Structural and Kinetic Characterization of a Novel N-acetylated Aliphatic Amine
Metabolite of the PRMT Inhibitor, EPZ011652

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List of nonstandard abbreviations: CoA, coenzyme A; LLOQ, lower limit of quantitation; m/z, mass-to-charge ratio; NAT, N-acetyltransferase; MRM, multiple reaction monitoring; UGT, UDP-glucuronosyltransferase
ABSTRACT

Pharmacokinetic and metabolite identification studies were conducted to understand the clearance pathways of EPZ011652 [(2-aminoethyl)(methyl)(3-[4-(propan-2-yloxy)phenyl]-1H-pyrazol-4-yl)methyl]amine], a potent protein arginine N-methyltransferase (PRMT) inhibitor. Metabolic clearance was the major pathway of EPZ011652 elimination in rats with structural elucidation of metabolites via LC-MS\textsuperscript{n} accurate mass measurement revealing the formation of a novel aliphatic N-acetylated metabolite (M1) located on the terminal nitrogen of the ethylenediamine sidechain. EPZ015564, a synthetic standard of the N-acetyl product, was prepared and was also generated by human and rat, but not dog hepatocytes. In rat hepatocytes, on incubation with EPZ011652 the concentration of EPZ015564 initially increased before decreasing with incubation time suggesting that the metabolite is itself a substrate for other metabolizing enzymes, in agreement with the identification of metabolites M2, M3 and M4 in rat bile, all N-acetylated metabolites, undergoing sequential phase I (demethylation, oxidation) or phase II (sulfation) reactions. Reaction phenotyping with recombinant human N-acetyltransferase (NAT) isoforms revealed that both NAT1 and NAT2 are capable of acetylating EPZ011652, although with different catalytic efficiencies. Kinetic profiles of EPZ015564 formation followed classical Michaelis-Menten behavior with apparent K\textsubscript{m} values of >1000 µM for NAT1 and 165 ± 14.1 µM for NAT2. The \textit{in vitro} intrinsic clearance for EPZ011652 by NAT2 (110 µL/min/mg) was 500-fold greater than by NAT1. In summary, we report the unusual N-acetylation of an aliphatic amine, and discuss the implications for drug discovery and clinical development.
INTRODUCTION

Achieving the appropriate balance of clearance and metabolic stability along with potency and permeability is an integral part of drug discovery efforts and multiparametric lead optimization. The optimization of metabolic stability is largely focused on the CYP and UDP-glucuronosyltransferase (UGT) mediated pathways, since these routes represent the vast majority of clearance pathways for drugs (Guengerich et al., 2005; Rowland et al., 2013). The role of physicochemical properties on the propensity for clearance by these enzymes is well recognized, as is the importance of physicochemistry in overall hit and lead candidate quality (Gleeson, 2008). With medicinal chemistry efforts driving towards compounds with low CYP and UGT turnover, recent years have seen a sharp rise in the reported involvement of some of the other, perhaps lesser studied, drug metabolizing enzymes such as aldehyde oxidase (Pryde et al., 2010; Garattini and Terao, 2011), monoamine oxidase (Gaweska and Fitzpatrick, 2011), sulfotransferases (James and Ambadapadi, 2013), and N-acetyltransferases (Sim et al., 2014). Human N-acetyltransferase 1 (NAT1) and 2 (NAT2) are important cytosolic phase II enzymes that catalyze the transfer of acetyl groups from endogenous acetyl-coenzyme A (CoA) to a range of arylamine, heterocyclic amine, and hydrazine substrates, including many common carcinogens and therapeutic agents (Hein, 2000; Sim et al., 2014). Human NAT1 and NAT2 have different but overlapping substrate specificity profiles, and no clear structural motif determines substrate specificity for each isoform. In general, p-aminobenzoic acid (PABA) and p-aminosalicylic acid are considered substrates for human NAT1. In contrast, sulfamethazine, procainamide and isoniazid are acetylated primarily by human NAT2. Other compounds are substrates for both human NAT1 and NAT2, with the best known example being 5-
aminosalicylic acid (Kawamura et al., 2005; Minchin et al., 1992; Sim et al., 2014). However, aliphatic amines have not generally been reported to be human NAT1 or NAT2 substrates.

EPZ011652 is a relatively unselective protein arginine N-methyltransferase (PRMT) 1, 6 and 8 inhibitor that was the starting point for a PRMT6 lead optimization effort (Mitchell et al., 2015). PRMT6 is a member of the PRMT family which comprises 45 enzymes that are known or presumed to catalyze protein arginine N-methylation reactions on histones and other proteins. Site-specific histone methylation is critical for chromatin-mediated regulation of gene transcription, and is often genetically altered in human cancers (Copeland, 2013). PRMT6 overexpression has been reported in several cancer types including melanoma (Limm et al., 2013) and bladder, lung and prostate carcinoma (Yoshimatsu et al., 2011; Vieira et al., 2014) suggesting that inhibition of PRMT6 may have therapeutic utility and supporting development of small molecule inhibitors.

During the course of lead optimization, a putative N-acetyl metabolite (M1) of EPZ011652 was observed in in vivo and in vitro samples. The aim of this study was to elucidate the structure of this metabolite and characterize the kinetics and enzymology of this reaction, since the N-acetylation of aliphatic amines represents an unusual biotransformation and has not been widely reported to be mediated by the NAT1 or NAT2 enzymes.
MATERIALS & METHODS

Materials.

EPZ011652 [(2-aminoethyl)(methyl)({3-[4-(propan-2-yloxy)phenyl]-1H-pyrazol-4-yl}methyl)amine] and EPZ015564 (authentic reference standard of EPZ011652 N-acetyl metabolite, M1, N-[2-[methyl({3-[4-(propan-2-yloxy)phenyl]-1H-pyrazol-4-yl}methyl)amino]ethyl}acetamide) were synthesized by Epizyme (Cambridge, MA; Mitchell et al, 2015). The $^1$H NMR spectrum of EPZ015564 is shown in Supplemental Figure 1. All other reagents were purchased from sources as described below.

Pharmacokinetic Study in Rats.

All animal studies were performed in accordance with the AAALAC International and NIH guidelines standards. Male Sprague-Dawley rats (193-267 g; Vital River Laboratory Animal Technology Co., Ltd.; n=3 per group) were treated with a single dose of EPZ011652 at 1 mg/kg (Group 1) or 10 mg/kg (Group 2; bile-duct cannulated) by intravenous tail vein injection (1 or 5 mg/mL in saline). Animals were weighed prior to dose administration and Group 2 animals were placed into metabolic cages to allow for urine collection. Approximately 300 μL of blood were taken from animals via jugular vein cannulation at pre-specified time intervals for up to 24 h (predose, 0.05, 0.167, 0.5, 1, 2, 4, 8 and 24 h). Blood samples were transferred into K$_2$-EDTA tubes and placed on wet ice prior to centrifugation at 4°C (2000 g, 5 min) to obtain plasma within 60 min after sample collection. Urine and bile samples were collected on ice at intervals of 0-4 h, 4-8 h, 8-12 h and 12-24 h from the animals in Group 2. All samples were stored at -70 ± 10°C prior to protein precipitation and analysis. Typically, samples were injected onto a LC-
MS/MS system (API 4000, AB Sciex, Framingham MA) using a Shimadzu Shim-pack XR-ODS III column (2.2 µm; 2.0 × 50 mm). Mobile phase A was 95% acetonitrile in 4 mM ammonium acetate buffer, mobile phase B was 5% acetonitrile in 4 mM ammonium acetate buffer with a gradient of 5% A to 90% A in 1.3 minutes, maintained at 90% A for 0.5 min. The injection volume was 2 µL, and the flow rate was 0.6 mL/min. The retention time of EPZ011652 was ~1.1 min. The ionization was conducted by electrospray in the positive ion mode using the multiple reaction monitoring (MRM) transition [M+H]+ m/z 289.3/215.0. Standard calibration curves were constructed by analyzing a series of control plasma, bile or urine aliquots containing 100 ng/mL dexamethasone as internal standard and 0.5 ng/mL (Group 1) or 2.0 ng/mL (Group 2) to 1000 ng/mL EPZ011652. Three levels of quality controls were also included in the analysis (1, 50 and 800 ng/mL for Group 1 and 3, 50 and 800 ng/mL for Group 2). The concentration of EPZ011652 in each unknown sample was determined by solving the linear calibration curve equation for each corresponding drug/internal standard ratio. Pharmacokinetic parameters were calculated by non-compartmental methods using Phoenix WinNonlin 6.2 (Certara, St-Louis, MO). Terminal half-life values were determined by regression of at least 3 data-points in the later phase of the time-concentration profile. Parameters are presented as mean ± SD were applicable. Parent excretion in urine or bile was calculated as the % dose excreted = sum of (urine or bile concentration * urine or bile volume), divided by dose.

Metabolite Identification in Rat Plasma and Bile.

Aliquots of individual rat plasma and bile (0 to 4 h time-points) were pooled for metabolite identification studies. The 0-4 h collection interval corresponded to 88% of the plasma AUC0-inf and so was deemed representative of the overall metabolite profile. Plasma samples were
quenched with 1 volume of methanol followed by centrifugation 4°C (16100 g, 15 min). One volume of acetonitrile was added to bile samples prior to centrifugation. Metabolites were identified using a Q-TOF Premier exact mass spectrometer (Waters, Milford, MA). Chromatography separation was performed using an ACUITY UPLC system with a binary solvent manager using 50 mM ammonium acetate in water (A) and 0.1% formic acid in acetonitrile (B) as the mobile phase with the following gradient: 0 to 24 min: gradient from 95% A/5% B to 95% B at a flow rate of 400 μL/min. The analytical column used was a Nucleodur C18 Gravity (5 μm, 2 x 50 mm; Macherey-Nagel, Bethlehem, PA). The injection volume was 10 μL. The mass spectrometer was operated with a desolvation temperature of 300°C, desolvation gas at 500 L/h, source temperature of 100°C, cone gas at 20 L/h and the spray capillary was set to 2.5 kV. A prescreening full scan from 50 to 950 amu (positive ion electrospray) was performed for comprehensive analysis of metabolites. The possible chemical structures of the metabolites were deduced based on their MS$^1$ and MS$^2$ spectra in addition to their exact mass.

**Metabolite Profiling in Liver S9.**

Following identification of M1 in rat bile, EPZ011652 was incubated with human and rat S9. Male pooled human (10 donors) and Sprague-Dawley rat liver S9 were obtained (Bioreclamation IVT, Baltimore, MD). EPZ011652, 10 μM, was added to 100 mM phosphate buffer, pH 7.4, containing 1.0 mg/mL S9 and pre-warmed to 37°C for 5 min. Reactions were initiated by the addition of 100 μM Acetyl-CoA and UDPGA. Reactions were incubated at 37°C for 90 minutes and terminated by the addition of one volume of ice-cold acetonitrile. A negative sample, without co-factor was also incubated for 90 min. Samples were centrifuged at 2000g, 4°C, for 10
min, and the supernatant was used for LC-MS/MS analysis and metabolite identification using the method described above.

**N-acetylation of EPZ011652 in Human, Rat and Dog Hepatocytes.**

Pooled mixed gender cryopreserved human, pooled male Sprague-Dawley rat and pooled male Beagle dog hepatocytes (Bioreclamation IVT, Baltimore, MD) were thawed and re-suspended at 1 million viable cells/mL in incubation buffer [10 g/L Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO), 26 mM NaHCO₃, 9 mM HEPES, 2.22 mM fructose, pH 7.4]. Duplicate reactions were initiated by the addition of 10 μM EPZ011652 in a final volume of 0.5 mL. Reactions were incubated at 37°C for up to 120 minutes, with sampling of 50 μL at 15, 30, 45, 60 and 120 min, and terminated by the addition of 3 volumes of methanol containing 1 μM tolbutamide as internal standard (IS). Formation of p-acetamidobenzoic acid from incubations with 40 μM p-aminobenzoic acid (PABA), and formation of N-acetyl sulfamethazine from incubations with 40 μM sulfamethazine, were used as positive controls for NAT1 and NAT2, respectively. Diltiazem and diclofenac depletion was also assessed for each species as a marker of phase I and II reactions and were in concordance with internal historical values (data not shown). All samples were centrifuged at 2500g, 4°C, for 20 min, and supernatants were analyzed by LC-MS/MS (MRM mode). Parent depletion was monitored and in parallel, standard curves were prepared to measure the formation of EPZ015564 (M1, LLOQ 3 nM), p-acetamidobenzoic acid (LLOQ 12.5 nM) and N-acetyl sulfamethazine (LLOQ 4 nM). In vitro t₁/₂ values were determined by plotting the natural logarithm of the analyte/IS peak area ratios versus time, with the slope of the linear regression (-k) converted to in vitro t₁/₂ values where t₁/₂ =0.693/k.
Experimental half-lives were transformed to the corresponding scaled intrinsic clearance values (in units of mL/min/kg) as below:

\[ \text{CL}_{\text{int}} = 0.693/\text{in vitro} \ t_{1/2} * \text{mL incubation/million cells} * \text{hepatocellularity} * \text{LWPBW} \]

where hepatocellularity is 120, 240 and 120 million cells/gram liver for rats, dogs and humans, respectively, and LWPBW is grams liver/kg body weight (Davies and Morris, 1993).

**Human NAT1 and NAT2 phenotyping.**

cDNA expressed human NAT1 and NAT2 enzyme preparations (Corning Life Sciences, The Netherlands) (0.1 mg/mL), 0.1 M phosphate buffer, pH 7.4, and 1 µM EPZ011652 (0.25% final DMSO concentration) were pre-incubated at 37°C prior to the addition of 1 mM acetyl CoA to initiate the reaction. The final incubation volume was 500 µL. A control incubation was included where 0.1 M phosphate buffer was added instead of acetyl-CoA. Two control compounds, p-aminobenzoic acid and isoniazid were included for the respective NAT isoforms. Compounds were incubated for up to 45 min, with samples taken at 0, 5, 15 and 30 minutes. At each time point, 50 µL samples were removed and the reactions were stopped by the addition of 100 µL methanol containing internal standard. The incubation plates were centrifuged at 2,500 rpm for 20 min at 4°C to precipitate the protein, prior to LC-MS/MS analysis. Analysis was carried out on a Xevo-TQ MS Triple Quadrupole mass spectrometer (Waters Ltd, Elstree, Herts, UK). This system used a Waters Aquity™ HSS T3 (1.8µm, 2.1 x 50 mm) column maintained at 70°C running a solvent gradient of 10 mM ammonium acetate in deionised water (Eluent A) and methanol (Eluent B). A gradient of 0% to 95% B was run over a 2.15 min period and this condition was maintained for 40 seconds at a flow rate of 0.6 mL/min. The ionization was
conducted by electrospray, in the positive ion mode, using the MRM of [M+H]^+ m/z 331.2/173.0 for EPZ015564 and m/z 289.2/173.0 for EPZ011652.

**Characterization of EPZ011652 N-acetylation by recombinant human NAT1 and NAT2.**

To determine the time- and concentration-linearity of EPZ015564 formation, 1 µM EPZ011652 was added in duplicate to 0.05 to 1.0 mg/mL NAT1 or 6.25 to 100 µg/mL NAT2 in 0.1 M phosphate buffer, pH 7.4, and pre-incubated at 37°C prior to the addition of 1 mM acetyl-CoA to initiate the reaction. The final incubation volume was 0.5 mL. Compounds were incubated for 60 min, with samples taken at 0, 5, 15, 30 and 45 minutes. Samples were processed and analyzed as described above. For K_m and V_max determinations, EPZ011652 (1, 2.5, 5, 10, 25, 50, 75, 100, 250, 500 µM for NAT1; 1, 5, 10, 25, 50, 100, 200, 400, 600, 800 µM for NAT2) plus a vehicle control (0.25% DMSO NAT1; 0.4% DMSO NAT2) were incubated with either 250 µg/mL NAT1 or 12.5 µg/mL NAT2 at 37°C. Incubations were initiated with the addition of acetyl-CoA (1 mM) and were terminated by removing an aliquot of the incubation into methanol. Reaction times were 30 min for NAT1 and 15 min for NAT2. Standard curves were prepared in protein concentration matched control-insect cell cytosol to allow quantification of the rate of metabolite formation. Following centrifugation at 2500 rpm for 30 min at 4°C to precipitate protein, the sample supernatants were diluted with deionised water containing internal standard prior to LC-MS/MS analysis using the method described above for NAT phenotyping.

Non-linear regression analysis of enzyme kinetic data was carried out using the enzyme kinetics module of SigmaPlot version 11.0 (Systat Software Inc. Chicago, IL), using models for the Hill equation, substrate activation, substrate inhibition, Michaelis-Menten and isozyme kinetics. The goodness of fit criteria comprising of visual inspection of the data, squared correlation
coefficient ($R^2$) and corrected Akaike’s Information Criterion (AICc) were utilized to select the most appropriate model. $CL_{int}$ values were calculated as $V_{max}/K_m$. 
RESULTS

Pharmacokinetic Study in Rats. Male Sprague-Dawley rats administered a single dose of EPZ011652 at 1 mg/kg or 10 mg/kg by intravenous (iv) bolus injection showed similar, high clearance (CL) of 96.2 ± 16.3 mL/min/kg and 73.7 ± 13.6 mL/min/kg, respectively. Likewise, similar volumes of distribution at steady state (Vss) were observed that were several-fold greater than total body water at 7.93 ± 1.44 L/kg and 8.10 ± 2.12 L/kg, respectively (Table 1). AUC0-t and AUC0-inf, the areas under the curve to the last measurable data point and extrapolated to infinity, respectively, increased in a dose-proportional manner. Following 10 mg/kg iv administration, renal CL (1.51 ± 0.906 mL/min/kg) and biliary CL (0.050 ± 0.029 mL/min/kg) were minor pathways of excretion for EPZ011652, with only 8.29 ± 4.24% of the parent recovered in urine and less than 1% of the parent recovered in bile after 24 hours.

Identification of the Major Metabolites of EPZ011652 in Rat in vivo, and Human and Rat in vitro. The protonated molecular ion of EPZ011652 was m/z 289.201. Figure 1 shows the fragmentation spectrum of EPZ011652, with structurally diagnostic ions observed at m/z 215.120, 173.069, 146.059 and 75.091. As more metabolites were observed in rat bile (m/z 331.213, 347.208, 355.108 and 369.123) than in plasma (m/z 355.108 and 369.123), structural elucidation of the major metabolites was performed using the 0 to 4h pooled rat bile sample. The 0-4 h interval represented 88% of the plasma AUC0-inf. A representative extracted ion chromatogram for M1, M2, M3 and EPZ11652 in rat bile is presented in Figure 2. Figure 3 illustrates the MS² spectrum of the N-acetylated metabolite, M1 (m/z 331.213). M1 has a mass shift of +42 Da compared to EPZ011652, corresponding to an acetylation reaction. The
diagnostic fragment ion at \( m/z \) 117.103 indicates that the acetyl group is located on the terminal amine (daughter ion 75.1 + 42 = 117.1). The proposed metabolite scheme for EPZ011652 in rat is shown in Figure 4, with the identified metabolites summarized in Table 2. Following identification of M1 (\( m/z \) 331.213) in rat bile, this metabolite was also observed following incubation of EPZ011652 in both human and rat liver S9 fractions supplemented with acetyl-CoA (not present in a control sample in the absence of cofactor).

The protonated molecular ion of M2 was \( m/z \) 347.208, indicating a mass shift of +58 Da. A diagnostic fragment ion at \( m/z \) 117.103 indicated that the acetylation was located on the terminal amine, similarly to M1. In addition, a fragment ion of \( m/z \) 231.121 suggested a mono-hydroxylation of the isopropyl group (EPZ011652 fragment 215.1 + 16 Da), also supported by the presence of the unmodified fragment ion at \( m/z \) 173.069.

The protonated molecular ion of M3 was \( m/z \) 369.123, indicating a mass shift of +80 Da. The diagnostic fragment ion at \( m/z \) 117.103 indicates that EPZ011652 is acetylated on the terminal amine, as observed for M1 and M2. The ion at \( m/z \) 253.027 implicates a sulfate of the desisopropyl metabolite.

The protonated molecular ion of M4 was \( m/z \) 355.108, indicating a mass shift of +66 Da. The diagnostic fragment ion at \( m/z \) 103.094 indicates that as for M1, M2 and M3, an acetyl group was conjugated on the terminal amine, but in contrast to the other metabolites, an \( N \)-demethylation (-14 Da) occurred on the tertiary amine. As observed for M3, the fragment ion 253.027 suggests a sulfate of the desisopropyl metabolite of EPZ011652.
Neither M2, M3 nor M4 were observed in human and rat liver S9 incubations and additionally no evidence of glucuronidation was seen in rat bile or in liver S9 fractions supplemented with UDPGA.

**M1 (EPZ015564) Formation in Pooled Human, Rat and Dog Hepatocytes.** EPZ011652 showed a high scaled clearance (59 mL/min/kg) in rat hepatocytes, correlating well with the rat *in vivo* clearance. A moderate scaled clearance in dog hepatocytes (15 mL/min/kg) and low clearance (<5 mL/min/kg) in human hepatocytes were also observed (Table 3). Using EPZ015564, the synthesized authentic reference standard of M1, enabled the concentration of the metabolite to be monitored over time as summarized in Table 3. In rats, EPZ015564 concentration declined with incubation time, with a peak level of 74 nM at 15 minutes, likely due to sequential metabolism, as was observed *in vivo*. In contrast, EPZ015564 was detected only after 45 minutes of incubation in human hepatocytes and increased with incubation time up to 9 nM at 120 min. EPZ015564 was not detected in dog hepatocyte incubations, in line with the absence of NAT positive control metabolites, *p*-acetamidobenzoic acid and *N*-acetyl sulfamethazine formation in this species.

**Human NAT1 and NAT2 Phenotyping.** Formation of EPZ015564 (M1) from incubations with EPZ011652 was minimal in the presence of human NAT1, with a conversion of less than 1% of the initial parent concentration (3.1 nM EPZ015564 at 45 min). In contrast, *p*-aminobenzoic acid, a known NAT1 substrate had a half-life of less than 2 minutes under the same incubation conditions. EPZ011652 *N*-acetylation was much more extensive in the presence of NAT2 compared to NAT1, with more than 50% of the initial parent concentration converted to EPZ015564 within 45 minutes (final concentration of 527 nM). Under similar incubation
conditions, the positive control isoniazid had a half-life of less than 2 min in the presence of NAT2.

Characterization of EPZ011652 N-acetylation by Recombinant Human NAT1 and NAT2.

Time- and protein-linearity of EPZ015564 (M1) formation from incubations with 1 µM EPZ011652 and recombinant human NAT1 and NAT2 are presented in Supplemental Figure 2. EPZ015564 formation was linear as a function of time between 30 and 60 minutes for all NAT1 protein concentrations tested, and was linear between 45 and 60 minutes up to 50 µg/mL for NAT2. EPZ015564 formation deviated from linearity at NAT1 concentrations of ≥ 0.5 mg/mL and at NAT2 concentrations greater than 50 µg/mL. Based on these observations, linear conditions, with less than 5% of parent depletion, could be selected for K_m and V_max determinations; 0.25 mg/mL NAT1 for 30 mins and 12.5 µg/mL NAT2 for 15 mins. The model of best fit for the observed formation of EPZ015564 was the classical Michaelis-Menten model for both isoforms of NAT. For NAT1 the K_m was calculated to be 1321 ± 262 µM with a corresponding V_max 0.288 ± 0.0443 nmol/min/mg protein (Figure 5; R^2 = 0.991), extrapolated above the top concentration tested and hence underdetermined. For NAT2 the K_m was 165 ± 14.1 µM, with a V_max of 18.2 ± 0.517 nmol/min/mg protein (R^2 = 0.988). Comparison of V_max/K_m ratios indicated that the in vitro intrinsic clearance for EPZ015564 formation by NAT2 (110 µL/min/mg) was 500-fold greater than that by NAT1 (0.22 µL/min/mg).
DISCUSSION

During the course of lead optimization, a putative \(N\)-acetyl metabolite (M1) of EPZ011652 was observed \textit{in vivo} and \textit{in vitro}. The aim of this study was to elucidate the structure of this metabolite and characterize the kinetics and enzymology of this reaction, since the \(N\)-acetylation of aliphatic amines represents an unusual biotransformation and has not been widely reported to be mediated by NAT1 or NAT2 enzymes.

EPZ011652 exhibited a high clearance in rat, which was explored further in a bile duct cannulated rat experiment to determine the mechanisms of elimination. Excretion of parent compound was demonstrated to be low, and metabolite profiling was undertaken to identify the metabolic liabilities in an effort to guide optimization of this chemical series. Metabolite identification studies in rat bile and rat and human liver S9 samples with structural elucidation via accurate mass measurement and diagnostic fragment ions (LC/MS\textsuperscript{n}) revealed the formation of a novel, \(N\)-acetylated metabolite (M1) of EPZ011652. An authentic synthetic standard of M1, namely EPZ015564, was used to characterize this uncommon enzymatic reaction. In addition to EPZ015564, three other \(N\)-acetylated metabolites were observed in rat bile, demonstrating that \(N\)-acetylation is a major pathway of clearance for EPZ011652 in this species. The observed secondary metabolites and deciphering the exact metabolic sequelae represent an interesting case for further study but were not the focus of this work.

Formation of EPZ015564 was also observed in pooled human and rat hepatocytes, but not in dog hepatocytes, in line with the lack of acetylation capacity of known NAT1 and NAT2 substrates.
in this species (Collins, 2001). This was expected, as NATs are expressed in humans and rats, but not in dogs due to an absence of the NAT genes (Trepanier et al., 1997), and suggests that NATs are the primary enzymes implicated in the formation of EPZ015564. The high intrinsic clearance of EPZ011652 in rat hepatocytes correlated well with the systemic clearance observed in an *in vivo* pharmacokinetic study and was roughly equivalent to hepatic blood flow. In contrast, the scaled clearance of the compound was moderate in dog hepatocytes and low (<5 mL/min/kg) in human hepatocytes. In rat hepatocytes, the concentration of EPZ015564 increased transiently before decreasing with incubation time suggesting that this primary metabolite is a substrate for other metabolizing enzymes. This observation is in agreement with the identification of metabolites M2, M3 and M4 in rat bile, implicating phase I (dealkylation, oxidation) or phase II (sulfation) sequential reactions. In contrast, EPZ015564 formation increased with incubation time in human hepatocytes, implying no further secondary metabolism of the *N*-acetyl metabolite.

From the assays with human recombinant NATs, it can clearly be seen that NAT1 and NAT2 are capable of acetylating EPZ011652, although with different catalytic efficiencies. The kinetic parameters measured for these two isoforms show at least an 8-fold lower $K_m$ and a 63-fold higher $V_{max}$ with NAT2 than for NAT1, resulting in a 500-fold higher intrinsic clearance by NAT2 (110 μL/min/mg). This is in agreement with the recent report of *N*-acetylation of the aminoethyl group of etamicastat mediated by NAT2, with a lower affinity for NAT1 (Loureiro et al., 2013). Although NAT1 intrinsic clearance is much lower, it could still play a role in non-hepatic tissues as NAT1 mRNA is uniformly expressed whereas NAT2 in mostly expressed in
the liver and gut (Husain et al., 2007a, 2007b). Similar findings have been noted for certain heterocyclic amines, with the N-acetylation of a piperazine moiety recently reported (Rawal et al., 2008). UK-469,413 had good physicochemical properties and was slowly metabolized by CYP in rat and human liver microsomes. However, in rat in vivo the compound was highly cleared and subsequently shown to be rapidly acetylated in rat liver cytosol to an N-acetylpiperazine metabolite that was the major circulating metabolite in rat plasma in vivo. Using specific inhibitors, correlation analysis and expressed human NAT enzymes, the compound was shown to be a NAT2 substrate. The current study further adds to these observations that NAT isozymes have the capacity to N-acetylate substrates other than the classical arylamine and hydrazine motifs. The apparent \( K_m \) of 165 \( \mu \)M observed with human NAT2 is in the same range as reported for sulfamethazine, an arylamine commonly used as a positive control for NAT2 acetylation (\( K_m \) of 110 to 600 \( \mu \)M; Delomenie et al, 1997; Hickman et al., 1995; Meyer et al., 2014; Zang et al., 2007) and isoniazid, a well characterized hydrazine substrate (Hickman et al., 1995). Thus, although N-acetylation of an aliphatic amine is an uncommon reaction, NAT2 affinity for EPZ011652 was in the same range as that reported for classical arylamine and hydrazine substrates such as sulfamethazine and isoniazid. It should also be noted that there are other enzymes capable of N-acetylation of aliphatic amines, in particular Spermidine/Spermine-\( N^1 \)-Acetyltransferase and Thialysine Acetyltransferase, which typically N-acetylate various endogenous polyamine substrates (Hyvönen et al., 2013). These enzymes may also have a role in the N-acetylation of aliphatic and cyclic amine xenobiotics (Hyvönen et al., 2013; Bras et al., 2001).
The two NAT genes are 87% homologous and are located at 8p22, a chromosomal region commonly deleted in human cancers. The sequencing of NAT1 and NAT2 genes has revealed a number of allelic variants that affect activity of both genes in vivo, providing a genetic understanding for the long known functional polymorphism in NAT2 activity and, more recently, in NAT1 activity. This genetic variation modulates the acetylator status of individuals and therefore can impact their predisposition to toxicity and disease. Since the capacity of NAT2 varies in humans, manifest as rapid, intermediate and slow acetylator phenotypes (Hein, 2000), N-acetylation as the major elimination pathway can lead to variability in exposure in humans. This was the basis for the recent mechanistic work on the enzymology of etamicastat clearance, wherein high variability in etamicastat exposures in a rising, multiple dose study in healthy volunteers was associated with the polymorphic NAT2-mediated N-acetylation pathway (Nunes et al., 2010; Loureiro et al., 2013). The extent of systemic exposure to etamicastat in NAT2 slow acetylators was 1.5–6.7 times higher than in NAT2 fast acetylators, whereas exposure to etamicastat N-acetyl metabolite was 1.5–3.5 times higher in rapid NAT2 acetylators, thus etamicastat pharmacokinetics were markedly affected by the NAT2 phenotype (Nunes et al., 2010).

In addition to the clinical implications of this pathway, it has consequences for drug discovery and design. In our program, efforts were focused on perturbing this metabolic pathway and the N-acetylation reaction could be blocked by alkyl substituents on the terminal nitrogen, with the N-methyl analogs showing superior PK with retention of potency compared to EPZ011652 (Mitchell et al., 2015). However, this N-acetylation pathway does represent another challenge in
the balance of drug properties during lead optimization, as N-alkyl substituents are often substrates for CYP dealkylation reactions. In addition, the variable expression of NAT enzymes across species and the absence of active enzyme in dog confound the appropriate selection of toxicology species, interspecies scaling approaches and the prediction of human clearance. In some cases, the N-acetyl metabolite can be a substrate for N-deacetylases or chemical hydrolysis leading to a futile cycling of acetyl and desacetyl products, further complicating studies of drug disposition (Gao et al., 2006). In our studies there was no evidence for the back-conversion of EPZ015564 to EPZ011652 via deacetylation, suggesting the potential for futile acetylation is low.

In conclusion, we report the unusual N-acetylation of an aliphatic amine mediated predominantly by human NAT2, and observed both in vivo and in vitro in Sprague Dawley rats. This study adds to a growing body of evidence that aliphatic and cyclic amines can also be substrates of the NAT enzymes with implications for drug design, interspecies scaling and projection of human pharmacokinetics as well as the clinical implications of drug clearance by a polymorphic enzyme.
Authorship Contributions

Participated in research design: Rioux, Mitchell, Tiller, Ribich, Moyer, Copeland, Chesworth, Waters

Conducted experiments: Tiller, Plant, Shaw, Frost

Performed data analysis: Rioux, Mitchell, Tiller, Plant, Shaw, Frost, Waters

Wrote or contributed to the writing of the manuscript: Rioux, Copeland, Waters
REFERENCES


Gao W, Johnston JS, Miller DD, Dalton JT (2006) Interspecies differences in pharmacokinetics and metabolism of S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-


FIGURE LEGENDS

Figure 1. MS$^1$ and MS$^2$ spectra of EPZ011652 with assignment of diagnostic fragment ions. Note the position of the site of protonation is arbitrary. The ion $m/z$ 577.394 is the [2M+H]$^+$ molecular ion.

Figure 2. Representative extracted ion chromatogram for M1, M2, M3 and EPZ011652 in rat bile (0 to 4h) after a 10 mg/kg iv dose.

Figure 3. MS$^2$ spectrum of EPZ015564 (M1; +42 Da metabolite) from rat bile sample (0 to 4h) after a 10 mg/kg iv dose. Note the position of the site of protonation is arbitrary.

Figure 4. Proposed metabolic scheme for EPZ011652 as observed in rat bile (0 to 4h) after a 10 mg/kg iv dose.

Figure 5. Michaelis-Menten model fit for $K_m$ and $V_{max}$ determination of EPZ011652 N-acetylation mediated by (A) human recombinant NAT1 and (B) human recombinant NAT2 enzyme. Mean ± SD, n=3.
TABLES

Table 1. Pharmacokinetic parameters for EPZ011652 following iv bolus administration to Sprague-Dawley rats. Expressed as mean ± SD, n=3. nd: not determined

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 mg/kg iv naive</th>
<th>10 mg/kg iv bile-duct cannulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL (mL/min/kg)</td>
<td>96.2 ± 16.3</td>
<td>73.7 ± 13.6</td>
</tr>
<tr>
<td>CLrenal (mL/min/kg)</td>
<td>nd</td>
<td>1.51 ± 0.906</td>
</tr>
<tr>
<td>CLbile (mL/min/kg)</td>
<td>nd</td>
<td>0.050 ± 0.029</td>
</tr>
<tr>
<td>Vss (L/kg)</td>
<td>7.93 ± 1.44</td>
<td>8.10 ± 2.12</td>
</tr>
<tr>
<td>AUC0-8h (h*ng/mL)</td>
<td>174 ± 31.3</td>
<td>2295 ± 418</td>
</tr>
<tr>
<td>AUC0-inf (h*ng/mL)</td>
<td>177 ± 32.1</td>
<td>2313 ± 420</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>1.38 ± 0.191</td>
<td>1.83 ± 0.319</td>
</tr>
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</table>
Table 2. Summary of metabolites of EPZ011652 observed in rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mass shift (Da)</th>
<th>m/z</th>
<th>Product ions (m/z)</th>
<th>Rat Plasma (0-4h)</th>
<th>Rat Bile (0-4h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>0</td>
<td>289.201</td>
<td>215.120, 173.069, 146.059, 75.091</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>M1</td>
<td>+42</td>
<td>331.213</td>
<td>215.120, 173.069, 117.101</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>M2</td>
<td>+58</td>
<td>347.208</td>
<td>231.120, 173.069, 117.101</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>M3</td>
<td>+80</td>
<td>359.123</td>
<td>253.027, 173.069, 117.103</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>M4</td>
<td>+66</td>
<td>355.108</td>
<td>253.027, 173.069, 103.094</td>
<td>√</td>
<td></td>
</tr>
</tbody>
</table>

Bold ions represent diagnostic fragment ions specific to metabolites.
Table 3. Clearance of EPZ011652 and formation of EPZ015564 (M1) in pooled male Sprague-Dawley rat, Beagle dog and mixed gender human hepatocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>EPZ011652 CL&lt;sub&gt;int&lt;/sub&gt; (μL/min/million cells)</th>
<th>Scaled CL (mL/min/kg)</th>
<th>EPZ015564</th>
<th>15 min</th>
<th>45 min</th>
<th>90 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>79</td>
<td>59</td>
<td>√</td>
<td>√</td>
<td>BLQ</td>
<td>BLQ</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>4.5</td>
<td>15</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>&lt; 2.1</td>
<td>&lt; 4.9</td>
<td>BLQ</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean of duplicate incubations performed in presence of 3 μM EPZ011652 for up to 120 minutes. BLQ: below limit of quantitation (3.0 nM).
Figure 2

**M3**  
*m/z* 369.123

**M2**  
*m/z* 347.208

**M1**  
*m/z* 331.213

**EPZ011652**  
*m/z* 289.201
Figure 3
Figure 4

O-dealkylation and sulfation

EPZ011652

m/z 289.201

N-acetylation

EPZ015564

M1

m/z 331.213

Hydroxylation

M2

m/z 347.208

O-dealkylation, sulfation

N-demethylation

M3

m/z 369.123

N-demethylation

M4

m/z 355.108

Hydroxylation

N-demethylation

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Figure 5