Species Difference in Glucuronidation Formation Kinetics with a Selective mTOR Inhibitor

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ABSTRACT

The mammalian target of rapamycin (mTOR) is a protein kinase that shows key involvement in age-related disease and promises to be a target for treatment of cancer. In the present study, the elimination of potent ATP-competitive mTOR inhibitor 3-(6-amino-2-methylpyrimidin-4-yl)-N-(1H-pyrazol-3-yl)imidazo[1,2-b]pyridazin-2-amine (compound 1) is studied in bile duct-cannulated rats, and the metabolism of compound 1 in liver microsomes is compared across species. Compound 1 was shown to undergo extensive N-glucuronidation in bile duct-catheterized rats. N-glucuronides were detected on positions N1 (M2) and N2 (M1) of the pyrazole moiety as well as on the primary amine (M3). All three N-glucuronide metabolites were detected in liver microsomes of the rat, dog, and human, while primary amine glucuronidation was not detected in cynomolgus monkey. In addition, N1- and N2-glucuronidation showed strong species selectivity in vitro, with rat, dog, and human favoring N2-glucuronidation and monkey favoring N1-glucuronide formation. Formation of M1 in monkey liver microsomes also followed sigmoidal kinetics, singling out monkey as unique among the species with regard to compound 1 N-glucuronidation. In this respect, monkeys might not always be the best animal model for N-glucuronidation of uridine diphosphate glucuronosyltransferase (UGT) 1A9 or UGT1A1 substrates in humans. The impact of N-glucuronidation of compound 1 could be more pronounced in higher species such as monkey and human, leading to high clearance in these species. While compound 1 shows promise as a candidate for investigating the impact of pan-mTOR inhibition in vivo, opportunities may exist through medicinal chemistry efforts to reduce metabolic liability with the goal of improving systemic exposure.

Introduction

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and a member of the phosphatidylinositol-3-OH kinase (PI3K) family (Lamming et al., 2013; Johnson et al., 2013). mTOR is active when associated with protein complexes that have differing functions and sensitivities to inhibition by rapamycin. mTOR complex 1 (mTORC1) is acutely sensitive to allosteric inhibitor rapamycin, while mTORC2 is acutely resistant, but ultimately, affected to some extent by chronic exposure to rapamycin. The biologic functions of the mTOR complexes are to sense cellular availability of nutrients and other physiologically important signals such as amino acids, glucose, oxygen, WNT ligands, cAMP, and insulin. As a result of these signals, mTORC1 activates translation and cell growth, while mTORC2 activates production of the cytoskeleton and phosphorylation of oncogenic AKT protein kinase. In particular, mTORC1 has been considered as a key modulator of several processes related to ageing and age-related disease. Indeed, inhibition of mTOR by rapamycin had been found to extend life span in a number of species, including mammals, with biologic effects similar, but not necessarily identical, to those found following calorie/nutrient restriction (Fok et al., 2013; Johnson et al., 2013; Lamming et al., 2013). The mechanism(s) behind extended life span are still under investigation; however, a number of possible mechanisms have been proposed, including anticancer effects, reduced translation, increased autophagy, support of functional stem cells, modulation of immune response, reduced inflammation, and improved mitochondrial function.

While use of mTOR inhibitors as treatments for extending life span or for other age-related diseases in humans is still theoretical, rapamycin and analogs everolimus and temsirolimus have been approved for use in the treatment of a limited number of cancers (Benjamin et al., 2011). However, rapamycin and the analogs studied only incompletely inhibit mTORC1 phosphorylation, and selective inhibition of mTORC1 can increase AKT signaling by reducing negative feedback in the PI3K-mTORC2-AKT pathway. Therefore, second-generation mTOR inhibitors, particularly ATP-competitive mTOR inhibitors that can more potently inhibit both mTORC1 and mTORC2, are being investigated for use in the oncology space, as well as to further elucidate the role of mTOR in ageing and disease (Benjamin et al., 2011; Schenone et al., 2011).

Previously, Peterson et al. (2012) described the discovery of a series of selective imidazopyridazine mTOR inhibitors. In particular, compound 1 (Fig. 1) was found to have an IC50 against mTOR of 13 nM, with 58× selectivity over PI3K. Compound 1 exhibited moderate clearance following an intravenous dose to rats (2.3 l/h/kg), and approxmately 100% bioavailability following an oral dose. Solubility

ABBRVIATIONS: BDC, bile duct-catheterized; CL, clearance; CLint, intrinsic clearance; g-HMBC, gradient-heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; PI3K, phosphatidylinositol-3-0H kinase; PK, pharmacokinetics; ROESY, rotating-frame Overhauser effect spectroscopy; UDP, uridine diphosphate; UDPGA, uridine-5′-diphosphoglucuronic acid; UGT, UDP glucuronosyltransferase.
and bioavailability were greatly improved with compound 1 as compared with its imidazopyridine analog. Compound 1 shows promise as a tool for examining mTOR inhibition in vivo. However, little is known about the disposition of compound 1 across species. In the present study, the elimination of compound 1 is studied in bile duct–cannulated rats, and the metabolism of compound 1 in liver microsomes is compared across species.

**Materials and Methods**

Compound 1 (>98% purity) was synthesized by the Medicinal Chemistry Department at Amgen, Inc. (Cambridge, MA) (Peterson et al., 2012). [14C]-compound 1 (57.5 mCi/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). All other compounds and reagents were obtained from commercial sources as appropriate. Liver microsomes isolated from male Sprague-Dawley rats (n > 100), pooled male beagle dogs (n = 5), pooled male cynomolgus monkey (n = 10), and pooled male and female human (n = 50) were purchased from BD Biosciences (San Jose, CA). Recombinant cDNA-expressed human uridine diphosphate glucuronosyltransferase (UGT) isoforms UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 were obtained from BD Biosciences, San Jose, CA.

**Excretion Studies in Bile Duct–Catheterized Rats.** All animal procedures were conducted under protocols approved by the Amgen (Cambridge) Institutional Animal Care and Use Committee. Three male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). The rats were housed in a humidity- and temperature-controlled environment subject to a 12-hour light:12-hour dark cycle and had access to water and a standard laboratory diet ad libitum. Following a 1-week acclimation period, rats had Silastic catheters implanted in the bile duct and proximal duodenum using aseptic surgical techniques. The externalized catheters were protected with a Covance infusion harness (Instech Solomon, Plymouth Meeting, PA) and connected to permit recirculation of bile. The bile duct–catheterized (BDC) rats were placed in Nalge metabolism cages (Nalge Company, Rochester, NY) and given access to food and water ad libitum. The rats were fasted overnight prior to dosing; food was returned 2 hours post-dose. On the second day postsurgery, the catheters were connected to a dual channel swivel (Instech Solomon) for the collection of bile and the infusion of artificial bile (25 mM sodium taurocholate:150 mM NaCl:7 mM KCl, 2 ml/h) (Klaassen, 1974). Each BDC rat was administered a single dose of [14C]-compound 1 (5 mg/kg, 100 μCi/kg PO). Bile, feces, and urine samples were collected at specified intervals through 48 hours post-dose into preweighed bottles. The volume of bile and urine was determined gravimetrically, assuming a density of 1 g/ml.

**Isolation of N-Glucuronides from In Vitro Incubations.** Ten milligrams of compound 1 (100 μM) were incubated with rat, dog, or monkey liver microsomes (2 mg protein/ml) in phosphate buffer (0.1 M, pH 7.4), with 10 mM MgCl2, 5 mM uridine-5′-diphosphoglucuronic acid (UDPGA), and 50 μg alamethicin/mg microsomal protein for 4 hours at 37°C. The incubation mixture was extracted using an OASIS reverse-phase cartridge (Waters Corporation, Milford, MA). The acetonitrile wash was evaporated and the sample was redissolved in high-performance liquid chromatography (HPLC) mobile phase and separated using the system described under liquid chromatography–mass spectrometry (LC-MS) analysis of BDC and in vitro samples. The HPLC eluate was split 1/20 with one part going to the MS and the rest to a Gilson FC 204 fraction collector (Gilson, Inc., Middleton, WI). The fraction containing the isolated glucuronide was dried, redissolved in HPLC solvent, and subjected to further purification using a new HPLC column. The purity of the final product was determined by LC-MS (>90% for M1 and M2; >80% for M3).

**Structural Elucidation of N-Glucuronide Metabolites by NMR.** All NMR spectra were acquired on a Bruker Avance II spectrometer system operating at a proton frequency of 600.13 MHz. The parent and isolated metabolites were dissolved in d6-dimethylsulfoxide, and NMR data were collected in 1.7-mm tubes using a Bruker 5-mm TCI CryoProbe. Proton and
carbon assignments were made by using standard 1-D and 2-D pulse sequences. All NMR spectra were collected at a temperature of 26°C.

**In Vitro Metabolism of Compound 1.** For the identification of glucuronide metabolites in liver microsomes, radiolabeled compound 1 (10 μM, 28 mCi/mmol) was incubated with rat, dog, monkey, and human liver microsomes (1 mg protein/ml) in phosphate buffer (0.1 M, pH 7.4) with 10 mM MgCl₂ and 50 μg alamethicin/mg microsomal protein for 1 hour at 37°C with and without 5 mM uridine-5′-diphosphoglucuronic acid. The reaction mixtures were quenched with an equal volume of acetonitrile and centrifuged at 16,000 g for 5 minutes. The supernatant was diluted with an equal volume of 0.1% formic acid in water and analyzed by LC-MS/Radiolabelling following the method described under LC-MS analysis of BDC and in vitro samples.

The depletion of compound 1 in liver microsomes in the presence of NADPH was determined. Compound 1 (0.1 or 1 μM) was incubated with rat, dog, monkey, or human liver microsomes at 37°C and in the presence of 1 mM NADPH, and 10 mM MgCl₂. Samples were collected from the incubations at 0, 5, 10, 20, 30, and 40 minutes of incubation, and were quenched with an equal volume of acetonitrile and centrifuged at 14,000 rpm for 5 minutes. The supernatant was diluted with an equal volume of 0.1% formic acid in water and analyzed by LC-MS/MS.

**LC-MS Analysis of BDC and In Vitro Samples.** For metabolite identification in radiolabeled or nonradiolabeled samples from BDC and in vitro experiments, the LC-MS/MS system consisted of a Shimadzu LC-20AD HPLC system (Shimadzu America, Torrance, CA) and a Thermo LTQ Orbitrap mass spectrometer (Thermo Electron Corp., San Jose, CA), and a Radiomatic 625 Flow Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Downers Grove, IL). The LTQ-Orbitrap was equipped with an API2 source and Xcalibur version 2.0 software (Thermo Electron Corp.). All mass spectra were acquired in the high-resolution mode using 30,000 resolving power. Exact mass measurement was accomplished using external calibration. Electrospray ionization with positive ion detection was used. The source temperature was set at 250°C, and the ion spray voltage was held at 4.5 kV. Pooled rat bile (0–48 hour, 5 μl), pooled rat urine (0–48 hours, 100 μl), or in vitro samples were loaded onto an YMC-AQ C18 column (YMC America, Inc., Torrance, CA) 4.6 × 250 mm, 5-μm particle size, 120-Å pore size). The metabolites were

![Fig. 2](image)

**Fig. 2.** Radiocromatographic profile of [14C]-compound 1 and metabolites in bile, bile treated with glucuronidase, and urine from bile duct–cannulated male rats following a 5 mg/kg oral dose of [14C]-compound 1.
separated using a gradient solvent system consisting of two components: solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile). The percentage of solvent B was held at 5% for 3 minutes, then was increased in a linear fashion from 5% to 30% B over 37 minutes, and from 30% to 95% B in the next 10 minutes. The flow rate was set at 1.0 ml/min, and the column eluate was split 1:20, with one part channeled to the ion source and the rest to the flow scintillation analyzer (waste for nonradiolabeled samples or fraction collector for isolation). The MS/MS spectra were recorded by collision-induced dissociation of \([M + H]^+\) species.

Samples from compound 1 depletion and \(N\)-glucuronide formation kinetics studies were analyzed by multiple reaction monitoring on an LC-MS/MS system consisting of dual Shimadzu LC-10AD HPLC pumps and a DGU-14A degasser (Shimadzu, Columbia, MD), a CTC PAL autoinjector (Leap Technologies, Carrboro, NC), and an API4000 MS system equipped with an electrospray ion source and operated by the Analyst software package (Applied Biosystems, Foster City, CA). The source temperature was set to 400°C, and the ion spray voltage was set to 4.5 kV. Chromatography was conducted on a YMC ODS-AQ (50 × 4.6 mm, 5 μm) analytical column with a 0.5 μm PEEK guard filter, using the mobile phase solvents A and B described above at a flow rate of 0.7 ml/min. The percentage of solvent B was increased in a linear fashion from 2% to 18% over 7 minutes, then from 18% to 90% in the next 0.5 minutes and held at 90% for the next 2 minutes, when it was then returned to 2%. The LC eluent was diverted from the ion source to waste for the first 3 minutes of each sample run. Compound 1 (\(m/z \) 308 → 250), \(N\)-glucuronides (\(m/z \) 484 → 308), and internal standard (\(m/z \) 322 → 264) were detected in positive ion mode.

Modeling of \(N\)-Glucuronide Metabolite Formation Kinetics. To determine which kinetic model(s) to use, Eadie-Hofstee plots were created using the reaction velocity data from each metabolite in liver microsomes of each species. Based on the appearance of the data in this plot, one of the following models (below) was used to describe the data. Kinetics parameters were fit by nonlinear regression, and metabolism formation was simulated using GraphPad Prism v6.02 (GraphPad Software Inc., San Diego, CA). In addition, the choice of model was supported by comparison against possible competing models using the model comparison feature in GraphPad Prism. Preferred models were confirmed based on difference in Akaike information criterion corrected for small sample size. Metabolite formation with an apparently linear Eadie-Hofstee plot was described by a single-enzyme model assuming standard Michaelis-Menten kinetics (one-site),

\[
v = \frac{V_{\text{max}} \times C}{K_m + C}
\]

where \(v\) is the reaction velocity, \(V_{\text{max}}\) is the maximum reaction velocity, \(K_m\) is the concentration required to achieve 50% of the maximum reaction velocity, and \(C\) is the concentration of compound 1 in the incubation.

Metabolite formation where the Eadie-Hofstee plot appeared biphasic was described by a two-enzyme model (two-site),

\[
v = \frac{V_{\text{max,h}} \times C}{K_{m,h} + C} + \frac{V_{\text{max,l}} \times C}{K_{m,l} + C}
\]

where the subscripts \(h\) and \(l\) indicate \(V_{\text{max}}\) and \(K_m\) parameters for the high- and low-affinity enzymes, respectively. Metabolite formation where the Eadie-Hofstee plot appeared biphasic with an apparent decrease in \(v/[S]\) values was described by a single-enzyme model with substrate inhibition (one-site SI),

\[
v = \frac{V_{\text{max}} \times C}{K_m + \frac{1}{4}C}
\]
where $K_{m1}$ is the inhibition constant for the substrate inhibition. Metabolite formation where the Eadie-Hofstee plot appeared triphasic with an apparent decrease in $v$ with decreasing $v$/$[S]$ values was described by a two-enzyme model with substrate inhibition (two-site SI),

$$v = \frac{V_{\text{max1}} \cdot C}{K_m + C \cdot (1 + \frac{C}{K_{m1}})}$$

Eq. 3

where the substrate inhibition is considered to occur against the low-affinity enzyme. Finally, non-Michaelis-Menten kinetics displaying cooperativity was described by

$$v = \frac{V_{\text{max1}} \cdot C}{K_{m1} + C \cdot (1 + \frac{C}{K_{m1}})}$$

Eq. 4

$$v = \frac{V_{\text{max2}} \cdot C}{K_{m2} + C} + \frac{V_{\text{max1}} \cdot C}{K_{m1} + C \cdot (1 + \frac{C}{K_{m1}})}$$

where $n$ is the Hill coefficient.

### Results

**Metabolism and Excretion of $[^{14}$C]-Compound 1 in BDC Rats.** Following oral administration of $[^{14}$C]-compound 1, approximately 52% of the radioactivity was recovered in bile, with another 10% recovered in urine, and 14% recovered in feces after 48 hours. Radiochromatograms of bile and urine are shown in Fig. 2. The amount of radioactivity in bile and urine attributable to compound 1 was minimal (<5% of dose). Several major metabolite peaks were visible in the bile and/or urine, labeled M1–M4. M1–M3 were detected in both bile and urine, and were found to have an accurate mass (m/z) of 484.169 amu, corresponding to direct glucuronidation of compound 1 at three distinct locations of the parent molecule. The product ion spectra (Fig. 3) of the glucuronides were similar and produced the parent (m/z 308.138) and the fragments of the parent (within < 4 ppm). Therefore, the exact positions of the glucuronides could not be elucidated using the product ion data. M4 was detected in bile but not urine, and was found to have an accurate mass (m/z) of 500.164 amu, corresponding to oxidation and glucuronidation of the parent. Subsequent analysis revealed that treatment of bile samples with glucuronidase caused the degradation of M4 to M5, found to have an accurate mass (m/z) of 324.132 amu, and corresponding to hydroxylation of compound 1. M4 was then concluded to be an −O-glucuronide of compound 1. M1–M3 were stable in the presence of glucuronidase.

**Structural Elucidation of N-Glucuronide Metabolites by NMR.** To elucidate the structures of metabolites formed by direct glucuronidation of compound 1, M1–M3 were isolated and purified by fraction collection. The NMR studies of the isolated glucuronide metabolites were conducted by dissolving the metabolites (within 0.50 ppm) in dimethyl sulfoxide-d$_6$ and placing the samples in 1.7-mm tubes for data collection. The proton and carbon assignments were obtained from the 1D (1H) and 2D—g-DQOSY, g-HSQCdept, gradient–heteronuclear multiple bond correlation (g-HMBC), rotating-frame Overhauser effect spectroscopy (ROESY)—spectra collected on the samples (Table 1). The NMR spectra are consistent with the structures shown in Fig. 1. Glucuronide 1 (M1) was attached to N2 of the pyrazole ring and demonstrated by connections in the g-HMBC spectrum between H1’ and C3. In the ROESY spectrum of glucuronide 1, a cross-peak was observed between the anomeric proton H1’ and the secondary amine labeled 6. The attachment of glucuronide 2 (M2) was verified by g-HMBC connections between H1’ and C5. The ROESY spectrum of glucuronide 2 presents cross-peaks between glucuronides protons H1’ and H2’ to H5. The attachment of a glucuronide at position 1 or 2 of the pyrazole moiety induced a shift in the double bonds of that ring to accommodate the substitution. In glucuronide 1, a double bond exists between N1 and C5. This double bond deshields carbon 5 and causes an ~10 ppm shift of the carbon resonance. This effect was also observed in glucuronide 2 for C3. These chemical shift changes were used to track substitutions of many synthetic analogs at both positions of the pyrazole ring. For glucuronide 3 (M3), ROESY cross-peaks were observed between H21 and an anomic proton at 5.30 ppm and two other glucuronide methine protons at 3.91 and 3.51 ppm. Based on the results of the LC-MS and NMR data the scheme for the metabolism of compound 1 in rats was proposed as shown in Fig. 1.

**Metabolism of Compound 1 in Liver Microsomes.** Experiments were conducted using rat, dog, monkey, and human liver microsomes to investigate the similarity of metabolism of compound 1 across species. Preliminary experiments in the presence of UDPGA revealed that all three N-glucuronides observed in BDC rats were formed from rat liver microsomes with similar relative abundances, M1 > M2 > M3 (Fig. 4). This suggests that liver microsomes are a suitable enzyme source for investigating N-glucuronide formation from compound 1 in vitro. Looking across species, M1 and M2 were formed by liver microsomes from all species tested, albeit to different extents. Formation of M3 was detected in rat, dog, and human, but not monkey, liver microsomes. No other glucuronides were detected with liver microsomes in vitro. At a concentration of 10 μM, formation of M1 was substantially greater than formation of M2 or M3 in dog and human liver microsomes. However, M2 was the major metabolite in monkey liver microsomes. Experiments with liver microsomes fortified with NADPH indicated that compound 1 undergoes rapid oxidative metabolism in dog and monkey liver microsomes, while that in rat and human liver microsomes occurs more slowly (Table 2).
Metabolite formation experiments were conducted to further characterize the apparent species dependence in N-glucuronide formation. Concentration dependence in formation kinetics was described by a number of different kinetic models, depending on the appearance of Eadie-Hofstee plots shown in Fig. 5 (M1), Fig. 6 (M2), and Fig. 7 (M3). M1 formation was most accurately described by two-enzyme kinetic models in rat, dog, and human liver microsomes, indicating that M1 is most likely primarily formed by two major UGT isoforms within each of these species. In dog, a substrate inhibition effect was also required to describe the decreasing reaction velocity at the highest compound 1 concentrations tested. The model fit best when substrate inhibition was applied to the low-affinity binding site, rather than the high-affinity binding site or both binding sites simultaneously. Formation of M1 in monkey liver microsomes displayed atypical kinetics and was described by a sigmoid function incorporating a Hill slope of 1.54 ± 0.03, indicating positive cooperativity. V_max values for M1 formation showed considerable variation across the species tested (Table 3). M2 formation was most accurately described by single-enzyme kinetic models. Models for M2 formation in dog, monkey, and human liver microsomes fit best when a substrate inhibition effect was included. Substrate inhibition was not required to fit the formation of M2 in rat liver microsomes. Values for V_max and K_m showed a 10-fold or greater variation across species. One-site kinetic models were also fit to the formation of M3. Some cases (rat and dog liver microsomes) required a substrate inhibition effect. M3 was not detected in incubations with monkey liver microsomes.

**Formation of N-Glucuronides in Human UGTs.** M1 was formed principally by UGT1A1 and UGT1A9 (Fig. 8). M1 was also formed to a minor extent in UGT1A3, UGT1A7, and UGT1A8. Both M2 and M3 were principally formed by UGT1A9. M2 was formed to a minor extent in UGT1A1 and UGT1A3. M3 was formed to a minor extent in UGT1A1, UGT1A3, UGT1A4, and UGT1A8. No glucuronides were detected in incubations with UGT1A6, UGT1A10, UGT2B4, UGT2B7, UGT2B15, or UGT2B17. In addition, no other glucuronides (besides

**TABLE 2**
Depletion of compound 1 in liver microsomes fortified with NADPH

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Rat (µl/min/mg)</th>
<th>Dog (µl/min/mg)</th>
<th>Monkey (µl/min/mg)</th>
<th>Human (µl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>86</td>
<td>307</td>
<td>554</td>
<td>69</td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>97</td>
<td>209</td>
<td>18</td>
</tr>
</tbody>
</table>
M1–M3) were observed in incubations with isolated human UGTs. Interestingly, some concentration dependence was observed in the metabolic profile of compound 1 when incubated with UGT1A9. At low concentrations (0.1 μM), M2 was the main metabolite formed from UGT1A9, while at high concentrations (10 μM), M1 was the major metabolite.

Discussion

The mTOR inhibitor compound 1 was extensively glucuronidated in rats. Substantial quantities of N-glucuronides, but minimal parent (<5% of dose), were found in both urine and bile of BDC rats dosed orally with compound 1. Although compound 1 contains several possible locations for direct N-glucuronidation, glucuronidation was detected only on N1 and N2 of the pyrazole moiety, as well as on the primary amine (N23). No glucuronidation was detected on the secondary amine (N6) or tertiary amines (N8, N10, N11, N17, or N19). In addition, other glucuronides species such as carbamoyl glucuronides (Schaefer, 2006) were not detected in rat bile or urine. However, it cannot be ruled out that such glucuronides exist, perhaps in quantities that are below the detection limits of radiochemical or LC-MS detection. Interestingly, the relative abundance of the three N-glucuronides were similar between urine and feces, with M1 being the major metabolite in urine and bile, and M2 and M3 formed to a smaller, though significant, extent. Since minimal (≤10% of parent) N-glucuronides were detected in rat plasma following an oral dose of compound 1 (unpublished data), it is possible that the N-glucuronides found in urine were formed by UGT enzymes in the kidney, perhaps by the same enzymes responsible for their formation in the liver. However, given the wide tissue expression of UGT mRNA and activity in rats, their formation by other extrahepatic tissues cannot be ruled out at this time (Shelby et al., 2003; Shiratani et al., 2008). Since the total quantity of dose excreted in urine was minor relative to that in bile (10% versus 52%, respectively), and oxidative metabolism was also a minor pathway, further detailed investigation of compound 1 metabolism was focused on N-glucuronide formation in liver microsomes, particularly, given available documentation that species differences in N-glucuronidation of aromatic N-heterocycles can be highly compound dependent (Kaivosaari et al., 2011).

When incubated in rat liver microsomes in the presence of UDPGA, Compound 1 formed the same three glucuronides found in vivo to similar proportions, M1 > M2 > M3 (Fig. 4). This helped to establish confidence that liver microsomes were a reasonable enzyme source for investigating N-glucuronidation of compound 1. Qualitative and

Fig. 5. Formation of M1 from compound 1 in incubations of rat (A), dog (B), monkey (C), and human (D) liver microsomes fortified with UDPGA. Inserts in each panel are the Eadie-Hofstee diagrams of the same data displayed in the main reaction velocity plots.
Quantitative species differences were found in the metabolism of compound 1 by liver microsomes, as were there some similarities. Firstly, all three N-glucuronides were detected in liver microsomes from dogs and humans. However, while M1 and M2 were detected in liver microsomes from monkeys, M3 was not detected. No other N-glucuronides were detected in liver microsomes from all species tested.

Secondly, M3 was consistently the relatively minor metabolite of the three, across three of the species tested (rat, dog, and human), or undetected in monkey (Fig. 4). However, at low concentrations (10 μM or less), M1 was the major metabolite formed in rat, dog, and human liver microsomes, while M2 was the major metabolite formed in monkey liver microsomes. This species selectivity found in monkeys is unique when compared with other recent examples of triazole and pyrazole N-glucuronidation. In the case of FYX-051, the ratios of N1- to N2-glucuronide formation in vivo were quite similar in dogs, monkey, and humans (Nakazawa et al., 2006). In the case of JNJ-10198409, the N1-glucuronide was prominently formed in both monkey and human liver microsomes, while the N2-glucuronide was not detected in either species (Yan et al., 2006). The reasons for the unique species selectivity with compound 1 are not currently known.

Compound 1 was found to be metabolized primarily by UGT1A9 and UGT1A1 in humans in vitro. UGT1A1 is more or less conserved across species given its important role in bilirubin metabolism (Bosma et al., 1994; King et al., 1996). However, species differences have been reported in the activity of UGT1A9. Dog and rat apparently lack meaningful propofol glucuronidation activity, and UGT1A9 is a pseudogene in rats (Soars et al., 2001a, b; Shiratani et al., 2008). In addition, monkey and human UGT1A9 orthologs were found to be 93% homologous, and structurally and functionally similar (Albert et al., 1999). Such findings have supported the belief that rats and dogs are not predictive of glucuronidation by UGT1A9 substrates, and that monkeys might be a better model for this purpose. However, the present findings with compound 1 suggest that this might not always be the case. Given this unique species selectivity, compound 1 might be a useful probe to investigate such species differences in UGT functionality.

Thirdly, detailed kinetic analysis of N-glucuronide formation can shed further light on the apparent species differences and similarities. M1 formation was biphasic in rat, dog, and human liver microsomes, indicating that two enzymes, one high affinity and one low affinity, are responsible for M1 formation in these species in vitro (Fig. 5). In humans this conclusion is supported by reaction phenotyping experiments, which confirmed that UGT1A1 and UGT1A9 predominantly

Fig. 6. Formation of M2 from compound 1 in incubations of rat (A), dog (B), monkey (C), and human (D) liver microsomes fortified with UDPGA. Inserts in each panel are the Eadie-Hofstee diagrams of the same data displayed in the main reaction velocity plots.
formed M1 in vitro. If UGT1A1 is assumed to be one of the UGTs responsible for M1 formation in rats and dogs, another UGT, besides UGT1A9, might be implied as the second enzyme, given that UGT1A9 activity was found to be lacking in rats and dogs. Since other human UGTs were found to have minor activity in compound 1 N-glucuronidation, perhaps rat or dog orthologs of these UGTs are more important in

![Fig. 7. Formation of M3 from compound 1 in incubations of rat (A), dog (B), and human (C) liver microsomes fortified with UDPGA. Inserts in each panel are the Eadie-Hofstee diagrams of the same data displayed in the main reaction velocity plots.](image)

### TABLE 3

Best fit for the model parameters describing the formation of N-glucuronides from compound 1 in rat, dog, monkey, and human liver microsomes fortified with UDPGA

<table>
<thead>
<tr>
<th>N-Glucuronide/Model Parameter</th>
<th>Rat</th>
<th>Dog</th>
<th>Monkey</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>Two-site</td>
<td>Two-site SI</td>
<td>Sigmoid</td>
<td>Two-site</td>
</tr>
<tr>
<td>$v_{\text{max},h}$ (pmol/min*mg)</td>
<td>1.34 ± 0.25</td>
<td>0.362 ± 0.107</td>
<td>217 ± 5</td>
<td>33.5 ± 11.3</td>
</tr>
<tr>
<td>$k_{\text{m},h}$ (μM)</td>
<td>0.787 ± 0.248</td>
<td>1.42 ± 0.40</td>
<td>26.2 ± 0.9</td>
<td>3.55 ± 0.88</td>
</tr>
<tr>
<td>$v_{\text{max},l}$ (pmol/min*mg)</td>
<td>2.60 ± 0.20</td>
<td>1.63 ± 3.20</td>
<td>N.A.</td>
<td>44.6 ± 9.5</td>
</tr>
<tr>
<td>$K_{\text{s},l}$ (μM)</td>
<td>19.0 ± 5.5</td>
<td>65.2 ± 178.3</td>
<td>N.A.</td>
<td>22.7 ± 9.6</td>
</tr>
<tr>
<td>$n$</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td><strong>M2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>One-site</td>
<td>One-site SI</td>
<td>One-site SI</td>
<td>One-site SI</td>
</tr>
<tr>
<td>$v_{\text{max}}$ (pmol/min*mg)</td>
<td>1.82 ± 0.04</td>
<td>0.0965 ± 0.0171</td>
<td>213 ± 4</td>
<td>14.5 ± 0.5</td>
</tr>
<tr>
<td>$k_{m}$ (μM)</td>
<td>2.42 ± 0.20</td>
<td>15.0 ± 4.7</td>
<td>7.76 ± 0.31</td>
<td>25.4 ± 1.4</td>
</tr>
<tr>
<td>$K_{s,zol}$ (μM)</td>
<td>N.A.</td>
<td>91 ± 111</td>
<td>98.9 ± 5.3</td>
<td>86.4 ± 6.4</td>
</tr>
<tr>
<td><strong>M3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>One-site SI</td>
<td>One-site SI</td>
<td>N.A.</td>
<td>One-site SI</td>
</tr>
<tr>
<td>$v_{\text{max}}$ (pmol/min*mg)</td>
<td>0.0696 ± 0.0021</td>
<td>0.323 ± 0.023</td>
<td>N.A.</td>
<td>0.258 ± 0.008</td>
</tr>
<tr>
<td>$k_{m}$ (μM)</td>
<td>1.14 ± 0.11</td>
<td>10.6 ± 1.5</td>
<td>N.A.</td>
<td>15.0 ± 1.4</td>
</tr>
<tr>
<td>$K_{s,zol}$ (μM)</td>
<td>375 ± 93</td>
<td>271 ± 86</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not applicable; SI, substrate inhibition.
these species. In monkey liver microsomes, M1 was formed in a fashion consistent with single enzyme sigmoidal kinetics, requiring a Hill slope, indicative of positive cooperativity or autoactivation. This type of kinetics was unique among the species and compared with M2 formation in monkey liver microsomes. It is not currently known whether monkey UGT1A1 or UGT1A9 contributed to this kinetics, or what the specific nature of the mechanism behind the kinetics might be. Nevertheless this observation supports the uniqueness of monkeys among species in the N-glucuronidation of compound 1. Aside from some clear capacity differences across species, formation of M2 and M3 were more similar across species, following single enzyme kinetics (Fig. 6 and Fig. 7). The finding in human liver microsomes was supported by additional reaction phenotyping experiments, where M2 and M3 were formed principally by a single UGT, UGT1A9. Generally, capacity for compound 1 N-glucuronidation was much higher in human and monkey liver microsomes than in rat or dog liver microsomes, but the metabolic profiles in rats and dogs were qualitatively more similar to that in humans in vitro.

Reaction phenotyping experiments with compound 1 also have potential clinical relevance. Since compound 1 N-glucuronidation occurred predominantly by UGT1A1 and UGT1A9, care must be taken to avoid potential drug interactions with potent inhibitors of these enzymes. However, having more than one UGT responsible, in addition to any oxidation metabolism, may help limit effects of inhibition of any single UGT. In addition, since UGT1A1 is highly expressed in the human intestine (Rowland et al., 2013), inhibition of UGT1A1, or known polymorphisms, could impact the bioavailability of compound 1, if bioavailability is limited by metabolism by UGT1A1 in the gut. Further in vitro and physiologic modeling studies might be warranted to clarify the potential impact of UGT inhibition or polymorphisms.

In summary, compound 1 was shown to undergo extensive N-glucuronidation in BDC rats. N-glucuronides were detected on positions N1 (M2) and N2 (M1) of the pyrazole moiety, as well as on the primary amine (M3). All three N-glucuronide metabolites were detected in liver microsomes of the rat, dog, and human, while primary amine glucuronidation (M3) was not detected in monkey. In addition, N1- and N2-glucuronidation showed strong species selectivity in vitro, with rats, dogs, and humans favoring N2-glucuronidation and monkeys favoring N1-glucuronide formation. Formation of M1 (N2-glucuronidation) in monkey liver microsomes also followed sigmoidal kinetics, singling out monkey as unique among the species as far as compound 1 N-glucuronidation. In this respect, monkey might not always be the best animal model for N-glucuronidation of UGT1A9 or UGT1A1 substrates in humans. The impact of N-glucuronidation could be more pronounced in higher species such as monkey and human, leading to high clearance in these species. While compound 1 shows promise as a candidate for investigating the impact of pan-mTOR inhibition in vivo, opportunities may exist through medicinal chemistry efforts to potentially reduce metabolic liability with the goal of improving systemic exposure.

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Authorship Contributions
Participated in research design: Berry, Colletti, Teffera, Zhao.
Conducted experiments: Berry, Colletti, Krolikowski, Liu.
Performed data analysis: Berry, Colletti, Liu, Krolikowski, Teffera.
Wrote or contributed to the writing of the manuscript: Berry, Krolikowski, Teffera, Zhao.
References


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