Metabolism of a GPCR modulator including two major 1,2,4-oxadiazole ring-opened metabolites and a rearranged cysteine-piperazine adduct

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Metabolism involving oxadiazole or piperazine substructure

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ABBREVIATIONS:

ABT, 1-aminobenzotriazole; AO, aldehyde oxidase; BDC, bile-duct cannulated; ESI, electrospray ionization; EtOAc, ethyl acetate; FMO, flavin-containing monooxygenase; HLM, human liver microsomes; LC or HPLC, high performance liquid chromatography; MAD, multiple ascending dose; MeCN, acetonitrile; MeOH, methanol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; P450, cytochrome P450; RLM, rat liver microsomes; THF, tetrahydrofuran; TK, toxicokinetic; TFA, trifluoroacetic acid; XO, xanthine oxidase.
ABSTRACT

Metabolites of a G protein-coupled receptor (GPCR) modulator, containing 1,2,4-oxadiazole and piperazine substructures, were identified in vitro in human, rat and dog hepatocyte incubates and in vivo in rat plasma, bile, urine and feces using ^14C radiolabeled parent compound. Furthermore, exposure coverage for the major circulating metabolites in human at steady state by preclinical species used in drug safety assessment was determined using pooled plasma samples collected from a human multiple ascending dose study and the 3-month rat toxicokinetic study. Metabolites M1 and M2 formed by opening the 1,2,4-oxadiazole ring were observed as major metabolites, both in vitro and in vivo across species. The carboxylic acid metabolite M2 was presumably formed via reductive N-O bond cleavage of the oxadiazole ring and subsequent hydrolysis. However, the mechanism for the formation of the unusual N-cyano amide metabolite M1 remains uncertain. Neither M1 nor M2 has any on-target active as parent drug P. In rat bile, rearranged Cys-piperazine and Gly-Cys-piperazine adducts were observed as minor metabolites, involving the formation of a 5-membered heteroaromatic imidazole derivative from a 6-membered piperazine ring. This finding supports a previously reported mechanism in the literature regarding glutathione detoxification for piperazine bioactivation products.
Introduction

Compound P (Fig. 1) is a GPCR modulator in development intended for CNS indications. It has the molecular weight of 487 and contains a 3-(piperazin-1-ylmethyl)-1,2,4-oxadiazole substructure connecting to a substitute aromatic group (referred as to Ar- group hereafter) (Fig. 1). The Ar- group is not disclosed due to proprietary reason and would appear more lipophilic than the disclosed region. However, almost all of the biotransformation of compound P occurred in either the oxadiazole or the piperazine ring region.

The metabolism involving 1,2,4-oxadiazole ring opening has been of interest in the literature (Lan et al., 1973; Gyarmati et al., 1976; Dalvie et al., 2002; Yabuki et al., 1993; Allan et al., 2006; Bateman et al., 2006; Tsalta et al., 2011). The oxadiazole ring can undergo NADPH-dependent reductive cleavage of the N-O bond and then subsequent hydrolysis, to produce various ring opened products such as amides and carboxylic acids, etc. (Scheme 1a) (Lan et al., 1973; Gyarmati et al., 1976; Allan et al., 2006; Tsalta et al., 2011). It is worth noting that the reductive N-O bond cleavage for the oxadiazole could also occur chemically, e.g. by reaction with ferrous hydroxide (Lan et al., 1973). Reductive N-O cleavage is also common to the biotransformation of isoxazole, another 5-membered heteroaromatic ring with an N-O bond (Dalvie, 2002; Zhang D. et al., 2007). However, there would be no hydrolysis steps after reductive cleavage of N-O bond of the isoxazole, when compared with the oxadiazole (Scheme 1a). A ring-opened N-cyano amide metabolite produced from a 3,5-substituted 1,2,4-oxadiazole ring as illustrated in Scheme 1 b is a very unusual ring opening product, first reported as a major rat biliary metabolite of calcium channel antagonist SM-6586 (Yabuki et al., 1993). A similar metabolite was also found as a minor metabolite of a phosphodiesterase-4 inhibitor (L-454,560) in liver microsomes of several species (Bateman et al., 2006). In both reports, the unusual N-cyano amide metabolites were characterized by $^1$HNMR. Yabuki et al. verified their finding using a synthesized standard (Yabuki et al., 1993). The mechanism for the formation of N-cyano amide is still unknown. A review article once suggested that the
formation of an N-cyano amide from SM-6586 could be initiated by oxidation instead of reduction, e.g., hypothesized hydroxylation at the saturated carbon at 3-substitution of the oxadiazole derivative (Dalvie et al., 2002).

Two ring opened products of 1,2,4-oxadiazole, a carboxylic acid and a N-cyano amide metabolite, were observed as major metabolites of compound P in vitro and in vivo, in particular, in plasma samples from human and from preclinical species used in drug safety assessment. Understanding the level of metabolite exposure coverage during safety testing has been considered by regulatory authorities as one of the imperative drug metabolism issues in modern drug development (U.S. Food and Drug Administration, 2008; Baillie, 2009; Ma et al., 2010; Ramanathan et al., 2010; Nedderman et al., 2011). The Guidance on Safety Testing of Drug Metabolites published by the U.S. Food and Drug Administration (FDA) in February 2008 recommends that the safety assessment of major drug metabolites be completed before large-scale clinical trials are initiated. In the present study, the exposure coverage for major circulating human metabolites of compound P was determined using pooled residual plasma samples collected from a human multiple ascending dose (MAD) study vs. 3-month rat toxicokinetic (TK) studies.

Materials and Methods

Materials. Cryopreserved human hepatocytes were purchased from CellzDirect (Lot # Hu777 and Hu778, both Caucasian male). The cells underwent thawing, washing, and resuspension before incubation. The viability of the human hepatocytes post-thaw was 69% and 75% for Lot Hu777 and Hu778, respectively. Wistar Han rat hepatocytes were freshly prepared by Veterinary Medicine in AstraZeneca Wilmington. The rat hepatocyte viability was 95%. Cryopreserved male Beagle dog hepatocytes were purchased from CellzDirect. The cells underwent thawing, washing, and resuspension before incubation. The viabilities of dog hepatocytes post-thaw were determined as 95%.
Pooled human liver microsomes, cytosol and S9 fractions were supplied by BD Gentest (Woburn, MA). Human cDNA-expressed P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) and FMO enzymes (FMO1, FMO2, or FMO3) were made by BD Gentest, from insect cells infected with recombinant baculovirus containing a cDNA insert for individual human P450 and FMO enzymes. All human P450 enzymes were co-expressed with human P450 reductase and some of them were co-expressed with cytochrome b5.

Enzyme redox co-factors NADPH, NADP, NADH and NAD, aldehyde oxidase (AO) reduction cofactors 2-hydroxypyrimidine, 1-methylnicotinamide and benzaldehyde, xanthine oxidase (XO) reduction cofactor xanthine, P450 inhibitor 1-aminobenzotriazole (ABT), FMO inhibitor methimazole, and AO oxidation positive control substrate phthalazine were obtained from Sigma (St. Louis, MO). NADPH regenerating system was acquired from BD Biosciences (Woburn, MA).

**Synthesis of N-cyano amide 1** (Depicted in Scheme 2): A solution of 0.21 mmol of acid 2, 0.16 mL (1.2 mmol) of triethylamine and 102 mg (0.32 mmol) of TBTU in 1.5 mL of DMF was degassed with N2 for 1 min and 162 mg (3.85 mmol) of cyanamide was added. The solution was stirred for 1 h and was then added to 10 mL of 1 M HCl. The solution was extracted 3 times with 10 mL of CHCl3. The combined organics were concentrated to dryness and purified by RP-HPLC (30x100 mm Gemini NX C18, 18 to 35% MeCN-0.1 M aq NH4HCO3 (pH 10) over 10 min) to give 0.2 mmol of N-cyano amide 1 as a yellow solid. The compound was characterized using high resolution LC-MS and various NMR techniques.

**Synthesis of [14C]-P.** The route of radiolabel synthesis is depicted in Scheme 3.

Nitrile 4: A degassed solution of 1.17 mmol aryl bromide 3, 55 mg (52 mCi, 0.47 mmol) Zn(14CN)2, and 108 mg (0.093 mmol) Pd(PPh3)4 in 1.1 mL DMF was stirred and heated at 120 °C for 16 h. The solvent was removed at reduced pressure and the residue was purified by silica gel chromatography (20%
EtOAc:Hexanes). The combined product-containing fractions were concentrated to dryness to give 51 mCi at 87% radiochemical purity of nitrile 4.

Acid [14C]-2: A solution of 47 mCi (0.88 mmol) nitrile 4 in 1.9 mL MeOH was stirred as 177 mg (4.44 mmol) NaOH in 0.75 mL water was added slowly. The solution was heated at 75 °C for 16 h and after cooling to room temperature, the MeOH was removed. The crude reaction slurry was then diluted with 5 mL of 1 M HCl and was stirred for 3 h at room temperature. The solvent was removed by pipette and the residue was washed five times with 2 mL of 1 N HCl. The solid was crushed and rinsed four times with 5 mL water to give 30 mCi of [14C]-2 as a yellow solid (93% radiochemical purity). The combined water washes were acidified to give a further 11 mCi as a yellow precipitant (94% radiochemical purity).

Amide 5: A slurry of 30 mCi (0.56 mmol) acid [14C]-2 in 2 mL CH₂Cl₂ was stirred as 0.15 mL (1.68 mmol) oxalyl chloride and 5 µL DMF were added. After 2 h, the solvent was removed and the residue was taken up in 2 mL MeCN and was added to a slurry of 217 mg (0.84 mmol) (Z)-tert-butyl 4-(2-amino-2-(hydroxyimino)ethyl)piperazine-1-carboxylate and 170 mg K₂CO₃ in 4 mL MeCN. After stirring overnight, the reaction mixture was diluted with 20 mL of water and the solids were removed by filtration and were rinsed with 20 mL of water. They were then dissolved in 25 mL CHCl₃ and the resulting solution was extracted twice with 10 mL of water and then dried (Na₂SO₄). Filtration followed by concentration afforded 28 mCi of 5 at a radiochemical purity of 83%.

Oxadiazole 6: A solution of 23 mCi (0.42 mmol) piperazine 5 in 7 mL of THF was stirred under a vigorous flow of N₂ as 0.6 mL of a 1 M solution of NBu₄F in THF was added dropwise. After complete addition, the solution was stirred overnight. The solvent was then removed and the residue dissolved in 50 mL of EtOAc. The organic layer was washed with 20 mL saturated aq. NaCl and the organic layer was then dried over Na₂SO₄. After removing the drying agent, the solution was concentrated to dryness and the residue was dissolved in 4 mL of 80% MeCN-0.1% aq TFA and was applied to preparative HPLC (40-50% MeCN-0.1% TFA over 20 min; 21.2x250 mm Phenomenex Luna C18(2)). The combined...
product-containing fractions were extracted four times with EtOAc. The combined organic layers were dried (Na₂SO₄) and filtered, and the resulting solution was concentrated to dryness to give 17 mCi of 6 as a yellow oil (98% radiochemical purity).

[^14C]-P: A solution of 4.0 mCi (0.068 mmol) Boc amide 6 in 200 µL CH₂Cl₂ was stirred at room temperature as 100 µL TFA was added and the resulting solution was stirred for 2 h. The solution was concentrated to dryness and the residue taken up in 2.5 mL of 80% aq. MeCN. The resulting solution was applied to preparative HPLC (20 to 50% over 20 min MeCN-0.1% TFA on 21.2x250 mm Phenomenex Luna C18(2)) and the product containing fractions were combined and concentrated to removed the MeCN. The solution was then passed through a 100 mg Oasis HLB SepPak and was eluted with ethanol to give 2 mCi of[^14C]-P with a radiochemical purity of >99.5% and a specific activity of 58.5 mCi/mmol. ¹H NMR, LC-MS and co-elution of the product on two orthogonal HPLC columns confirm the identity of[^14C]-P.

Hepatocyte incubations.[^14C]-P was incubated at a nominal initial concentration of 10 µM in human, rat, and dog hepatocytes, respectively, at 2-million viable cells/mL. The specific activity of[^14C]-P was approximately 30 µCi/µmol, prepared by mixing labeled and unlabeled P at roughly equal molar ratio for automatic metabolite identification in LC-MS using the ^14C/^12C isotope ratio. The hepatocyte incubations were carried out in Williams E media containing 2 mM glutamine, supplemental ITS+ mixture (consisting of insulin, transferrin, and selenium) and 25 mM HEPES at 37°C for 2 hours. After 120 min incubation, two volume of MeCN was added to terminate metabolic reactions. The solvent in aliquots of supernatant of hepatocyte incubates was evaporated at 25°C using a centrifugal vacuum evaporator. The dry residues were reconstituted in 95%-5% water:MeCN containing 0.05% formic acid, then aliquots of reconstituted samples were injected in LC for metabolite profiling and identification. Aliquots of representative supernatant samples were also analyzed, to ensure there were no artifacts by the drying and reconstitution process.
Chemical inhibition of P450 activity in human and rat hepatocytes. Compound P (10 µM) was incubated in human or rat hepatocytes at 2 million cells/mL, in the presence or absence of 1 mM ABT at 37°C. ABT was pre-incubated with hepatocytes for 30 minutes to increase its inhibitory effect on the CYP enzymes. The reactions were started by adding the compound P and stopped by adding 2 volume of MeCN after 60 min incubation.

Human liver S9 fraction incubation. Compound P (10 µM) or M1 authentic standard (10 µM) was incubated with human liver S9 fraction (1 mg/mL protein) in triplicate at 37°C. Incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), 5 mM MgCl₂, and 1 mM of various cofactors (NADPH regenerating system, NADH, NAD, NADP, AO reduction cofactor 2-hydroxypyrimidine and 1-methylnicotinamide, and XO reduction cofactor xanthine). The incubations were terminated at 120 min by the addition of one volume of MeCN containing 0.1 µM hydroxytriazolam and 4-hydroxydiclofenac as internal standards for positive or negative electrospray ionization (ESI) LC-MS/MS quantitative analysis. Negative controls in the absence of cofactors were included in the experiment. Compound P was also incubated in the buffer without S9 to evaluate its chemical stability in the incubation conditions. Positive control compound phthalazine was incubated at 2 µM substrate concentration to verify the AO activity in liver fractions (Obach et al., 2004).

Anaerobic incubations with human S9 fraction. It has been reported that the AO catalyzed reduction reactions take place under anaerobic conditions (Sugihara et al., 1996). Thus, the incubation mixtures were bubbled with nitrogen gas for 20 seconds on the ice before addition of cofactors to initiate the reactions. Compound P (10 µM) was incubated with human liver S9 fraction (1 mg/mL protein) in triplicate at 37°C. Incubation mixtures also contained 0.1 M potassium phosphate buffer (pH 7.4), 5 mM MgCl₂, and 1 mM of various AO reduction cofactors: 2-hydroxypyrimidine, 1-methylnicotinamide, and benzaldehyde (Sugihara et al., 1996). The reactions were terminated after 30 min as above.
Human liver microsomal and cytosolic incubations. Compound P (10 μM) was incubated with pooled human liver microsomes (1 mg/mL protein) or cytosol (1 mg/mL protein) in triplicate at 37°C. Incubation mixtures also contained 0.1 M potassium phosphate (pH 7.4), 5 mM MgCl₂, and 1 mM redox cofactor (including NADPH, NADP, NADH, or NAD). Negative controls in the absence of cofactors were included in the experiment. Reactions were terminated at 60 min as above.

Incubations with human cDNA-expressed P450 or FMO enzymes. Compound P (1 μM) was incubated human P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) at 50 pmol/mL protein or FMO enzymes (FMO1, FMO2, and FMO3) at 250 μg/mL in triplicates at 37°C. Incubation mixtures also contained 0.1 M potassium phosphate buffer (pH 7.4), 5 mM MgCl₂, and NADPH regenerating system. Reactions were terminated after 120 min incubation as above. Metabolite formation in the presence of individual CYP was compared to the formation in vector-control Sf9 membranes, which was co expressed with cytochrome P450 reductase and cytochrome b5.

Rat plasma, bile and urine sample following single oral dose of [¹⁴C]-P. Rat mass balance study of [¹⁴C]-P was conducted by Charles River at Tranent, Edinburgh, UK. Residual plasma, bile, urine, and fecal samples, collected following a single 25 mg free base/kg oral dose of [¹⁴C]-P (incorporating 3.7MBq/kg) to male and female Han Wistar rats, were pooled to obtain a representative sample per sex per sample matrix in the present study. Plasma samples collected at different time points within 24h post dose were pooled in a commonly utilized “time-interval proportional” fashion (Hamilton et al., 1981; Hop et al., 1998), so that the pooled sample represented the mean concentration (or AUC) of the time period. Bile, urine or fecal homogenate samples were pooled in proportion to originally collected sample weights in the rat mass balance study, so that the pooled sample represented the cumulative excretion in each route. Aliquots of pooled plasma, bile or urine samples were mixed with two volumes of MeCN to precipitate proteins. The solvent in the supernatants was reduced under vacuum at 30°C using a
centrifugal vacuum evaporator. The residues were reconstituted in approximately 1/5 of original sample volumes. Aliquots of 5 g pooled fecal homogenates were extracted with 3 portions of 5 mL 90:10 v:v MeCN:water. The combined extracts were reduced to less than 1 mL under vacuum at 30°C, then reconstituted to approximately 1.5 mL by the addition of 80:20 water:MeOH.

**Human and rat plasma samples after multiple oral doses.** Residual human plasma samples collected over 24 hour on Day 13 following 12 consecutive once daily doses of 40 mg P were pooled to obtain 1 representative pooled sample for 7 dosed subjects and for 3 placebo subjects, separately. Residual rat plasma samples collected over 24 hours at the end of the 3 month rat TK study were pooled for the control, middle and high dose groups, respectively. Plasma samples collected at different time points within 24h post dose were pooled in a commonly utilized “time-interval proportional” fashion (Hamilton et al., 1981; Hop et al., 1998), so that the pooled sample represented the mean concentration (or AUC) of the time period. Aliquots of the plasma samples were mixed with 4 volumes of MeCN to precipitate proteins. The solvent in the supernatants was evaporated under vacuum at 30°C using a centrifugal vacuum evaporator. The dry residues of the plasma samples were reconstituted in 5:95 (v:v) MeCN:aqueous solvent (e.g., 26 mM ammonium formate pH 4.0 buffer) for metabolite profiling and identification using LC-UV-MS. Aliquots of 50 µL pooled plasma samples were mixed with 400 µL acenotreline containing 0.1 µM 4-hydroxydiclofenac as internal standard for LC-MS/MS quantitative analysis.

**LC-MS.** Table 1 summarizes LC-MS conditions for metabolite profiling or for quantitative analysis. Aliquots of 50 µL reconstituted samples (including hepatocyte incubates, rat in vivo samples, human MAD and the animal TK samples) were injected for metabolite profiling and identification. For the hepatocyte incubates of [14C]-P, data-dependent acquisition of collision induced dissociation (CID) product ions spectra were triggered by matching 12C/14C isotope pattern with that of the parent drug used in the incubations, e.g., ion intensity ratio of M:(M+2.003) = 1:0.95 (±0.07) as set in the present study.
High resolution MS scans were applied for all samples. The ion trap CID LC-MS/MS spectra were acquired for all metabolites detected in all samples. The MS\(^3\) CID spectra were acquired for metabolites in rat bile and urine.

Aliquots of 5 µL the supernatants of quenched incubation mixtures (including human liver microsomes, cytol and S9 fraction, human c-DNA expressed P450 and FMO enzymes) were injected for the LC-MS/MS quantitative analysis. Calibration solutions of authentic standards were prepared in corresponding blank matrices using authentic standard. In addition to the analysis of M1 and M2 in negative ion mode (Table 1), the parent drug P was also quantified separately using ion transitions 488.1→348.1 in positive ion mode. Quantitative analysis of M1 and M2 in pooled human MAD and TK plasma samples was performed similarly.

**Radioactivity Monitoring.** For \([^{14}C]\)-P metabolite profiling, approximately 90% of the LC eluate post UV cell was split into either a radio flow detector or a fraction collector for off-line counting. See Table 1 for summarized information. Radiochromatographic data was processed using Laura 3 software (LabLogic Systems, Sheffield, UK). Microsoft® Excel was used to construct the radiochromatograms from TopCount\(^\circledR\) acquired raw data for LC fractions of rat in vivo samples, however, the peak integration was done using Laura 3 software by inputting TopCount-acquired raw data into Laura 3 in LSC format.

**Results**

Fig. 2 shows radiochromatographic profiles of metabolites found in human, rat, and dog hepatocyte incubates after incubation with 10 µM \([^{14}C]\)-P. A total of 6 metabolites were observed in human or animal hepatocyte incubates, formed by either opening the 1,2,4-oxadiazole ring (M1, M2 and M3) or oxidation of the piperazine group (M4, M5 and M6) (Scheme 4). The two metabolites observed in human hepatocyte (M1 and M2) were also found in the hepatocyte incubates of rat and dog (Fig. 2). Phase II conjugate metabolites were not observed. The proposed metabolites in Scheme 4 were identified based
on the collective evidence from: (1) the same isotopic pattern of $^{14}$C-labeled vs. unlabeled [M+H]$^+$ ions as that of the parent drug used in incubation; (2) accurate mass of [M+H]$^+$ ions; (2) interpretation of LC-MS/MS product ion spectra with accurate mass of fragment ions; (3) the number of exchangeable hydrogen atoms in a metabolite molecule determined by H/D exchange. The experimental results of H/D exchange using D$_2$O mobile phase in LC-MS (Table 2) were useful in structural elucidation for mono-oxidation metabolites M4 and M5, and for the unusual ring opened metabolite M1. It was determined that the mono-oxidation of M4 occurred on a heteroatom and not on a carbon, since the number of exchangeable hydrogen in M4 remained unchanged from P (Table 2). However, it has not been determined whether M4 is an N-oxide or an N-hydroxyl metabolite (Scheme 4). The H/D exchange result for the 4-electron oxidation product M5 suggested 2 possibilities for its structure: no exchangeable proton (even after H/D exchange in D$_2$O solvent for 24 h prior to the LC-MS analysis), or no need for an ionizing proton (e.g., with an iminium carrying a fixed positive charge). Na$^+$ adduct ions of both C-14 labeled and unlabeled M5 were observed in LC-MS and verified with accurate masses, suggesting that M5 might not have a fixed positive charge. Therefore, the M5 structure is proposed to contain an imine and an N-oxide (Scheme 4). H/D experimental results for metabolite M1 were consistent with the N-cyano amide having an exchangeable -NH hydrogen, but does not support an alternative chemical structure like an intact 1,2,4-oxadiazole ring with a hydrogen at the 3-position as this H would not be exchangeable. Eventually, the identity of the N-cyano amide M1 and the carboxylic acid M2 were confirmed by LC-MS/MS comparison to authentic standards.

Although the unusual N-cyano amide metabolite M1 of compound P was observed as the major metabolite in human hepatocytes (Fig. 2a), only trace amounts (0.1%) of M1 were found in either human liver microsomes or cytosol when incubated in the presence of various co-factors. NADPH-dependent formation of M1 was observed in human liver S9 fraction with approximately 3.5% turnover from 10 μM substrate. Depletion of the parent drug was noticed in human liver microsomal incubate of P in the presence of NADPH, however only oxidations at the piperazine group were detected (e.g. M4 and a
piperazine-hydroxylation metabolite) by LC-MS and MS/MS metabolite identification. Based on the absence of M1 from human liver microsomal incubates, it was not surprising that M1 was not produced at a level above noise or negative control in expressed human P450 enzymes or FMO enzymes (including CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A5, FMO1, FMO3 and FMO5). FMO1 and FMO3 metabolized P, but only produced mono-oxidation metabolites at the piperazine group as detected by LC-MS and MS/MS metabolite identification of the FMO incubates. The carboxylic acid metabolite M2 was also monitored in the in vitro incubates of human liver microsomes, cytosol and S9 fractions, cDNA-expressed P450 and FMO enzymes. M2 was not detected in any of these in vitro incubations except of hepatocytes. In addition, incubation of synthetic M1 standard with human S9 fraction did not produce detectable M2.

The P450 enzyme inhibitor ABT or the FMO inhibitor methimazole did not abolish the NADPH-dependent formation of M1 in human liver S9 fraction. Use of ABT in human or rat hepatocyte incubation also did not inhibit the formation of M1 or M2 in the hepatocytes, where the turnover of M1 in the absence of the inhibitor was much higher than in the human S9 fraction. In the same experiment, ABT inhibited the metabolism of the CYP3A4 substrate midazolam, where more than 90% the formation of 1’-hydroxyl-midazolam metabolite was inhibited by ABT in both human and rat hepatocytes.

The contributions of AO to the metabolism of P in human liver S9 fraction were not substantiated under either aerobic or anaerobic conditions. M1 was not observed in these incubations, e.g., in the presence of various electron donors of AO, including 2-hydroxypyrimidine, 1-methylnicotinamide, and benzaldehyde (Sugihara et al., 1996).

[14C]-P was extensively metabolized in rat in vivo. The plasma metabolite profile of [14C]-P for the male rat is provided in Fig. 3, acquired with pooled 0-24 plasma samples following an oral dose of 25 mg free base/kg [14C]-P. Unchanged parent drug accounted for 7.7% and 18.7% of [14C]-P derived total radioactivity in pooled 0-24 h plasma samples of male and female rats, respectively. Three metabolites
(M1, M2 and M3), all of which resulted from opening the 1,2,4-oxadiazole ring, were detected in the plasma of both the male and the female. The pooling method adopted in this study gave a pooled sample in which the concentration was directly proportional to AUC (Hamilton et al., 1981; Hop et al., 1998). Therefore, the relative amounts of $^{14}$C-labeled metabolites shown in Fig 3 represent the exposure of 0-24 h post dose. M1, M2, and M3 represented 11.6%, 74.8%, and 1.7% of [14C]-P derived radioactivity in the 0-24 h male rat plasma (Figure 3). Similarly, M1, M2 and M3 accounted for 16.0%, 62.5%, and 1.2% of [14C]-P derived radioactivity in the female rat plasma (chromatogram not shown). In summary, qualitative similarity in the metabolism of [14C]-P was observed between rat plasma in vivo and hepatocytes in vitro (Fig. 3 vs. Fig. 2b).

Following oral administration of 25 mg/kg [14C]-P to bile-duct cannulated (BDC) rats, urinary excretion accounted for mean recoveries of 6.1% and 13% of administered radioactivity in male and female rats, respectively. Excretion of the administered radioactivity in feces was 56% in both male and female BDC rats. Biliary elimination accounted for 34% and 24% of the dosed radioactivity, in males and females, respectively.

The vast majority of administered [14C]-P radioactivity was excreted in the form of metabolites. The total of unchanged [14C]-P in the bile, urine and feces of BDC rats represented only approximately 1% of dosed radioactivity. Fig. 4 shows radiochromatographic metabolite profiles of [14C]-P in male rat bile, urine, and feces, acquired with pooled 0-48h samples collected following the oral dose to male BDC rats. Table 3 provides results for excreted metabolites in the percentage of dosed radioactivity, through the route of bile, urine and feces during 0-48 h post the oral dose of [14C]-P to male or female BDC rats, respectively. The values in Table 3 were calculated based on the percentage of a metabolite peak to the total radioactivity peak area in radiochromatograms, measured in the present study, and radioactivity recovery from each elimination route as described above.
The N-cyano amide M1 metabolite accounted for most of the total radioactivity excreted in bile, urine and feces of BDC rats, i.e. approximately 66% and 69% of the dose for males and females, respectively (Table 3). Carboxylic acid M2 was the next most abundant form of [14C]-P derived radioactivity excreted in bile, urine and feces (approximately 6% to 7% of the dose for males and females, respectively; Table 3). A number of minor metabolites of [14C]-P were also observed in rat bile and urine (Fig. 4a & b, Scheme 5, and Table 3). These included glucuronides M17a, b & c (of the same aromatic carboxylic acid metabolite M2), rearranged Cys-piperazine adduct M13, oxadiazole ring-opened product M3, piperazine ring-opened products (e.g., M10 and M11), and further oxidation products of the M1 and M2 major metabolites, etc (Scheme 5). In rat feces, the vast majority of [14C]-P derived radioactivity was found to be the N-cyano amide M1 (Fig 4c, Table 3), whereas unchanged P was not detected in the radiochromatogram (Fig 4c).

Fig. 5 shows CID product ion spectrum of [M+H]+ ions of parent drug P. Most of the fragmentations of P occurred in the 1,2,4-oxadiazole and the piperazine substructure regions (Fig. 5). Fragment ion m/z 348 produced by opening the 1,2,4-oxadiazole ring (Fig. 5) was also observed for all those metabolites with intact 5-Ar-oxadiazole substructure (spectra not shown here). Fragment ion m/z 406 was not assigned because it would be difficult to rationalize (Fig. 5). As indicated by accurate mass measurement, the m/z 406 ion had 2 additional hydrogen atoms than the m/z 404 ion. The m/z 406 ion gave further fragment ions m/z 365, 347 and 348 in MS³. The fragment ion m/z 460 behaved almost like a reductive oxadizole ring-opened product of the m/z 404 ion, possibly formed via rearrangement involving the piperazine in the CID of compound P.

Fig. 6 compares the product ion spectra of [M+H]+ ions of the N-cyano amide metabolite M1 and its authentic standard. The two spectra displayed perfect fingerprint like matches (Fig. 6a vs. b). In addition, identical retention times between the metabolite and authentic standard were observed under 2 different LC methods described in the Methods. The fragmentation of M1 differs dramatically from that of parent
compound P (Fig. 6 vs Fig 5), instead, it appeared somewhat similar to the carboxylic acid M2 (spectra not shown here).

Fig. 7 provides the product ion spectrum and spectral interpretation for M13, a rearranged Cys-piperazine adduct of compound P. The accurate mass measurements in LC-MS for M13 and rearranged Gly-Cys-piperazine adduct M14 were within 1 ppm error of the theoretical exact masses, i.e. the same accuracy as for parent P and other metabolites (data not shown here). The structures for M13 and M14 were proposed based on similar adducts of a piperazine-containing compound MB243 previously characterized using $^1$HNMR by Doss et al. (2005). The metabolism involved the ring contraction from a saturated 6-membered piperazine ring to an unsaturated 5-membered ring derivative (Doss et al., 2005). However, M13 and M14 contain a heteroaromatic imidazole (Scheme 5), instead of an imidazoline in those adducts of MB243 (Doss et al. 2005). The product ion spectrum of M13 shows some similar fragmentation patterns to what was reported for the rearranged Cys adduct of MB243 (Doss et al., 2005), e.g., the characteristic neutral loss of 131, producing fragment ion m/z 470 from [M+H]$^+$ ion of M13 (Fig. 7). In addition, fragmentation by neutral loss of CO$_2$ and by the loss of a H$_2$O plus a CO, forming respective fragment ions m/z 557 and 555, indicated a carboxylic group in M13 (Fig. 7). As seen in Fig 7, most of abundant fragment ions of M13 corroborate the proposed structure.

M17a, b & c in rat bile (Fig. 4a) were identified as migrated acyl glucuronides of the aromatic carboxylic acid M2. Metabolites M17a, b & c had identical accurate masses and produced the same fragment ions by CID. The intensity ratios of fragment ions of M17a, b & c were however distinct from one another (spectra not shown here), which is typical for isomeric compounds.

M12 and M18, two minor metabolites found in rat bile, were proposed to have formamide structures (Scheme 5). As an example of experimental evidence supporting the proposed structures, the further fragmentation in LC-MS$^3$ for M12, following CO neutral loss from the [M+H]$^+$ ions of M12, gave a MS$^3$ spectrum that was identical to the M10 MS/MS spectrum (spectra not shown here). These formamide
metabolites were shown not to be artifacts of chemical transformation of corresponding secondary amines by formic acid in the LC mobile phase, since M12 and M18 were also detected in LC-MS experiments using acetic acid or ammonium acetate buffer as mobile phase.

Fig. 8a overlays UV chromatograms of pooled human plasma samples collected over 24 h on Day 13 after 12 consecutive once daily doses of 40 mg P vs. placebo. The parent drug P reached the steady state before Day 13 (pharmacokinetic data not shown here). Fig. 8b provides a LC-MS accurate mass extraction ion chromatogram showing M1, M2, and M3 metabolite peaks in the plasma sample. It is evident from overlaid UV chromatogram (Fig. 8a) that M1 and M2 are major metabolites in the human plasma sample with exposure much higher than the parent P, whereas, M3 is a minor metabolite. The identities of major human metabolites N-cyano amide M1 and carboxylic acid M2 were confirmed in LC-MS/MS with their authentic standards, under both LC methods described in the Methods. The 0-24 h mean concentrations of M1 and M2 as determined in the pooled Day 13 plasma sample by LC-MS/MS quantitative analysis were 4.6 and 7.9 μM, respectively, whereas the mean concentration of unchanged P was only 0.24 μM. Hence, the exposure of M1 and M2 in human was 19 and 33 times higher than P, respectively, on a molar basis. It worth noting that neither M1 nor M2 showed any on-target activity as parent drug P, as determined by an in vitro bioassay.

All 3 human plasma metabolites (M1, M2, and M3) were already detected in rat plasma of the radiolabeled study (Figure 3). Exposure coverage for the major human metabolite M1 and M2 in rat oral TK study was determined, first by qualitative metabolite analysis of both pooled human MAD plasma and 3 month TK animal plasma samples using LC-UV-MS (data not shown), then confirmed quantitatively by LC-MS/MS bioanalysis of the human and rat plasma samples. The pooling method adopted in the present study gave a pooled sample in which the concentration of any analyte was directly proportional to its AUC, more specifically, the mean concentration across the pooled time range (Hamilton et al., 1981; Hop et al., 1998). The bioanalytical results of M1 and M2 in pooled plasma samples from the human
plasma and the rat TK plasma samples are provided in Table 4. The rat TK study overall had greater exposure of M1 and M2 than human, rat middle dose of 15 mg/kg that was the no observable adverse effect level gave greater exposure of M2 than human (Table 4). The results in Table 4 indicate the toxicological coverage of the major human metabolites M1 and M2 in at least one preclinical species.

Discussion

The unusual N-cyano amide metabolite (M1) would have been missed without the use of radiolabeled material, such as in the drug discovery process. The existence of the strongly retained M1 was discovered when low radioactivity recovery from the LC column was observed in early [14C]-P metabolite profiling of in vitro incubates. It was intriguing how simple solution-based chemistry affected the LC elution of M1. Currently, formic acid or TFA is often the top choice for mobile phase modifier in many LC-MS laboratories, due to: (i) the generally greater ionization efficiency in positive ion electrospray at low pH; and (ii) the unstable nature of some organic buffers due to microorganisms contamination, or simply inconvenience of preparing organic buffers. However, when formic acid or TFA was used in mobile phase with a C18 column, M1 was so strongly retained that it did not elute from the column, without being washed with 100% organic mobile phase at the end of LC gradient. The LC column used for the present study is still a C18 type column but with polar end-cap groups that may allow the elution of M1. The elution orders of M1 and M2 vs. P were noticeably affected by the pH of mobile phases. Both M1 and M2 eluted after P with 0.05% formic acid aqueous mobile phase (pH <2; Fig. 2 and Fig. 3). But with pH 4.0 formate buffer, M1 eluted before P (Fig. 4). When pH 5.0 acetate buffer was used, both M1 and M2 eluted ahead of P (chromatograms not shown). Compound P should be positively charged at piperazine at all these pH conditions. Observations on earlier elution of M1 at higher pH are consistent with the N-cyano amide as an organic acid, as the –NH can be deprotonated to give a negative charge that is stabilized by both the carbonyl and the cyano groups. However in low pH mobile phase such as 0.05%
Formic acid, the N-cyano amide group is neutral, thus along with the lipophilic Ar-group, the M1 molecule would be highly lipophilic and strongly retained on the HPLC column.

Possibly both cytosolic and membrane-bound enzymes contributed to M1 formation, because M1 was produced in human hepatocytes or in human liver S9 fraction in the presence of NADPH, but was not generated by either human liver microsomes or human liver cytosol alone. ABT did not inhibit the formation of M1 in hepatocytes and the S9 fraction, suggesting that cytochrome P450 enzymes are probably not involved, or at least not to a significant extent. M2 is believed to be formed via reductive N-O cleavage and subsequent hydrolysis (Lan et al., 1973; Allan et al., 2006). The absence of M2 from human liver S9 fraction incubates of the M1 authentic standard suggests that the carboxylic acid metabolite M2 may not be formed by hydrolysis of the N-cyano amide metabolite M1.

Doss et al. discovered unusual rearranged Cys-piperazine and Gly-Cys-piperazine adducts, after noticing that high levels of covalent binding of a piperazine-containing melanocortin subtype-4 receptor agonist MB243 in rat and human liver microsomes were attenuated by GSH. (Doss et al., 2005 and 2006). Based on their NMR structural characterization of isolated metabolites from rat bile, Doss et al. proposed a mechanism for the bioactivation of the piperazine ring that led to a ring contraction to afford an imidazoline derivative. Their mechanism has been adapted in Scheme 6 to rationalize the formation of the Cys adduct M13 and the Gly-Cys adduct M14 seen in rat bile. However, an additional dehydrogenation step occurred in the metabolism of compound P, which led to the aromatization and formation of an imidazole ring (Scheme 6), instead of an imidazoline as for MB243. This difference could possibly be due to steric hindrance of a bulky substituent group at a piperazine carbon ring in MB243 (Doss et al., 2005 and 2006) which might have prevented the dehydrogenation step from occurring. The absence of di-hydro intermediate from metabolic aromatization like the formation of M13 and M14 is not new in the literature, since two articles have previously described the absence of di-hydro intermediate from the formation of N-alkyl-quinolinium metabolites (Shaffer et al., 2001; Gu et al., 2006).
In summary, it appears that metabolic pathway leading to rearranged Cys-piperazine adduct originally discovered by Doss et al. is a common GSH detoxification mechanism for piperazine bioactivation products.

Two formamide metabolites M12 and M18 were observed as minor metabolites in rat bile (Scheme 4). We proved that these formamides were not artifacts in the LC-MS metabolite profiling process. However, it is unclear how formamide metabolites M12 and M18 were formed in the rat. Most recently, Shaffer et al. have also reported the detection of a formamide metabolite of a secondary amine compound in rat plasma and brain (Shaffer et al. 2009 and 2010).

Metabolite analysis to rationalize the safety of drug metabolites according to FDA’s recent guidance can involve 2 analytical issues: (i) detection of metabolites in complex biological matrixes such as human plasma at steady state without reliance on radiolabel, since the most readily available human plasma samples at steady state would be derived from a multiple dose study with unlabeled drug; (ii) to determine which circulating metabolites in human are significant enough for concern, and to quantitatively or semi-quantitatively compare plasma metabolites after multiple doses to human vs. preclinical species in the absence of radiolabel or often synthetic standards. Recent computer algorithms for background subtraction from LC/high-resolution MS data have been demonstrated to be effective approaches to uncover drug metabolites in complex biological matrixes (Zhang H. et al., 2008 and 2008; Zhu et al., 2009). Unlike radioactivity monitoring of metabolites where the physical property of radioactive nucleus (e.g., ¹⁴C) is not affected by biotransformation at molecular level, the LC-MS signal-response efficiency of metabolites varies, sometimes dramatically such as with the acidic metabolites M1 and M2 of the basic parent compound P in the present study. The present study has provided an example for that under certain circumstances, traditional UV detection can help to identify the major plasma metabolites, when parent drug and metabolites have reasonably strong UV absorption and the concentrations in plasma are not too low (Fig 8). Due to availability of authentic standards of both M1 and M2, quantitative
comparison for the plasma metabolites was not a big analytical issue after they were identified as the major metabolites in the human plasma. If the standards had not been available, it would still have been possible to obtain quantitative estimates for exposure coverage, e.g., by the use of radiolabeled M1 and M2 metabolites in rat samples as reference to correlate MS signal responses of unlabeled metabolites in the pooled human MAD and animal TK plasma samples. However, that would be out of the scope of this article.
Acknowledgments. We thank Dr. Magnus Halldin of AstraZeneca R&D Södertälje Sweden for insightful discussions and helpful information. The rat mass balance study was conducted by Charles River at Tranent, Edinburgh, UK.
Authorship Contributions

*Participated in research design:* Gu, Elmore, Zhou, and Grimm

*Conducted experiments:* Gu, Elmore, Lin, Luzietti, and Dorff

*Contributed new reagents or analytical tools:* Elmore and Dorff

*Performed data analysis:* Gu, Lin, and Luzietti

*Wrote or contributed to the writing of the manuscript:* Gu, Elmore, Zhou, and Grimm
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DMD #44636


Lan SJ, Weliky I and Schreiber EC (1973) *Xenobiotica* **3**:97-102


Legends for Schemes

Scheme 1. Two types of ring opening of 1,2,4-oxadiazole: reductive cleavage of N-O bond (a) and unknown cleavage leading to the formation of unusual N-cyano amide metabolite (b)

Scheme 2. Synthesis of the authentic standard of metabolite M1

Scheme 3. Synthesis of [14C]-P

Scheme 4. In vitro metabolites of [14C]-P in human, rat and dog hepatocytes

Scheme 5. Proposed biotransformation scheme of [14C]-P in the rat

Scheme 6. Plausible mechanism for the formation of rearranged Cys-piperazine adduct, adapted from the mechanism originally proposed by Doss et al. (2005) but with an additional dehydrogenation that led to the aromatization forming an imidazole in the present case

Legends for figures

Fig 1. Compound P containing 1,2,4-oxadiazole and piperazine substrutures

Fig 2. In vitro metabolite profiles of [14C]-P in human (a), rat (b) and dog (c) hepatocyte incubates

Fig 3. Male rat plasma metabolite profile of [14C]-P in pooled 0-24 h plasma sample following a 25 mg free base/kg oral dose

Fig 4. Male bile-duct cannulated (BDC) rat metabolite profile of [14C]-P in pooled 0-48 h bile (a), urine (b), and feces (c) samples following a 25 mg free base/kg oral dose

Fig 5. LC-MS/MS product ion spectrum acquired for [M+H]+ ions of the parent drug P

Fig 6. LC-MS/MS product ion spectra acquired for [M+H]+ ions of metabolite M1 (a) vs. the authentic standard compound 1 (b)

Fig 7. LC-MS/MS product ion spectrum acquired for [M+H]+ ions of rearranged Cys-piperazine adduct metabolite M13

Fig 8. UV (a) and LC-MS accurate-mass extracted ion chromatogram (b) showing metabolites in pooled 0-24 h human plasma collected following 12 consecutive once daily doses of 40 mg P.
<table>
<thead>
<tr>
<th>Type of Experiment</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>LC Instrument</th>
<th>Mass Spectrometer</th>
<th>Radioactivity detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte Metabolites of $[^{14}C]$-P</td>
<td>Synergi 4μ Hydro-RP 80A 2.0×150 mm (Phenomenex®, Torrance CA).</td>
<td>A: Water with 0.05% formic acid; B: MeCN with 0.05% formic acid. Linear gradients of 5%B to 30%B over 10 min, to 55%B over next 25 min, to 95%B over another 5 min and held for 5 more min. Flow rate: 1 mL/min.</td>
<td>Agilent 1100 Series with photodiode array (PDA) UV detector (Agilent Technologies, Santa Clara, CA)</td>
<td>Approx. 10% LC eluate to LTQ Orbitrap XL hybrid FT mass spectrometer (Thermo Scientific, San Jose, CA). Positive ESI.</td>
<td>β-RAM model 3 with a 500 μL liquid scintillation cell&lt;sup&gt;a&lt;/sup&gt; (IN/US Systems, Brandon, FL)</td>
</tr>
<tr>
<td>Rat Plasma Metabolites of $[^{14}C]$-P</td>
<td>The same as above</td>
<td>The same as above</td>
<td>Acquity&lt;sup&gt;TM&lt;/sup&gt; UPLC system with a PDA UV detector (Waters, Milford, MA)</td>
<td>The same as above</td>
<td>The same as above</td>
</tr>
<tr>
<td>Rat Bile, Urine and Feces Metabolites of $[^{14}C]$-P</td>
<td>The same as above</td>
<td>A: 26 mM ammonium formate pH 4.0 buffer; B: MeCN. Linear gradients of 5%B to 25%B over 10 min, to 40%B over next 50 min, to 95%B over another 5 min and held for 5 more min. Flow rate: 1 mL/min.</td>
<td>The same as above</td>
<td>The same as above. Both positive and negative ESI.</td>
<td>TopCount NXT&lt;sup&gt;TM&lt;/sup&gt; microplate scintillation counter following fraction collection&lt;sup&gt;b&lt;/sup&gt; (Perkin-Elmer, Waltham, MA)</td>
</tr>
<tr>
<td>Human MAD and Rat/Dog TK plasma Metabolites</td>
<td>The same as above</td>
<td>Both LC methods as above</td>
<td>The same as above. Wavelength 272 nm monitored and PDA spectra acquired.</td>
<td>The same as above. Positive ESI</td>
<td>N.A.</td>
</tr>
<tr>
<td>Quantitative Analysis of M1 and M2; in various &lt;i&gt;in vitro&lt;/i&gt; incubates, or in pooled MAD and TK plasma</td>
<td>Acquity&lt;sup&gt;TM&lt;/sup&gt; UPLC BEH C18 2.1×50 mm (Waters).</td>
<td>Consisting of 10 mM ammonium acetate and MeCN at the flow rate of 0.450 mL/min. A short gradient with 2 min total run time.</td>
<td>Acquity&lt;sup&gt;TM&lt;/sup&gt; UPLC system</td>
<td>API 4000 Qtrap (AB Sciex). Negative ion transitions m/z 388.1→184.1 &amp; m/z 364.1→320.1 for M1 &amp; M2, respectively.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A. Not applicable

<sup>a</sup> Using In-Flow<sup>TM</sup> 2:1 liquid scintillant (IN/US Systems)

<sup>b</sup> Into yttrium silicate scintillator-coated LumaPlate-96 plates (Perkin-Elmer)
Table 2. Electrospray ionization-generated molecular ions of compound P and metabolites in LC-MS before and after active H/D exchange

<table>
<thead>
<tr>
<th>[M + H/D]^+ (m/z)</th>
<th>Unlabeled</th>
<th>^14C-labeled</th>
<th>Number of exchangeable H</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>488 → 490</td>
<td>490 → 492</td>
<td>1</td>
</tr>
<tr>
<td>M1</td>
<td>390 → 392</td>
<td>392 → 394</td>
<td>1</td>
</tr>
<tr>
<td>M2</td>
<td>366 → 368</td>
<td>368 → 370</td>
<td>1</td>
</tr>
<tr>
<td>M3</td>
<td>365 → 368</td>
<td>367 → 370</td>
<td>2</td>
</tr>
<tr>
<td>M4</td>
<td>504 → 506</td>
<td>506 → 508</td>
<td>1</td>
</tr>
<tr>
<td>M5</td>
<td>502 → 503</td>
<td>504 → 505</td>
<td>0</td>
</tr>
<tr>
<td>M6</td>
<td>462 → 466</td>
<td>464 → 468</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3. Excretion of metabolites (in the percentage of dosed radioactivity) through the route of bile, urine and feces during 0-48 h post a 25 mg free base/kg oral dose of [14C]-P to male and female bile duct cannulated rats.

<table>
<thead>
<tr>
<th>MW</th>
<th>Male Bile</th>
<th>Urine</th>
<th>Feces</th>
<th>total</th>
<th>Female Bile</th>
<th>Urine</th>
<th>Feces</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>co w/ M3</td>
<td>&lt;0.1</td>
<td>Trace</td>
<td>co w/ M3</td>
<td>1.0</td>
<td>0.2</td>
<td>Trace</td>
<td>1.2</td>
</tr>
<tr>
<td>M1</td>
<td>389</td>
<td>9.1</td>
<td>3.5</td>
<td>53.1</td>
<td>65.7</td>
<td>8.6</td>
<td>9.3</td>
<td>51.0</td>
</tr>
<tr>
<td>M2</td>
<td>365</td>
<td>5.7</td>
<td>1.0</td>
<td>6.7</td>
<td>4.8</td>
<td>0.3</td>
<td>1.1</td>
<td>6.2</td>
</tr>
<tr>
<td>M3</td>
<td>364</td>
<td>&lt;1.3</td>
<td>co w/ P</td>
<td>0.3</td>
<td>&lt;1.6</td>
<td>0.1</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>M6</td>
<td>461</td>
<td>co w/ M18</td>
<td>co w/ M18</td>
<td>co w/ M18</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>M7a</td>
<td>381</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
<td></td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>M7b</td>
<td>381</td>
<td>0.8</td>
<td>0.2</td>
<td></td>
<td>1.0</td>
<td>0.4</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>M8</td>
<td>396</td>
<td>0.4</td>
<td></td>
<td>0.4</td>
<td></td>
<td>0.5</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>M9</td>
<td>515</td>
<td>0.7</td>
<td></td>
<td>0.7</td>
<td>0.4</td>
<td>0.1</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>M10</td>
<td>476</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
<td>0.8</td>
<td></td>
<td></td>
<td>0.8</td>
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<tr>
<td>M11</td>
<td>418</td>
<td>1.2</td>
<td></td>
<td>1.2</td>
<td>0.3</td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>M12</td>
<td>504</td>
<td>0.3</td>
<td></td>
<td>0.3</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>M13</td>
<td>600</td>
<td>1.0</td>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>M14</td>
<td>657</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>M15</td>
<td>501</td>
<td></td>
<td>co w/ M18</td>
<td></td>
<td>co w/ M18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M16a</td>
<td>405</td>
<td>0.8</td>
<td>0.2</td>
<td>1.0</td>
<td>0.7</td>
<td>0.3</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>M16b</td>
<td>405</td>
<td>0.6</td>
<td>0.1</td>
<td>0.7</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>M17a,b,c</td>
<td>541</td>
<td>3.7</td>
<td>3.7</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M18</td>
<td>418</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.6</td>
<td>&lt;0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M19</td>
<td>535</td>
<td>0.3</td>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
</tbody>
</table>

Trace only detected in LC-MS, but not in radiochromatogram
co w/ co-eluted with

The percentages of co-eluted M2 and M9 were determined by using results acquired with another LC method that separated M2 and M9.
Table 4. Analysis of major metabolites M1 and M2 in human and rat plasma samples collected after multiple oral doses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Original study</th>
<th>Dose level rank in original study</th>
<th>Once daily dose level</th>
<th>Collection Day</th>
<th>Number of time points for &quot;AUC proportional&quot; sample pooling</th>
<th>Number of subjects pooled</th>
<th>Measured concentration in pooled plasma sample (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>MAD</td>
<td>Intermediate</td>
<td>40 mg</td>
<td>13</td>
<td>12</td>
<td>7</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td></td>
<td></td>
<td>13</td>
<td>12</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>Rat</td>
<td>TK</td>
<td>Middle</td>
<td>15 mg/kg</td>
<td>92</td>
<td>6</td>
<td>3m + 3f</td>
<td>1.24</td>
</tr>
<tr>
<td>Rat (m)</td>
<td>TK</td>
<td>High</td>
<td>75 mg/kg</td>
<td>92</td>
<td>6</td>
<td>3</td>
<td>9.05</td>
</tr>
<tr>
<td>Rat (f)</td>
<td>TK</td>
<td>High</td>
<td>50 mg/kg</td>
<td>92</td>
<td>6</td>
<td>3</td>
<td>3.32</td>
</tr>
</tbody>
</table>

All doses are in free base equivalent.
N/A: not applicable
m: male
f: female
Scheme 1

(a) Oxadiazole ring structure

Reductive cleavage

\[ \text{H}_2\text{O} \quad \text{H}_2\text{O} \quad \text{H}_2\text{O} \]

1. \[ \text{CO}_2\text{R}_1 + \text{HN}_2\text{NH}_2 \]

2. \[ \text{CO}_2\text{R}_3 + \text{HN}_2\text{CO}_2\text{R}_3 \]

Compared with

(b) Isoxazole ring structure

Reductive cleavage

\[ \text{SM-6586} \]

\[ \text{L-454,560} \]
\[
\text{Scheme 2}
\]
Scheme 3
HHep, RHep and DHeP represent human, rat and dog hepatocytes, respectively.
Note: * indicates the location of $^{14}$C label
Scheme 6
Ar- = substituted aromatic ring

MW (of unlabeled) = 487

* denotes $^{14}$C labeled carbon

Figure 1
Figure 4
Figure 5

Note: ionizing proton not shown
Figure 6
Figure 7

Note: ionizing proton not shown
hydrogen rearrangement across cleaved bond not shown
X — background chromatographic peaks also present for placebo sample