Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

Jian Yu, James J. Folmer, Valerie Hoesch, James Doherty, James B. Campbell, Doug Burdette

Department of Discovery Drug Metabolism and Pharmacokinetics (J.Y., D.B.), Medicinal Chemistry (J.J.F., V.H), Neuroscience (J.D.), and Lead Generation (J.B.C)

AstraZeneca Pharmaceuticals LP, 1800 Concord Pike, Wilmington, DE 19850-5437
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b) Address all correspondence to:

Jian Yu, Ph.D.

Department of Discovery DMPK, AstraZeneca Pharmaceuticals LP

L-260C, PO Box 15437

1800 Concord Pike, Wilmington, DE 19850-5437 USA

Tel: (302)-885-0494, Fax: (302)-995-2201, E-mail: jian.yu@astrazeneca.com

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d) Abbreviations:

UPLC: ultra-performance liquid chromatography

LC/MS/MS: liquid chromatography-tandem mass spectrometry

CID: collision-induced dissociation

MRFA: L-methionyl-arginyl-phenylalanyl-alanine

P450: cytochrome P450

MS: mass spectrometry
Abstract

Studies on the biotransformation of isoxazole rings have shown that molecules containing a C3-substituted isoxazole or a 1,2-benzisoxazole can undergo a two-electron reductive ring cleavage to form an imine. In the absence of a C3-substituent, the isoxazole ring opens via deprotonation of the C3 proton followed by N-O bond cleavage to yield a α-cyanoenol analog. We report the identification of a novel bioactivation pathway of a 3,4-unsubstituted isoxazole in human liver microsomes. Following the enzyme-catalyzed cleavage of the 3,4-unsubstituted isoxazole ring of N-((2-isopropyl-7-methyl-1-oxoisooindolin-5-yl)methyl)isoxazole-5-carboxamide (P) in HLM, the formed α-cyanoenol (M1) condenses with formaldehyde to generate an α,β-unsaturated Michael acceptor intermediate (a cyanoacrolein derivative, VII), which further reacts with the cysteinyi thiol of glutathione to yield a GSH-adduct of a cyanoacrolein derivative (M3). The same adduct also is formed when M1, generated in 0.1 N NaOH aqueous solution, reacts with formaldehyde and GSH. ¹³C-labeled methanol was employed to confirm that methanol from the drug stock solution was oxidized by liver microsomal enzymes to formaldehyde and the carbon atom from methanol was finally incorporated in the corresponding GSH-adduct. The formation of isoxazole ring opened products (M1 and M2) in human liver microsomes is NADPH dependent. M1 and M2 were found in human liver microsomes preincubated with 1-aminobenzotriazole (1 mM) and NADPH (5 mM) at ~ 10% of the levels found in the samples in the absence of 1-aminobenzotriazole, suggesting that this biotransformation pathway is primarily catalyzed by cytochrome P450. The formation of M3 also was inhibited by ABT at a similar level.
Introduction

Several isoxazole-containing drugs, such as zonisamide (Stiff and Zemaitis, 1990; Nakasa et al., 1992, 1993; Sugihara et al., 1996), risperidone (Mannens et al., 1993; Mendermans et al., 1994), iloperidone (Mutlib, 1995), danazol (Davison, 1976), and leflunomide (Prakash and Jarvis, 1999; Dalvie et al., 2002; Kalgutkar et al., 2003) have been reported with regard to their biotransformation, which revealed extensive N-O bond cleavage in the isoxazole rings. Two mechanistic pathways have been proposed to account for the N-O bond cleavage of isoxazole rings during biotransformation process. The first mechanism occurs by two-electron reductive ring cleavage in the molecules containing a C3-substituted isoxazole or a 1,2-benzisoxazole to form an imine intermediate that is hydrolyzed to the final structure of 1,3-hydroxycarbonyl. For example, zonisamide undergoes two-electron reductive cleavage of N-O bond to a presumable imine intermediate which is hydrolyzed to the phenol (Scheme 1) (Stiff and Zemaitis, 1990; Nakasa et al., 1992, 1993; Sugihara et al., 1996). P450, aldehyde oxidase, and intestinal bacteria have been indicated to be involved in this biotransformation pathway. The greater electronegativity of the oxygen atom adjacent to the nitrogen atom in the isoxazole ring is believed to be the cause of its facile reductive cleavage of the N-O bond (Dalvie et al., 2002). The second mechanism of isoxazole ring opening functions via deprotonation of the C3 proton followed by N-O bond cleavage in the isoxazole without a C3-substituent (e.g., leflunomide) to yield the α-cyanoenol analogs (e.g., A771726) (Scheme 2) (Kalgutkar et al., 2003). CYP1A2, human plasma, whole blood, and serum albumin have been implicated to catalyze this biotransformation pathway in leflunomide.
C3-unsubstituted isoxazole ring also can easily undergo base-catalyzed opening through deprotonation of C3 proton in the isoxazole ring to generate the same α-cyanoenol or its tautomer 3-oxopropanenitrile analog (Wakefield and Wright, 1979; Perez and Grierson, 1996; Zipse et al., 1995) (Scheme 3). Studies on the ring transformation of 3,4-unsubstituted isoxazoles under chemical conditions (Scheme 3, I) reveal that the formed cyanoenols (II) or 3-oxopropanenitriles (III) can react in situ with aldehydes leading to the corresponding condensed products (IV). The un-isolated intermediate IV reacts with propanedinitrile affording 3,5-dicyano-4H-pyran-2-amine (V) following a Michael addition and a spontaneous cyclization (Eugster et al., 1963; Ciller et al., 1984, 1985). These reactions suggest that the 3,4-unsubstituted isoxazole ring opened product, α-cyanoenol, is reactive.

N-((2-Isopropyl-7-methyl-1-oxoisoindolin-5-yl)ethyl)isoxazole-5-carboxamide (P) is an exploratory CNS compound that contains a 3,4-unsubstituted isoxazole ring. The present studies describe the identification of its isoxazole ring opened products and the corresponding glutathione-adduct of a cyanoacrolein derivative formed in incubations of P with human liver microsomes in the presence of NADPH and glutathione (GSH) by liquid chromatography/tandem mass spectrometry (LC/MS/MS). The same GSH-adduct also was identified in the aqueous solution (pH = 7.5) containing GSH, formaldehyde, and the isoxazole ring opened products generated in the incubations of P with 0.1 N NaOH. In the present report, the mechanism of the biactivation of a 3,4-unsubstituted isoxazole leading to GSH-adduct formation is discussed.
Materials and Methods

Chemicals and Materials. Reduced L-glutathione (GSH), magnesium chloride, β-nicotinamide adenine dinucleotide 2’-phosphate (reduced, NADPH), ¹³C-labeled methanol, formaldehyde (37 wt.% in water), labetalol, and 1-aminobenzotriazole (1-ABT) were obtained from Sigma Life Science (St. Louis, MO). L-methionyl-arginyl-phenylalanyl-alanine (MRFA) was purchased from Research Plus Inc. (Manasquan, NJ). Ultramark 1621 mass spectrometry standard was purchased from Alfa Aesar (Ward Hill, MA). Pooled human liver microsomes (HLM, mixed gender, protein concentration 20 mg/ml) were purchased from BD Bioscience (Woburn, MA). N-((2-Isopropyl-7-methyl-1-oxoisindolin-5-yl)methyl)isoxazole-5-carboxamide (P) was obtained from AstraZeneca Pharmacy. The 2 mM stock solutions of P used for incubations were prepared in four solvents separately: methanol, ¹³C-labeled methanol, acetonitrile, and ethanol. All of the other chemicals and solvents were purchased from Sigma/Aldrich (St. Louis, MO) and were of analytical or high performance liquid chromatography (HPLC) grade.

Identification of Metabolites (M1, M2, M3, ¹³C-labeled M3, M5, and M6) of P in HLM. The metabolism of P was investigated in incubations with HLM. P (20 μM) was mixed with HLM (1 mg/ml), MgCl₂ (10 mM), and potassium phosphate buffer (100 mM, pH 7.4) in a final volume of 500 μl. The mixture was pre-incubated at 37 °C for 5 min. The reactions were initiated by the addition of NADPH (final concentration was 1 mM). Control experiments were conducted in the absence of NADPH. After incubation at 37 °C for 60 min, the reactions were terminated by the addition of 1 ml of acetonitrile, followed by vortexing and centrifugation at 14000 rpm (MIKRO 22R centrifuge, Andreas...
Hettich GmbH & Co. KG, Tuttlingen, Germany) at 4 °C for 6 min to pellet the precipitated protein. The resulting supernatant was transferred to another tube and evaporated to dryness under a steady stream of nitrogen at room temperature. The residue was reconstituted in 5% (v/v) acetonitrile-water and analyzed by LC/MS/MS. To study GSH conjugate profiles, incubations were performed in the presence of GSH (10 mM) to capture the potential nucleophilic reactive species. Control experiments were conducted in the absence of NADPH. Four drug stock solutions that contained methanol, 13C-labeled methanol, ethanol, and acetonitrile, respectively, were used in the incubations. To study the enzymes involved in the formation of isoxazole ring opened products (M1 and M2), incubations were performed with HLM (1 mg/ml) preincubated with 1-ABT (1 mM) and NADPH (5 mM) at 37 °C for 20 min to inhibit P450 activity (Dalmadi et al., 2003; Williams et al., 2003). The reactions were initiated by the addition of P in acetonitrile (final concentration was 20 μM). After incubation at 37 °C for 60 min, the reaction was terminated by the addition of 1 ml of ice-cooled acetonitrile containing 0.15 μM labetalol followed by vortexing and centrifugation at 14000 rpm (MIKRO 22R centrifuge, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 4 °C for 6 min to pellet the precipitated protein. The incubations were performed in triplicate. The resulting supernatant was transferred to another tube and evaporated to dryness under a steady stream of nitrogen at room temperature. The residue was reconstituted in 5% (v/v) acetonitrile-water and analyzed by LC/MS/MS. Control experiments were conducted in the absence of 1-ABT. To study the inhibition of M3 formation, incubations were performed in the presence of GSH (10 mM) with HLM preincubated with 1-ABT and NADPH under similar experimental conditions.
Formation of Isoxazole Ring Opened Products (M1, M2, and M4) of P and the Corresponding GSH-Adduct (M3) In the Absence of Any Enzymes. The formation of isoxazole ring opened products of P were also investigated in 0.1 N NaOH aqueous solution containing P. P (40 μM) was mixed with 0.1 N NaOH in a final volume of 400 μl. The mixture was incubated at 37 °C for 20 min. Two μl of the reaction mixture was analyzed by LC/MS/MS directly.

To study GSH conjugate profile, the pH value of the above incubation mixture was adjusted to pH 7.5 using 1 N HCl aqueous solution (~ 40 μl). Then GSH (1.44 mg, final concentration 10 mM) and formaldehyde solution (30 μl, final concentration 1M) were added. The mixture was left to stand at room temperature for 15 min to allow a sufficient reaction between formaldehyde and the isoxazole ring opened products. The resulting mixture was incubated at 37 °C for 20 min. Two μl of the supernatant was analyzed by LC/MS/MS directly. Drug stock solution containing acetonitrile was used. Control experiments were conducted in the presence of GSH but absence of formaldehyde.

LC/MS/MS Analysis. Metabolites and decomposition products were analyzed by LC/MS/MS on an Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm, with a VanGuard™ HSS T3 1.8 μm guard column, Waters, Milford, MA) at room temperature. The mobile phase consisted of water (solvent A, containing 25 mM HCO₂NH₄, pH = 5) and 95% acetonitrile in water (solvent B) and was delivered at a flow rate of 0.2 ml/min. The column was eluted with 5% B in A during the first 3 minute, followed by a linear solvent gradient, running from 5% B to 35% B in 12 min, 35% B to 90% B over next 2 min, held constant at 90% B for 3 min, returned to 5% B over 1 min. The column was
allowed to equilibrate at 5% solvent B for 5 min prior to the next injection. The first 2 min of LC run was diverted to waste. Sample temperature was kept at 5 °C.

Mass spectrometric analyses were performed on a Thermo LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA), which was interfaced to a Waters Acquity UPLC system (Milford, MA). MS analyses were conducted using a standard electrospray ionization source operating in positive ionization mode. Source operating conditions were: 4.0 kV spray voltage, 275 °C heated capillary temperature, 14 V capillary voltage, sheath and auxiliary gas flow at 40 and 10 arbitrary units of the manufacture, respectively. The full scan and MS/MS product ion scan mass spectra acquired at a resolving power of 30,000 (at m/z 400) and the data were centroid. The product ion scan activation parameters used an isolation width of 2 Da (for fragments with m/z values that were 1 Da different from the intended fragment, the isolation width was set to 1 Da), normalized collision energy of 30 to 40%, and an activation time of 30 milliseconds. The quantitative analysis was carried out by the measurement of the peak area of the extracted ion chromatograms using full scan mode and a mass window with an error of 5 ppm centering on the theoretical m/z of the isoxazole ring opened metabolites (M1 at m/z 314.1499 and M2 at m/z 332.1605) and labetalol (m/z 329.1860). The calculated values were mean ± S.D. of three experiments. Calibration of the instrument was performed using the standard LTQ calibration mixture with caffeine, the peptide L-methionyl-arginy1-phenylalanyl-alanine, and Ultramark 1621 dissolved in an acetonitrile:methanol:water solution containing 1% (v/v) acetic acid. The predicted chemical formula and calculated accurate mass were obtained from Chemdraw Ultra 8.0 (CambridgeSoft, Cambridge, MA) based on the proposed fragmentation pathways and
the putative fragment structures. The data were filtered in Qual Browser (Thermo Electron, San Jose, CA) based on accurate mass thresholds. In addition, the Qual Browser chemical formula calculator was used to provide chemical formula and experimental error values for molecular ions and product ions of P and its metabolites. Fragmentations were proposed based on plausible protonation sites, subsequent isomerization and even electron species, as well as bond saturation. Comparison between the parent and metabolite product ion spectra further aided in the identification of metabolite structures and modification site(s) in the parent molecule.
Results

Metabolite Profile of P in Human Liver Microsomes. The structures of the metabolites of P formed in liver microsomal incubations were characterized with respect to mass to charge ratio, chemical formula, and fragmentation pattern by LC/MS/MS as described in the Materials and Methods.

After a 60 min incubation of P in HLM in the presence of NADPH at 37 °C, two isoxazole ring opened products (M1 and M2), which co-eluted on the LC column at the retention time of 13.55 min, were identified (Fig. 1A). These metabolites were not found in the control experiments that were conducted in the absence of NADPH, suggesting that their formation was enzyme mediated. Mass responses (estimated by the ratio of the sum of peak areas of M1 and M2 to the peak area of the internal standard) of M1 and M2 from the incubations of HLM preincubated with 1-ABT (1 mM) and NADPH (5 mM) and from the incubations without 1-ABT were 0.128 ± 0.016 and 1.22 ± 0.17, respectively, suggesting that this biotransformation pathway is primarily catalyzed by cytochrome P450. While several hydroxylated metabolites with the modifications at the 5-(aminomethyl)-2-isopropyl-7-methylisoindolin-1-one moiety of P also were detected, these metabolites are not relevant to the isoxazole ring biotransformation process, therefore, are not discussed here. In the presence of NADPH and GSH and when the drug stock solution contained methanol (1% in the incubation mixture), a new metabolite M3 was detected (Fig. 1B). Under similar experimental conditions, when the drug stock solution contained ethanol (1% in the incubation mixture), two new metabolites, M5 and M6, were detected. These metabolites were not detected in the incubations that contained acetonitrile (1% in the incubation mixture) as the solvent of
drug stock solution. Table 1 summarizes the LC retention times ($R_t$), accurate masses, chemical formulae and postulated molecular structures of the isoxazole ring opened products of P and the corresponding GSH adducts identified in HLM and in incubates in the absence of any enzymes. In full-scan positive ion mode, metabolites M1, M2, M3, M5, and M6 form protonated molecular ion $[M+H]^+$ at m/z 314 (the same as that of the parent), 332 (incorporation of H$_2$O), 633 (incorporation of a glutathionyl plus a CH$_2$ moiety), 647 (incorporation of a glutathionyl plus a CH$_3$CH moiety), and 647 (incorporation of a glutathionyl plus a CH$_3$CH moiety), respectively (Table 1).

**Product Profile of P in Incubates in the Absence of Any Enzymes.** After a 20 min incubation of P in 0.1 N NaOH aqueous solution at 37 °C, in addition to the two isoxazole ring opened products M1 and M2, a new product M4, which was not observed in HLM, was also identified (Fig. 1C). In full-scan positive ion mode, metabolite M4 forms protonated molecular ion $[M+H]^+$ at m/z 314 (Table 1). When this incubation mixture was adjusted to pH 7.5 and formaldehyde and GSH were added, a product, which has the same LC retention time and mass fragment patterns as those of M3, was identified, providing strong evidence that this product is M3. This GSH-adduct was not detected in incubations performed in the absence of formaldehyde.

*N-((2-Isopropyl-7-methyl-1-oxoisooindolin-5-yl)methyl)isoxazole-5-carboxamide* (P). The collision-induced dissociation (CID) mass spectrum of the protonated molecular ion of P at m/z 314 revealed product ions at m/z 272, 219, 202, 187, 177, 160, and 148 (Fig. 2A; Supplemental Table S1). The characteristic product ion at m/z 272 is likely formed through a loss of an isopropyl moiety at the nitrogen atom of 7-methylisoindolin-1-one (Fig. 2A). The cleavage at the amide bond or N-C bond
between the 5,7-dimethylisoindolin-1-one and isoxazole-5-carboxamine moieties in the ion of m/z 272 leads to the formation of second-generation product ions at m/z 177 and 160 (Fig. 2B). The similar cleavages of the bonds in the [M+H]⁺ ion at m/z 314 generate ions at m/z 219 and m/z 202, respectively (Fig. 2A). These characteristic fragmentation patterns were applied toward the structural identification of the metabolites of P.

**Metabolite M1.** CID mass spectrum of the protonated molecular ion of M1 revealed product ions at m/z 272, 219, 202, 200, 187, 177, 160, 148, and 134 (Fig. 3; Supplemental Table S2). The major fragmentation pathways are similar to those of P. The formation of fragment ions at m/z 219 and 202 from the [M+H]⁺ ion of M1 suggests that the 5-(aminomethyl)-2-isopropyl-7-methylisoindolin-1-one moiety of the parent molecule P was intact, therefore, the modification in M1 was at the isoxazole ring.

**Metabolite M2.** CID mass spectrum of the protonated molecular ion of M2 revealed product ions at m/z 314, 290, 272, 247, 230, 229, 219, 205, 203, 202, 187, and 160 (Fig. 4A; Supplemental Table S3). The proposed fragmentation pathways are given in Scheme 4. Accurate mass measurement showed the chemical formula of the ion at m/z 314 as [C₁₇H₂₀N₃O₃]⁺ (Supplemental Table S3). Further fragmentation of this ion led to the formation of the second-generation product ions at m/z 272, 219, 202, 187, 177, 168, 148, and 134, which are consistent with those found with M1 (Fig. 3A, 4A and 4B). This information suggests that the [M+H]⁺ ion of M1 is generated in the ion source from the molecular ion of M2 following the loss of a H₂O (Scheme 4, pathway a). The ion at m/z 290 was generated through the loss of an isopropyl moiety from the molecular ion (Fig. 4A and 4C; Scheme 4, pathway b). The characteristic product ion at m/z 247 was likely generated through the loss of the isoxazole ring opened moiety and a H₂O (C₃H₅NO₂, 85

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Da) from the parent molecule (Fig. 4A and 4D; Scheme 4, pathway c). The ion at m/z 229 was generated through the loss of a C₃H₅NO₃ moiety from the molecular ion (Fig. 4A; Scheme 4, pathway d). The mass spectra of M2 suggest that a water molecule is added to M1 at the isoxazole ring opened moiety. The data also indicated that the 5-(aminomethyl)-2-isopropyl-7-methylisoindolin-1-one moiety in the molecule is likely intact. While efforts to separate M1 and M2 on different HPLC columns and with different mobile phases were not successful, it was found that the pH value of the mobile phase significantly affected the relative amount of M1 and M2 (Supplemental Fig. S1 and S2).

**Metabolite M3.** The CID mass spectrum of the molecular ion of M3 at m/z 633 revealed product ions at m/z 558, 504, 486, 326, 308, 233, and 179 (Fig. 5A; Supplemental Table S4). Accurate mass measurement showed the chemical formula of the ion at m/z 308 as [C₁₀H₁₈N₃O₆S]⁺ (Supplemental Table S4). Further fragmentation of this ion led to the formation of the second-generation product ions at m/z 272, 245, 233, 215, 179, 162, 144, and 116 (Fig. 5B), which is consistent with those found with glutathione molecule (Baillie and Davis, 1993). Accurate mass measurement of the ion at m/z 326 provided a chemical formula of [C₁₈H₂₀N₅O₃]⁺ (Supplemental Table S4), suggesting an incorporation of an extra carbon atom in the molecular ion of M1. CID of the ion at m/z 326 yielded second-generation product ions at 284, 202, and 160 (Fig. 5C). The ions at m/z 558, 504, and 486 are formed through the loss of glycyl, γ-pyroglutamic acid, and γ-pyroglutamic acid plus H₂O moiety, respectively (Fig. 5A) (Baillie and Davis, 1993). Therefore, the tandem mass spectra of M3 suggest the formation of a GSH-adduct of a molecule that incorporated an extra carbon atom to M1.
\textbf{13C-Labeled Metabolite M3.} When $^{13}$C-labeled methanol was used as a solvent to prepare drug stock solution (for subsequent use) in the liver microsomal incubation, a metabolite that has the protonated molecular ion at m/z 634 was detected at the same LC retention time and with similar fragment patterns as those of M3 (Supplemental Fig. S3). The ions at m/z 559, 505, 487, 327, and 285 observed with the product ions of $^{13}$C-labeled M3 were 1Da higher compared with the corresponding product ions of M3 at m/z 558, 504, 486, 326, and 285, respectively (Fig 5 and Supplemental Fig. S3). The results from this study suggest that the extra carbon atom detected in the molecule of M3 comes from the methanol of the drug stock solution.

\textbf{Metabolite M4.} The CID mass spectrum of the protonated molecular ion of M4 at m/z 314 revealed product ions at m/z 286, 272, 244, 219, 217, 202, 187, 177, and 160 (Fig. 6A; Supplemental Table S5). The proposed fragmentation pathways are shown in Scheme 5. The major ion at m/z 286 in the product ion mass spectra of M4 provides critical information for its structural elucidation (Fig. 6A). It is likely formed through a loss of a CO moiety from the molecular ion of M4 via an intra-molecular reaction (Fig. 6B; Scheme 5, pathways a). This fragment pathway is not observed in both M1 and M2. The product ion at m/z 272 is likely formed through a loss of an isopropyl moiety at the nitrogen atom of 7-methylisoindolin-1-one (Fig. 6A; Scheme 5, pathways b). The formation of ions at m/z 219 and m/z 202 suggests that the 5-(aminomethyl)-2-isopropyl-7-methylisoindolin-1-one moiety in the molecule is intact (Scheme 5, pathway c and d). The mass spectra of M4 suggested a 3-oxopropanenitrile tautomer of M1. The equilibrium between the keto and enol form of the isoxazole ring opened products (M1 and M4) is pH dependent (Supplemental Figure S1).
Metabolites M5 and M6. The mass spectra of M5 (Supplemental Figure S4; Supplemental Scheme S1) suggest the formation of the GSH-adduct of a cyanocrotonaldehyde derivative. M6 has similar fragment patterns as those found in M5, therefore, M6 was identified as the isomer/diasteromer of M5 (Supplemental Figure S5).
Discussion:

This study reports the identification of isoxazole ring opened products of $P$ and a GSH-adduct of a cyanoacrolein derivative (of $P$) formed via the condensation of formaldehyde and the isoxazole ring opened product in human liver microsomes. The mechanism of the formation of the GSH-adduct also is investigated.

The product ion mass spectra of M2 suggest the addition of H$_2$O at the double bond of the cyanoenol moiety of M1 (Scheme 6). The peak area ratio of M2 to M1 is 82, 2.7, and 0.33 for the same sample when the mobile phase is at pH 2.1, 5.0, or 7, respectively (Supplemental Fig. S1 and S2). The acid-catalyzed hydration of alkenes is an equilibrium process and the hydration of M1 (formation of M2) prefers lower pH value. The easy addition of H$_2$O to M1 indicates that the $\alpha$-cyanoenol moiety is very labile.

The pH value of the mobile phases also affected the relative amount of M4 in the LC run (Supplemental Fig. S1). At pH 7.0, only M1 and M2, but not M4, were detected as evidenced by the fact that the characteristic ion m/z 286 of M4 was not appeared in the product ion mass spectra of the protonated molecular ion at m/z 314. This suggests that the proton $\alpha$ to the cyano group in the keto form tautomer (M4) is active. It can be abstracted at higher pH and form the enol form M1 (Scheme 6).

The mass spectra of the molecular ion of M3 at m/z 633 suggest formation of a GSH-adduct. The formation of the second generation product ions at m/z 202 and 160 from the ion at m/z 326 suggests the 2-isopropyl-5,7-dimethylisoindolin-1-one moiety is intact. Studies with $^{13}$C-labeled methanol resulted in a 1 Da shift of the $[M+H]^+$ of M3 at 633 and its product ion mass spectra at m/z 558, 504, 486, 326, and 284, suggesting the
incorporation of a carbon from methanol to the molecule of M3. The chemical reaction among the isoxazole ring opened product, formaldehyde, and GSH also yields M3, suggesting the likely scenario (Scheme 6) that the methanol in the drug stock solution is oxidized by liver microsomal enzymes to formaldehyde which then reacts with the isoxazole ring opened product M1 to form an intermediate VI. Following a dehydration reaction, a cyanoacrolein derivative (α,β-unsaturated Michael acceptor) VII is formed and reacts with nucleophilic cysteiny1 thiol of GSH via 1,4- or 1,2-Michael addition to generate the final GSH-adduct M3. M3 may exist in equilibration between the enol and keto form. This mechanism is supported by the observation that when the drug stock solution contains ethanol, isomers/diasteromers of GSH-adducts of cyanocrotonaldehyde derivatives were detected (Supplemental Fig. S5 and S5; Supplemental Scheme S1).

In the absent of any enzymes, isoxazole ring can be cleaved easily by a basic reagent. The ring opening is especially facile in 5-substituted derivatives with C3-unsubstituted, where abstraction of the proton at C3 prompts the N-O bond cleavage (Wakefield and Wright, 1979). When 5-methylisoxazole and benzaldehyde were allowed to react with sodium ethoxide in dry ethanol at room temperature followed by acid treatments, Eugster et al. have successfully isolated the aldol condensation product, benzylidenecyanoacetone (Eugster et al., 1963). The condensation reaction between the isoxazole ring opened products and aldehydes has been used to bring about the transformation of 5-substituted isoxazoles into different kinds of heterocycles, such as furan and pyran derivatives (Ciller et al., 1984, 1985). When the isoxazole ring cleavage occurs during biotransformation, it is not surprising that the formed α-cyanoenol can further condense with an aldehyde if present in the in vitro system.
The formation of a GSH-adduct of cyanoacrolein or crotonaldehyde derivative identified in human liver microsomes suggests that the 3,4-unsubstituted isoxazole, or any compounds that can form 3-hydroxyacrylonitrile or 3-oxopropanenitrile derivatives through biotransformation, may be reactive. In the case of leflunomide, the C4 was substituted (Scheme 2). Following the isoxazole ring opening, it is impossible for A771726 to form an \( \alpha,\beta \)-unsaturated Michael acceptor intermediate through the reaction with formaldehyde or actaldehyde.

Metabolite M3 was not detected in human liver microsomes in the presence of NADPH and GSH when acetonitrile was used as a solvent to prepare the drug stock solution. This result not only supports the fact that methanol in the drug stock solution plays a necessary role in the formation of this type of GSH conjugate, but also suggests that solvent effects must be considered when this type of metabolic pathway is investigated.

**Authorship Contributions:**

Participated in research design: Yu

Conducted experiments: Yu and Hoesch

Performed data analysis: Yu, Folmer, and Hoesch

Wrote or contributed the writing of the manuscript: Yu, Burdette, Folmer, Campbell, and Doherty

Other: Burdette provided guidance for the research.
References


Footnotes

Send the reprint requests to:

Dr. Jian Yu
Department of Discovery DMPK
AstraZeneca Pharmaceuticals LP
L-260C
1800 Concord Pike
PO Box 15437
Wilmington, DE 19850-5437 USA
Tel: (302)-885-0494
Fax: (302)-995-2201
E-mail: jian.yu@astrazeneca.com
Legend of Figures

Scheme 1. Reductive isoxazole ring opening of zonisamide.

Scheme 2. Base-induced or enzyme-catalyzed isoxazole ring opening of leflunomide.

Scheme 3. Ring transformation of 3,4-unsubstituted isoxazoles under chemical conditions

Scheme 4. Proposed plausible fragmentation pathways of the molecular ion \([\text{M+H}]^+\) of the metabolite M2 (RI = 13.55 min) at m/z 332 (Fig. 4).

Scheme 5. Proposed plausible fragmentation pathways of the molecular ion \([\text{M+H}]^+\) of the metabolite M4 (RI = 12.69 min) at m/z 314 (Fig 6).

Scheme 6. Proposed metabolic pathways of the formation of metabolites M1, M2, and M3 from P in human liver microsomes in the presence of NADPH and GSH and the formation of M1-M4 under chemical conditions.

Figure 1. LC/MS extracted total ion chromatogram (TIC) of the isoxazole ring opened products of \(N-((2\text{-isopropyl-7-methyl-1-oxoisoindolin-5-yl})\text{methyl})\text{isoxazole-5-carboxamide (P)}\) and the corresponding GSH-adduct of a cyanoacrolein analog. Data were filtered to show only parent, isoxazole ring opened products and the corresponding GSH-adduct. A, TIC from the incubation of P (20 µM) with human liver microsomes in the presence of NADPH at 37 °C for 1 h, the drug stock solution contained acetonitrile.
B, TIC from the incubation of P (20 µM) with human liver microsomes in the presence of NADPH and GSH at 37 °C for 1 h, the drug stock solution contained methanol. C, TIC from the incubation of P (40 µM) with 0.1 N NaOH aqueous solution at 37 °C for 20 min.

Figure 2. A, Product ion mass spectrum obtained by CID of the molecular ion of N-((2-isopropyl-7-methyl-1-oxoisindolin-5-yl)methyl)isoxazole-5-carboxamide (P) (R_t = 17.06 min) at m/z 314 (see Table 1 and Supplemental Table S1). B, MS^3 spectrum of fragment ion m/z 272 observed in the product ion spectrum of P in A. The inserts show the proposed plausible fragmentation pathways of the molecular ion [M+H]^+ of P at m/z 314.

Figure 3. A, Product ion mass spectrum obtained by CID of the molecular ion of metabolite M1 (R_t = 13.55 min) at m/z 314 from NADPH-supplemented human liver microsomal incubations containing P (20 µM) (see Table 1 and Supplemental Table S2). B, MS^3 spectrum of fragment ion m/z 272 observed in the product ion spectrum of M1 in A. The inserts show the proposed plausible fragmentation pathways of the molecular ion [M+H]^+ of M1 at m/z 314.

Figure 4. A, Product ion mass spectrum obtained by CID of the molecular ion of metabolite M2 (R_t = 13.55 min) at m/z 332 from NADPH-supplemented human liver microsomal incubations containing P (20 µM) (see Table 1 and Supplemental Table S3, Scheme 4). B, MS^3 spectrum of fragment ion m/z 314 observed in the product ion
spectrum of M2 in A. C, MS$^3$ spectrum of fragment ion m/z 290 observed in the product ion spectrum of M2 in A. D, MS$^3$ spectrum of fragment ion m/z 247 observed in the product ion spectrum of M2 in A.

Figure 5. A, Product ion mass spectrum obtained by CID of the molecular ion of metabolite M3 (R$_t$ = 9.72 min) at m/z 633 from NADPH and GSH-supplemented human liver microsomal incubations containing P (20 µM) (see Table 1 and Supplemental Table S4). Drug stock solution contained CH$_3$OH. B, MS$^3$ spectrum of fragment ion m/z 308 observed in the product ion spectrum of M3 in A. C, MS$^3$ spectrum of fragment ion m/z 326 observed in the product ion spectrum of M3 in A. The inserts show the proposed plausible fragmentation pathways of the molecular ion [M+H]$^+$ of M3 at m/z 633. In the case of $^{13}$C-labeled M3, the molecular ion is at m/z 634 and the ions at m/z 559, 506, 487, 327, and 285 (Supplemental Fig. S3) were 1 Da higher compared with the corresponding ions of M3 at m/z 558, 504, 486, 326, and 284, respectively. * denotes the $^{13}$C-labeled atom.

Figure 6. A, Product ion mass spectrum obtained by CID of the molecular ion of metabolite M4 (R$_t$ = 12.69 min) at m/z 314 from the incubations of P (40 µM) with 0.1 N NaOH (see Table 1 and Supplemental Table S5, Scheme 5). B, MS$^3$ spectrum of fragment ion m/z 286 observed in the product ion spectrum of M4 in A.
Table 1. The LC retention times ($R_t$), accurate masses, elemental compositions and postulated molecular structures of the products of $N$-((2-isopropyl-7-methyl-1-oxoisindolin-5-yl)methyl)isoxazole-5-carboxamide (P) identified in human liver microsomes and under chemical conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_t$ (min)</th>
<th>Elemental composition</th>
<th>Measured mass (Da)</th>
<th>Mass error (ppm)</th>
<th>$\Delta M$</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>17.06</td>
<td>[C$<em>{17}$H$</em>{20}$N$_3$O$_3$]$^+$</td>
<td>314.1504</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>13.55</td>
<td>[C$<em>{17}$H$</em>{20}$N$_3$O$_3$]$^+$</td>
<td>314.1505</td>
<td>1.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>13.55</td>
<td>[C$<em>{17}$H$</em>{22}$N$_3$O$_4$]$^+$</td>
<td>332.1605</td>
<td>1.6</td>
<td>+18</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>9.72</td>
<td>[C$<em>{28}$H$</em>{37}$N$_6$O$_9$S]$^+$</td>
<td>633.2334</td>
<td>-0.51</td>
<td>+319</td>
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<tr>
<td>M4</td>
<td>12.69</td>
<td>[C$<em>{17}$H$</em>{20}$N$_3$O$_3$]$^+$</td>
<td>314.1506</td>
<td>2.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>10.82</td>
<td>[C$<em>{29}$H$</em>{39}$N$_6$O$_9$S]$^+$</td>
<td>647.2495</td>
<td>0.20</td>
<td>+333</td>
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<tr>
<td>M6</td>
<td>10.08</td>
<td>[C$<em>{29}$H$</em>{39}$N$_6$O$_9$S]$^+$</td>
<td>647.2495</td>
<td>0.20</td>
<td>+333</td>
<td></td>
</tr>
</tbody>
</table>
Scheme 1

Zonisamide

2e⁻, 2H⁺

P450

Aldehyde oxidase

Intestinal bacteria

Imine intermediate

H₂O

2-(Sulfamoylacetyl)phenol
Scheme 4

m/z 203

m/z 202

m/z 187

m/z 229

m/z 272

m/z 290

m/z 332

m/z 314

m/z 272

m/z 247

m/z 230

m/z 205

m/z 160

m/z 187

-C₂H₇NO

-H⁺

-H₂O, -C₃H₆

-C₃H₆NO₃

-H₂O

-H⁺
Figure 2

A) MS², m/z 314 →

B) MS³, m/z 314 → 272
Figure 3

A) MS², m/z 314 →

B) MS³, m/z 314 → 272
Figure 4

A) MS², m/z 332 →

B) MS³, m/z 332 → 314

C) MS³, m/z 332 → 290

D) MS³, m/z 332 → 247
Figure 5

A) MS², m/z 633 →

B) MS³, m/z 633 → 308

C) MS³, m/z 633 → 326
A) MS², m/z 314 →

B) MS², m/z 314 → 286
Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

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**Supplemental Data**

**Table S1.** List of accurate mass measurements and elemental compositions of the product ions of N-((2-isopropyl-7-methyl-1-oxoisindolin-5-yl)methyl)isoxazole-5-carboxamide P (Rt = 17.06 min) following CID

<table>
<thead>
<tr>
<th>Fragment ion (m/z)</th>
<th>Elemental composition</th>
<th>Measured mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>314</td>
<td>[C\textsubscript{17}H\textsubscript{20}N\textsubscript{3}O\textsubscript{3}]\textsuperscript{+}</td>
<td>314.1504</td>
<td>314.1499</td>
<td>1.5</td>
</tr>
<tr>
<td>272</td>
<td>[C\textsubscript{14}H\textsubscript{14}N\textsubscript{3}O\textsubscript{3}]\textsuperscript{+}</td>
<td>272.1035</td>
<td>272.1030</td>
<td>2.0</td>
</tr>
<tr>
<td>219</td>
<td>[C\textsubscript{13}H\textsubscript{19}N\textsubscript{2}O\textsubscript{3}]\textsuperscript{+}</td>
<td>219.1493</td>
<td>219.1492</td>
<td>0.50</td>
</tr>
<tr>
<td>202</td>
<td>[C\textsubscript{13}H\textsubscript{16}NO\textsubscript{2}]\textsuperscript{+}</td>
<td>202.1227</td>
<td>202.1226</td>
<td>0.29</td>
</tr>
<tr>
<td>187</td>
<td>[C\textsubscript{10}H\textsubscript{13}N\textsubscript{2}]\textsuperscript{+}</td>
<td>187.0992</td>
<td>187.0992</td>
<td>0.18</td>
</tr>
<tr>
<td>177</td>
<td>[C\textsubscript{10}H\textsubscript{13}N\textsubscript{2}O\textsubscript{2}]\textsuperscript{+}</td>
<td>177.1022</td>
<td>177.1022</td>
<td>-0.22</td>
</tr>
<tr>
<td>160</td>
<td>[C\textsubscript{10}H\textsubscript{10}NO\textsubscript{2}]\textsuperscript{+}</td>
<td>160.0756</td>
<td>160.0757</td>
<td>-0.57</td>
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<tr>
<td>148</td>
<td>[C\textsubscript{9}H\textsubscript{10}NO\textsubscript{2}]\textsuperscript{+}</td>
<td>148.0755</td>
<td>148.0757</td>
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</table>
Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

Jian Yu, James J. Folmer, Valerie Hoesch, James Doherty, James B. Campbell, Doug Burdette

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Table S2. List of accurate mass measurements and elemental compositions of the product ions of M1 ($R_t = 13.55$ min) following CID

<table>
<thead>
<tr>
<th>Fragment ion ( (m/z) )</th>
<th>Elemental composition</th>
<th>Measured mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>314</td>
<td>([C_{17}H_{20}N_3O_3]^+)</td>
<td>314.1505</td>
<td>314.1499</td>
<td>1.8</td>
</tr>
<tr>
<td>272</td>
<td>([C_{14}H_{14}N_3O_3]^+)</td>
<td>272.1036</td>
<td>272.1030</td>
<td>2.3</td>
</tr>
<tr>
<td>219</td>
<td>([C_{13}H_{19}N_2O]^+)</td>
<td>219.1495</td>
<td>219.1492</td>
<td>1.4</td>
</tr>
<tr>
<td>202</td>
<td>([C_{13}H_{16}NO]^+)</td>
<td>202.1229</td>
<td>202.1226</td>
<td>1.3</td>
</tr>
<tr>
<td>200</td>
<td>([C_{12}H_{10}NO_2]^+)</td>
<td>200.0709</td>
<td>200.0706</td>
<td>1.5</td>
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<tr>
<td>187</td>
<td>([C_{12}H_{13}NO]^+)</td>
<td>187.0993</td>
<td>187.0992</td>
<td>0.72</td>
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<td>177</td>
<td>([C_{16}H_{13}N_2O]^+)</td>
<td>177.1023</td>
<td>177.1022</td>
<td>-0.22</td>
</tr>
<tr>
<td>160</td>
<td>([C_{10}H_{10}NO]^+)</td>
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<td>160.0757</td>
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<tr>
<td>148</td>
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<td>134</td>
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Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

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**Table S3.** List of accurate mass measurements and elemental compositions of the product ions of M2 ($R_t = 13.55$ min) following CID

<table>
<thead>
<tr>
<th>Fragment ion (m/z)</th>
<th>Elemental composition</th>
<th>Measured mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Mass error (ppm)</th>
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<tbody>
<tr>
<td>332</td>
<td>$[\text{C}<em>{17}\text{H}</em>{22}\text{N}_3\text{O}_4]^+$</td>
<td>332.1610</td>
<td>332.1605</td>
<td>1.6</td>
</tr>
<tr>
<td>314</td>
<td>$[\text{C}<em>{17}\text{H}</em>{20}\text{N}_3\text{O}_3]^+$</td>
<td>314.1504</td>
<td>314.1499</td>
<td>1.5</td>
</tr>
<tr>
<td>290</td>
<td>$[\text{C}<em>{14}\text{H}</em>{16}\text{N}_3\text{O}_4]^+$</td>
<td>290.1140</td>
<td>290.1135</td>
<td>1.6</td>
</tr>
<tr>
<td>272</td>
<td>$[\text{C}<em>{14}\text{H}</em>{14}\text{N}_3\text{O}_3]^+$</td>
<td>272.1034</td>
<td>272.1030</td>
<td>1.6</td>
</tr>
<tr>
<td>247</td>
<td>$[\text{C}<em>{14}\text{H}</em>{19}\text{N}_2\text{O}_2]^+$</td>
<td>247.1445</td>
<td>247.1441</td>
<td>1.6</td>
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<tr>
<td>230</td>
<td>$[\text{C}<em>{14}\text{H}</em>{16}\text{NO}_2]^+$</td>
<td>230.1179</td>
<td>230.1176</td>
<td>1.5</td>
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<tr>
<td>229</td>
<td>$[\text{C}<em>{14}\text{H}</em>{17}\text{N}_2\text{O}]^+$</td>
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<td>1.1</td>
</tr>
<tr>
<td>219</td>
<td>$[\text{C}<em>{13}\text{H}</em>{19}\text{N}_2\text{O}]^+$</td>
<td>219.1495</td>
<td>219.1492</td>
<td>1.4</td>
</tr>
<tr>
<td>205</td>
<td>$[\text{C}<em>{11}\text{H}</em>{13}\text{N}_2\text{O}_2]^+$</td>
<td>205.0973</td>
<td>205.0972</td>
<td>0.7</td>
</tr>
<tr>
<td>203</td>
<td>$[\text{C}<em>{13}\text{H}</em>{17}\text{NO}]^{2+}$</td>
<td>203.1307</td>
<td>203.1305</td>
<td>1.2</td>
</tr>
<tr>
<td>202</td>
<td>$[\text{C}<em>{13}\text{H}</em>{16}\text{NO}]^+$</td>
<td>202.1228</td>
<td>202.1226</td>
<td>0.8</td>
</tr>
<tr>
<td>187</td>
<td>$[\text{C}<em>{11}\text{H}</em>{11}\text{N}_2\text{O}]^+$</td>
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<tr>
<td>160</td>
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<td>160.0757</td>
<td>0.06</td>
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</table>
Table S4. List of accurate mass measurements and elemental compositions of the product ions of M3 ($R_t = 9.72$ min) following CID

<table>
<thead>
<tr>
<th>Fragment ion (m/z)</th>
<th>Elemental composition</th>
<th>Measured mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>633</td>
<td>[C$<em>{28}$H$</em>{37}$N$_6$O$_9$S]$^+$</td>
<td>633.2334</td>
<td>633.2337</td>
<td>-0.51</td>
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<tr>
<td>558</td>
<td>[C$<em>{26}$H$</em>{32}$N$_5$O$_7$S]$^+$</td>
<td>558.2029</td>
<td>558.2017</td>
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<tr>
<td>504</td>
<td>[C$<em>{23}$H$</em>{30}$N$_5$O$_6$S]$^+$</td>
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<td>504.1906</td>
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<tr>
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<tr>
<td>326</td>
<td>[C$<em>{18}$H$</em>{20}$N$_3$O$_3$]$^+$</td>
<td>326.1503</td>
<td>326.1499</td>
<td>1.2</td>
</tr>
<tr>
<td>308</td>
<td>[C$<em>{10}$H$</em>{18}$N$_3$O$_6$S]$^+$</td>
<td>308.0914</td>
<td>308.0911</td>
<td>1.0</td>
</tr>
<tr>
<td>233</td>
<td>[C$<em>8$H$</em>{13}$N$_2$O$_4$S]$^+$</td>
<td>233.0593</td>
<td>233.0591</td>
<td>1.1</td>
</tr>
<tr>
<td>179</td>
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<td>179.0485</td>
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</table>
Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

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Table S5. List of accurate mass measurements and elemental compositions of the product ions of M4 ($R_t = 12.69$ min) following CID

<table>
<thead>
<tr>
<th>Fragment ion (m/z)</th>
<th>Elemental composition</th>
<th>Measured mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>314</td>
<td>$[C_{17}H_{20}N_3O_3]^+$</td>
<td>314.1506</td>
<td>314.1499</td>
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</tr>
<tr>
<td>272</td>
<td>$[C_{16}H_{14}N_3O_3]^+$</td>
<td>272.1036</td>
<td>272.1030</td>
<td>2.3</td>
</tr>
<tr>
<td>244</td>
<td>$[C_{13}H_{14}N_3O_2]^+$</td>
<td>244.1085</td>
<td>244.1080</td>
<td>1.8</td>
</tr>
<tr>
<td>219</td>
<td>$[C_{15}H_{19}N_2O]^+$</td>
<td>219.1495</td>
<td>219.1492</td>
<td>1.4</td>
</tr>
<tr>
<td>217</td>
<td>$[C_{13}H_{17}N_2O]^+$</td>
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<td>0.74</td>
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<tr>
<td>202</td>
<td>$[C_{15}H_{16}NO]^+$</td>
<td>202.1229</td>
<td>202.1226</td>
<td>1.3</td>
</tr>
<tr>
<td>187</td>
<td>$[C_{12}H_{13}NO]^+$</td>
<td>187.0993</td>
<td>187.0992</td>
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<td>$[C_{10}H_{10}NO]^+$</td>
<td>160.0757</td>
<td>160.0757</td>
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</table>
Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

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Figure S1. LC/MS extracted total ion chromatogram (TIC) of the isoxazole ring opened products of \( N-((2\text{-isopropyl-7-methyl-1-oxoisoindolin-5-yl})\text{methyl})\text{isoxazole-5-carboxamide} (P) \) on a UPLC column using different mobile phases (see Supplemental Fig.S2). The sample was generated in 0.1 N NaOH solution as described in the Materials and methods. Data were filtered to show only parent and isoxazole ring opened products (M1, M2, and M4). The UPLC column, flow rate, and the gradient are the same as those described in the Materials and Methods section. A, The mobile phase consisted of water (solvent A, containing 0.1% formic acid, pH = 2.1) and acetonitrile (solvent B, containing 0.1% formic acid). B, The mobile phase consisted of water (solvent A, containing 25 mM HCO2NH4, pH = 5.0) and 95% acetonitrile in water (solvent B). C, The mobile phase consisted of water (solvent A, containing 25 mM CH3CO2NH4, pH = 7.0) and 95% acetonitrile in water (solvent B). No M4 was detected.
Elucidation of a Novel Bioactivation Pathway of a 3,4-Unsubstituted Isoxazole in Human Liver Microsomes: Formation of a Glutathione Adduct of a Cyanoacrolein Derivative Following Isoxazole Ring Opening

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**Figure S2.** LC/Full scan mass spectrum of the isoxazole ring opened products of \( N-((2\text{-isopropyl-7-methyl-1-oxoisindolin-5-yl})\text{methyl})\text{isoxazole-5-carboxamide} \) (P) on a UPLC column using different mobile phases (see Supplemental Fig. S1). A, The molecular ions of M1 and M2 are showed at 13.79 min. The peak area ratio of M2 to M1 is 82 (see Fig. S1A). The mobile phase consisted of water (solvent A, containing 0.1% formic acid, pH = 2.1) and acetonitrile (solvent B, containing 0.1% formic acid). B, The molecular ions of M1 and M2 are showed at 13.67 min. The peak area ratio of M2 to M1 is 2.7 (see Fig. S1B). The mobile phase consisted of water (solvent A, containing 25 mM HCO_2NH_4, pH = 5.0) and 95% acetonitrile in water (solvent B). C, The molecular ions of M1 and M2 are showed at 12.75 min. The peak area ratio of M2 to M1 is 0.33 (see Fig. S1C). The mobile phase consisted of water (solvent A, containing 25 mM CH_3CO_2NH_4, pH = 7.0) and 95% acetonitrile in water (solvent B).
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Figure S3. A, Product ion mass spectrum obtained by CID of the molecular ion of the ^13^C-labeled M3 at m/z 634 from NADPH and GSH-supplemented human liver microsomal incubations containing P (20 μM), the drug stock solution contained ^13^C-labeled CH₃OH. B, MS³ spectrum of fragment ion m/z 327 observed in the product ion spectrum of ^13^C-labeled M3 in A.
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**Figure S4.** A, Product ion mass spectrum obtained by CID of the molecular ion of metabolite M5 ($R_t = 10.82$ min) at m/z 647 from NADPH and GSH-supplemented human liver microsomal incubations containing $P$ (see Supplemental Scheme S1). Drug stock solution contained CH$_3$CH$_2$OH. B, MS$^3$ spectrum of fragment ion m/z 308 observed in the product ion spectrum of M5 in A. C, MS$^3$ spectrum of fragment ion m/z 340 observed in the product ion spectrum of M5 in A.
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Figure S5. A, Product ion mass spectrum obtained by CID of the molecular ion of metabolite M6 ($R_t = 10.08$ min) at $m/z$ 647 from NADPH and GSH-supplemented human liver microsomal incubations containing P (see Supplemental Scheme S1). Drug stock solution contained CH$_3$CH$_2$OH. B, MS$^3$ spectrum of fragment ion $m/z$ 308 observed in the product ion spectrum of M6 in A. C, MS$^3$ spectrum of fragment ion $m/z$ 340 observed in the product ion spectrum of M6 in A.
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**Scheme S1.** Proposed plausible fragmentation pathways of the molecular ion [M+H]$^+$ of the metabolite M5 ($R_t = 10.82$ min) at m/z 647 (see Supplemental Fig. S4).