and Wahlstrom, 2008; Foti et al., 2010; Huang et al., 2008; Huang et al., 2007; Lewis, 2010; Zhang et al., 2010; Zhang et al., 2009). Currently accepted methods for determining the enzymes involved in an NME’s metabolism 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). Conversely, it is equally as important to understand the potential of the NME to inhibit the metabolism of other drugs currently on the market. Generating 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_i value of greater than 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 (PPARα) 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; Randinitis et al., 1986). Subsequent studies have identified gemfibrozil 1-O-acyl glucuronide as both a competitive inhibitor as well as heme-modifying ligand of CYP2C8 in vitro (Baer et al., 2009; Jenkins et al., 2011; Ogilvie et al., 2006). Furthermore, an increased focus has recently been placed on the assessment of circulating metabolites for both inhibition potential as well as other biochemical properties such as target potency. While characterization of circulating metabolites is guided by such strategies as U.S. Food and Drug Administration’s Guidance for Industry: Safety Testing of Drug Metabolites, there are no recommendations on the assessment of the drug interaction potential of circulating metabolites. Recent efforts utilizing the Metabolism and Transport Drug Interaction Database have noted that of 129 drug interactions (greater than 20% increase in AUC) involving the CYP 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 previously reported (Davis et al., 2012). 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 pre-clinical species would 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 manuscript supports the previous assessment and also implicates 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 (Gaedigk et al., 2006; Lee et al., 2010; Paine et al., 2006; Wu et al., 1996), While CYP2J2 accounts for approximately 1 to 2 percent of total CYP 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 anti-histamine 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; Lee et al., 2010; Matsumoto et al., 2002). Furthermore, as with CYP3A4, the enzyme is inhibited by ketoconazole (Stresser et al., 2004).

The determination of enzyme kinetics parameters, a more mechanistic approach to identifying the relative contributions of each CYP 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 that indicates CYP 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 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 and NADPH, which indicates that NADPH-dependent metabolism may reduce the amount of aglycone (AMG 853) available to the UGTs for glucuronidation. Interestingly, when only UDPGA was included in the incubation, the amount of M1 formed was still dependent on the CYP3A5 genotype status of each donor. Higher formation rates were observed for M1 in donors homozygous for the CYP3A5*1 allele (high expression) and lower in those homozygous for the CYP3A5*3 allele (low expression). A plausible explanation may be the effects of CYP-UGT heterodimerization often observed in vitro (Fremont et al., 2005; Takeda et al., 2009), though it is important to note that it may also be related to variation in amount of UGT protein in each lot of liver microsomes.

Using $V_{\text{max}}/K_m$ as a measure of the intrinsic contribution of an enzyme to a metabolic pathway, the highest intrinsic activities observed in recombinant CYPs for M2 was with CYP2J2, followed by CYP3A5. An approximately 5.7-fold higher $V_{\text{max}}/K_m$ value was observed using recombinant CYP3A5 as compared to CYP3A4, which indicates a greater contribution from CYP3A5 for the formation of M2. This is supported by the decreased formation of M2 in HLM genotyped to be CYP3A5 *3/*3 as compared to 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 CYPs tested, though the relative difference between contributing isoforms was not as great as that noted for M2. While CYP3A and CYP2J2 are known to be expressed in the lung (Hukkanen et al., 2002; Zeldin et al., 1996), the relatively small $V_{\text{max}}/K_m$ values observed in
HLuM would 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 HLuM 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 (Filppula et al., 2011; Karonen et al., 2010; Karonen 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 Cmax or AUC values of paclitaxel, rosiglitazone or montelukast was observed. While AMG 853 had low micromolar Ki values against CYP2C8 in vitro, one must also take into account that the free fraction of AMG 853 in human plasma was previously determined to be 0.003. As such, one could expect the free in vivo concentrations of AMG 853 to be well below the observed in vitro Ki values and would not anticipate a clinically significant drug interaction.

Though AMG 853 exhibits a 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 non-selective 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, as the predicted change in AUC was near the point of clinical relevance and the effects of many CYP3A inhibitors may also manifest on 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 over-prediction in C<sub>max</sub> and AUC ratios that were estimated can most likely be accounted for by the multiple clearance pathways of AMG 853. While 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 are accounted for. Based on 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 over-prediction of drug interaction values. As such, 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 manuscript describes the reaction phenotyping and drug interaction studies undertaken to support the development of AMG 853, a novel inhibitor of the D-prostanoid (DP) and chemoattractant receptor-homologous molecule expressed on T-helper cells (CRTH2) G-protein coupled receptors. *In vitro* studies implicated CYP2C8, CYP2J2, CYP3A and multiple UGT isoforms 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. Based on 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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Author Contribution

Participated in research design: Foti, Pearson, Wong, Zalikowski, Banfield, Rock, Wahlstrom, Amore

Conducted experiments: Foti, Wong, Davis, Boudreaux, Prokop

Contributed new reagents or analytic tools: None

Performed data analysis: Foti, Pearson, Wong, Zalikowski, Boudreaux, Prokop, Emery

Wrote or contributed to the writing of the manuscript: Foti, Zalikowski, Banfield, Wienkers, Amore
References


Marcel Dekker, Inc., New York.


Figure Legends

Figure 1. Structures of AMG 853, M1 (acyl glucuronide), M2 (t-butyl hydroxylation) and M3 (cyclopropyl hydroxylation).

Figure 2. Formation of M1, M2 and M3 from AMG 853 in recombinant enzymes. UGT1A1, UGT1A3 and UGT2B7 were responsible for the formation of M1 (Fig. 2a) while CYP2C8, CYP2J2 and CYP3A exhibited the highest rates of formation for M2 and M3 (Fig. 2b and 2c). Recombinant data was confirmed by inhibition of M2 and M3 formation using selective chemical inhibitors in HLM (Fig. 2d).

Figure 3. Formation of M1 under V_max conditions in pooled HLM and individual donors genotyped to be CYP3A5 *1/*1 or CYP3A5 *3/*3. Formation of M1 was dependent on the CYP3A5 genotype and higher when only UDPGA was included in the incubation as compared to both UDPGA and NADPH.

Figure 4. 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). M2 exhibited a dependence on the CYP3A5 genotype, with the highest formation rates in donors genotyped to be CYP3A5 *1/*1 (Fig. 4a). No dependence on the CYP3A5 genotype status was observed for M3 (Fig 4b).

Figure 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 was fit to a competitive inhibition model for paclitaxel (K_i = 1.1 µM) and rosiglitazone (K_i = 6.0 µM) and a biphasic inhibition model for montelukast (K_i = 1.8 µM). M1 data was fit to a linear-mixed inhibition model for paclitaxel (K_i =
2.7 µM) and rosiglitazone (Ki = 6.9 µM) and a biphasic inhibition model for montelukast (Ki = 7.3 µM).

**Figure 6.** Simcyp prediction of the potential drug interaction between ketoconazole and AMG 853 in a healthy population. Increases of 1.91-fold and 1.90-fold were predicted for the AMG 853 AUC and C_{max} values, respectively.

**Figure 7.** Observed plasma concentrations of AMG 853 in healthy volunteers in the presence or absence of ketoconazole. Mean AMG 853 AUC estimates increased 1.19-fold in the presence of ketoconazole while mean C_{max} values increased 1.18-fold.
Table 1. Kinetic parameters of AMG 853 M1, M2 and M3 formation in HLM, HLuM, and recombinant enzymes.

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Metabolite</th>
<th>In Vitro System</th>
<th>$S_{50}$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</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>
</tr>
<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>
</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>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Metabolite</th>
<th>In Vitro System</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ *</th>
<th>$K_i$ (µM)</th>
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</thead>
<tbody>
<tr>
<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>0.044 ± 0.001</td>
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<td>HLM CYP3A5*3/*3</td>
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<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>
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<td>rCYP2J2</td>
<td>4.53 ± 0.32</td>
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<td>rCYP3A4</td>
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<td>rCYP3A5</td>
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<td>rCYP3A7</td>
<td>10.0 ± 0.77</td>
<td>0.260 ± 0.0005</td>
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</table>

*HLMs: nmol/min/mg; rCYPs: nmol/min/nmol
### Table 2. Inhibition of probe substrate activity by AMG 853 or M1 in HLM.

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Probe</th>
<th>Test Article</th>
<th>$K_i$ (µM)</th>
<th>Inhibition Model</th>
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<td>CYP1A2</td>
<td>Phenacetin</td>
<td>AMG 853</td>
<td>&gt; 30</td>
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<tr>
<td></td>
<td>(7.5 – 60 µM)</td>
<td>M1</td>
<td>&gt; 30</td>
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<td>CYP2B6</td>
<td>Bupropion</td>
<td>AMG 853</td>
<td>&gt; 30</td>
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<tr>
<td></td>
<td>(25 – 200 µM)</td>
<td>M1</td>
<td>&gt; 30</td>
<td></td>
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<td>CYP2C8</td>
<td>Paclitaxel</td>
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<td>1.1 ± 0.2</td>
<td>Competitive</td>
</tr>
<tr>
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<td>(3.5 – 30 µM)</td>
<td>M1</td>
<td>2.7 ± 0.5</td>
<td>Linear-Mixed</td>
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<td>Rosiglitazone</td>
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<td>6.9 ± 1.4</td>
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<td>Montelukast</td>
<td>AMG 853</td>
<td>1.8 ± 1.0</td>
<td>Biphasic</td>
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<td>(0.035 – 0.75 µM)</td>
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<td>7.3 ± 3.3</td>
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<td>AMG 853</td>
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<td>(5 – 40 µM)</td>
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<td>CYP2C19</td>
<td>(S)-Mephenytoin</td>
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<td>CYP2D6</td>
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<td>AMG 853</td>
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<td>&gt; 30</td>
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<td>CYP2E1</td>
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<td></td>
<td>(25 – 250 µM)</td>
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<td>CYP3A4</td>
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<td>(0.75 – 7.5 µM)</td>
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<td>Testosterone</td>
<td>AMG 853</td>
<td>&gt; 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(25 – 200 µM)</td>
<td>M1</td>
<td>&gt; 30</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. 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. Values in parentheses indicate the 5th and 95th percentiles for the predicted changes in AUC and $C_{\text{max}}$.

<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 KTZ</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>
Figure 1

AMG 853

M2

M1

M3
Figure 2

A. Formation of M1 in Recombinant UGTs

B. Formation of M2 in Recombinant CYPs

C. Formation of M3 in Recombinant CYPs

D. Inhibition of M2 and M3 Formation in HLM
Figure 5

AMG 853

6-OH-Paclitaxel vs Paclitaxel (uM)

- 0 uM
- 2 uM
- 4 uM
- 8 uM
- 16 uM

AMG 853 M1

6-OH-Paclitaxel vs Paclitaxel (uM)

- 0 uM
- 2 uM
- 4 uM
- 8 uM
- 16 uM

AMG 853

Desmethylrosiglitazone vs Rosiglitazone (uM)

- 0 uM
- 2 uM
- 4 uM
- 8 uM
- 16 uM

AMG 853 M1

Desmethylrosiglitazone vs Rosiglitazone (uM)

- 0 uM
- 2 uM
- 4 uM
- 8 uM
- 16 uM

AMG 853

36-OH-Montelukast vs Montelukast (nM)

- 0 uM
- 2 uM
- 4 uM
- 8 uM
- 16 uM

AMG 853 M1

36-OH-Montelukast vs Montelukast (nM)

- 0 uM
- 2 uM
- 4 uM
- 8 uM
- 16 uM
Figure 6