Formation of A Novel Purine Metabolite through CYP3A4 Bioactivation and Glutathione Conjugation

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Abstract: Background: The study of novel sites of metabolism is important in understanding new mechanisms of biotransformation of a particular moiety by metabolic enzymes. This information is valuable in designing metabolically-stable compounds with drug-like properties. It may also provide insights into the existence of active and reactive metabolites.

Methods: We utilized small scale incubations to generate adequate amounts of the metabolite of interest. After purification, LC-MS/MS and Proton Nuclear Magnetic Resonance (\textsuperscript{\textit{1}}H-NMR) were utilized to unequivocally assign the novel site of glutathione conjugation on the purine ring system.

Results: A proposed novel site of glutathione conjugation was investigated on a diaminopurine-containing molecule. It was demonstrated that the formation of the glutathione conjugate at the C-6 position of the purine ring system was due to the bioactivation of the compound to a di-imine intermediate by CYP3A4, followed by the nucleophilic addition of glutathione.

Conclusion: S-glutathionylation at C-6 position of a purine was proven unequivocally. This previously unreported mechanism constitutes a novel biotransformation for purines.

Keywords: Biotransformation, CYP, drug discovery, glutathione, mass spectrometry, metabolite identification, metabolism, novel, NMR, purine, structural elucidation.

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1. INTRODUCTION

In the fast-paced and competitive environment of early drug discovery, there is a need to identify, profile, and nominate a compound that possesses both pharmacological activity and drug-like properties for further development. Newly synthesized compounds are routinely screened for inherent target inhibition potency and acceptable metabolic stability [1]. Although there are many parameters that need to be optimized in the discovery stage, it is safe to assume that a compound with good metabolic stability across preclinical species and human-based \textit{in vitro} assays has a higher probability of success in achieving adequate systemic exposures with the desired duration [2, 3]. Other factors of importance include presence of human-unique metabolites and reactive metabolites, both of which complicate the development process. In order to de-risk for metabolic instability and presence of reactive or human-unique metabolites, intensive multi-species metabolite profiling is conducted \textit{in vitro}. Generally, liquid chromatography-mass spectrometry is used to elucidate the molecular structure of each metabolite. There are, however, instances where mass spectrometry is not sufficient in fully determining the metabolic soft spots on a molecule due to inadequate fragmentation data [4]. In such cases, after isolation of a metabolite structural elucidation has been conducted using Nuclear Magnetic Resonance (NMR) in preclinical [5] and clinical settings [6].

Glutathione (GSH) conjugation is a Phase II biotransformation reaction aimed at metabolism and clearance of xenobiots. Glutathione is a tripeptide, \textgamma-L-Glutamyl-L-CysteinylGlycine, that is synthesized by living cells and functions as a prominent antioxidant that protects essential cellular components and their functions from reactive oxygen species [7]. Glutathione conjugates are generated either through catalysis by cytosolic and microsomal glutathione S-transferases or through non-enzymatic conjugation with highly electrophilic moieties containing carbon, sulfur, nitrogen or oxygen [8]. Glutathione conjugates generated in the liver are transported into plasma and bile. Glutathione conjugates in the plasma are cleared by the kidney and excreted in the urine [9]. In most cases, the formation of glutathione-S-conjugates results in cellular detoxification of free radical intermediates, however, in some instance glutathione-S-conjugates can result in bioactivation leading to generation of potentially toxic metabolites [10]. Several groups have reported novel sites of glutathione conjugations in dioxin metabolites [11] \textit{via} stable quinone methide intermediates [12].

The novel metabolite reported herein resulted by glutathione conjugation at the C-6 position of the dioxin metabolite. We demonstrated that the parent
molecule was bioactivated to a di-imine electrophilic intermediate by CYP3A4, which then underwent a nucleophilic attack by glutathione. The position of the glutathione conjugation was confirmed by high-resolution mass spectral analysis and 1H-NMR analysis of the metabolite after isolation using liquid chromatography. The novelty associated with this finding is two-fold: 1) a direct conjugation of GSH to the aromatic carbon (C-6) in the purine ring system has not been reported before, and therefore, the mechanism for such biotransformation has never been investigated, 2) the proposed mechanism involved oxidative bioactivation of C-6 catalyzed by CYP3A4 which is novel by itself since previously reported oxidations on this ring system were non-P450 mediated.

2. MATERIALS AND METHODS

2.1. Materials

Monkey liver S9 fraction was obtained from BD Biosciences Pharmingen (San Diego, CA). Trizma pre-set crystals, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), alamethicin, buspirone, potassium phosphate buffer, Krebs-Henseleit buffer powder, trypan blue, and uridine 5′-diphosphoglucuronic acid (UDPGA) were obtained from Sigma (St. Louis, MO). LC-MS grade acetonitrile (ACN), water, and methanol (MeOH) were purchased from EM Science (Gibbstown, N.J.). Deuterated acetonitrile and deuterated methanol were obtained from Cambridge Isotopes Laboratories Inc. (Andover, MA). NMR tubes were acquired from Kimble/Chase Life Science (Vineland, NJ). Human recombinant CYP (Supersomes) CYP3A4, CYP2C9, CYP2C19, CYP1A2, CYP2D6, CYP2C8, and CYP2E1 were purchased from BD Biosciences (San Jose, CA). Cryopreserved pooled mouse, rat, dog, monkey, and human hepatocytes together with hepatocyte thawing medium were obtained from Bioreclamation IVT (Baltimore, MD). The test article (diamine purine containing parent compound) was synthesized in Celgene.

2.2. Hepatocytes Incubation for Metabolite Identification

Two microliters of a 0.5 mM solution of the test article was added to 333 microliters of suspension consisting of one million viable cells per mL of mouse, rat, dog, monkey, or human hepatocytes in Krebs-Henseleit buffer. The samples were incubated for 2 hours at 37°C in a regular incubator (humidified, 95% air, 5% CO2) from NuAire (Plymouth, MN). After 2 hours, 100 microliters of the incubation medium were obtained from Bioreclamation IVT (Baltimore, MD). The test article (diamine purine containing parent compound) was synthesized in Celgene.

2.3. CYP Phenotyping

Five microliters of a 0.5 mM solution of test article was added to 495 microliters of suspension consisting of 50 pmol/mL of individual recombinant P450 CYP1A2, CYP3A4, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 supersomes in 100 mM potassium phosphate buffer, pH=7.4. Twenty five microliters of 100 mM glutathione and twenty five microliters of 20 mM NADPH were added to start the incubation. The samples were incubated at 37°C for 30 minutes in a Precision incubator from Thermo Scientific (Waltham, MA). After 30 minutes of incubation, the reaction was terminated by the addition of 1 milliliter of ice-cold acetonitrile with buspirone as internal standard. Formation of glutathione conjugate was determined by mass spectrometry. The contribution of each CYP isoform to the generation of the glutathione conjugate was determined by the relative quantitation of glutathione conjugate by each isoform compared to that of CYP3A4.

2.4. Time-Dependent CYP Inhibition

Time-dependent CYP inhibition study involved the comparison between CYP IC50’s from two incubations: one with and one without pre-incubation (30 minutes) with the test article at various concentrations.

Inhibition without pre-incubation was conducted by adding 1.25 µL of the test article with concentrations ranging from 0 to 12 mM to 225 microliters of 0.055 mg/mL human liver microsomes in 100 mM potassium phosphate with 5 mM magnesium chloride buffer, pH=7.4; resulting in a final concentration of 0 to 30 µM of the test article. The incubation was initiated by adding twenty five microliters of 0.5 mM testosterone and 10 mM NADPH to the mixture. The samples were incubated for 5 minutes at 37°C with gentle shaking in a Precision incubator from Thermo Scientific (Waltham, MA).

Inhibition with pre-incubation was conducted by adding 1.25 µL of the test article with concentrations ranging from 0 to 12 mM to 200 microliters of 0.060 mg/mL human liver microsomes in 100 mM potassium phosphate with 5 mM magnesium chloride buffer, pH=7.4; resulting in a final concentration of 0 to 30 µM of the test article. Twenty five microliters of 10 mM NADPH was added to initiate the 30 minute pre-incubation. At the end of the pre-incubation period, twenty five microliters of 0.5 mM testosterone and 10 mM NADPH was added to the mixture. The samples were incubated for 5 minutes at 37°C with gentle shaking in a Precision incubator from Thermo Scientific (Waltham, MA).

The samples from both assays were quenched with ice-cold acetonitrile at 2 fold the incubation volume. Formation and quantitation of 6β-hydroxy-testosterone from these samples were determined using a standard calibration range (0.0063 to 5 µM) in identical media and detected by mass spectrometry.

2.5. In-vitro Metabolite Generation and Processing

Two milligrams of the parent compound was incubated for 1 hour with 2 mg/mL monkey liver S9 fractions in 500 milliliters Tris buffer solution containing the following cofactors: 1 mM of NADPH and 0.5 mM reduced glutathione. Following incubation, the sample was quenched with 3 volumes of ice-cold acetonitrile. The sample was dried down using a GeneVac (Stoneridge, NY) centrifuge evaporator at 40°C for 14 hours and reconstituted in 22 milliliter of 20% acetonitrile in water after incubation.

2.6. LC/UV/MS Conditions

Samples were injected into an Agilent (Santa Clara, CA) 1260 Infinity liquid chromatography with a Waters XSelect,
HSST3 Prep column (150 X 10 mm i.d., 5 µm). The flowrate was 1 mL/minute. The samples were eluted with LC/MS-grade water for mobile phase A and LC/MS-grade acetonitrile for mobile phase B for 35 minutes. The mobile phase gradient used was 0 to 3 min: 5% B; 3-5 min: 25% B; 5-30 min: 75% B; 30-32 min: 95% B, and 32-35 min: 5% B.

The eluted peaks were collected using Agilent UV detector at 336 nanometers and an Agilent fraction collector. Waters Synapt QTOF-MS was used to confirm the identity of peaks of interest from the first injection. Settings for the mass spectrometer were electrospray ionization in positive-mode using sensitivity mode; mass to charge range: 50-1200; capillary voltage: 3 kV; source temperature: 120°C; sampling cone: 40; extraction cone: 5; desolvation gas temperature: 500; cone gas flow: 10 L/hr; desolvation gas flow: 890 L/hr; LockSpray Analyte: Leucine Enkephalin (m/z = 556.2771). Fractions for each compound were pooled in a previously weighed vessel. The solvent was removed with a slow stream of nitrogen. The bottles were preweighed to determine the quantity of samples before resuspending in 400 microliters of deuterated DMSO or deuterated methanol in preparation for NMR acquisition. 810 µg of metabolite was isolated using this method.

2.7 NMR Spectroscopy

Parent compound (2 mg) and its glutathione conjugated metabolite (810 µg) were dissolved in DMSO-d₆ and transferred to individual NMR tubes. Two drops of D₂O were added and the samples were incubated at 20°C for 24 hours, prior to collecting NMR data. NMR spectra were obtained on Bruker (Billerica, MA) instrument 500 MHz Ultrashield. Chemical shifts (δ) were reported in ppm. Chemical shifts for ¹H were reported relative to trimethylsilane (TMS) at 80.0. General parameters for a ¹H NMR experiments were: 45° pulse width, 1-second relaxation window, sweep width of 6.4 KHz, acquisition of scans at 20°C, operation at ambient temperature and line broadening (0.2 Hz) which was used to increase signal-to-noise ratio. The ¹H-NMR data was acquired using 32 scans for the parent molecule and 13,000 scans for the glutathione metabolite.

3. RESULTS AND DISCUSSIONS

Glutathione conjugation is a common metabolic reaction involved in the metabolism and clearance of xenobiotics. The formation of this conjugated metabolite could be through...
instantaneous reaction with an activated intermediate or facilitated by glutathione S-transferase [13, 14]. Unexpected glutathione conjugated metabolites have previously been investigated and reported on different scaffolds [15].

In the course of lead optimization in an internal discovery program it was revealed that for a diaminopurine-containing compound, of the over forty Phase I and Phase II metabolites identified (data not shown) in mouse, rat, dog, monkey, and human hepatocytes an unusual glutathione conjugate was the prominent metabolite (Fig. 1). Therefore, a detailed structural analysis of this metabolite was warranted.

Monkey hepatocytes generated a disproportionately higher levels of this metabolite compared to the other species (Fig. 1). Therefore, monkey liver S9 fraction was used to generate sufficient quantity of the glutathione metabolite. After 60 minutes of incubation with monkey liver S9, the LC-UV chromatogram (336 nm) revealed elution of a minor peak at 19.85 minutes and a predominant peak at 20.29 minutes (Fig. 2). Accurate mass and fragmentation data confirmed the minor peak to be due to the parent compound and the predominant peak to be due to the glutathione conjugated metabolite (MH⁺ + 305.0678).

The MS² data suggested presence of a sulfur (31.9720 amu's) on the diaminopurine moiety. However, the exact position of the sulfur could not be determined solely based on mass spectrometry data due to lack of informative secondary fragmentations. Nevertheless, the data suggested that the formation of this metabolite was mediated through a nucleophilic attack by the sulphydryl moiety of the GSH onto the purine ring system.

We believed the C-6 on purine moiety to be the best and most plausible position for addition of sulphydryl subsequent to activation of C-6. This carbon had previously been implicated as a metabolically labile site. However, the existing literature reported only on oxidation of this carbon by aldehyde oxidases (AO) [16, 17] and xanthine oxidases (XO) [18]. Several observations ruled out AO or XO as the culprit enzymes involved in any way in generation of our glutathione conjugate. One such observation was the fact that our in vitro generation of this metabolite required addition of NADPH and GSH in the S9 incubation mixture and was therefore likely to be P450 mediated. Subsequently, in a CYP profiling study involving 7 CYP isozymes we were able to show that the bioactivation step was predominantly orchestrated by CYP3A4 (Fig. 3). Due to its apparent novelty, a NMR study was launched to better understand the type of oxidation and unequivocally assign the position of GSH conjugation in this metabolite.

To enable our ¹H-NMR analysis, 810 µg of the glutathione metabolite was isolated using liquid chromatography (see Materials and Methods).

A careful inspection of the relevant ¹H-NMR chemical shifts in the down field aromatic region placed the site of glutathione conjugation on C-6 of the purine ring system,
unequivocally (Figs. 4 and 5). As discussed above, our mass spectrometry data supported the linkage to be through a thioether bond. In the parent ¹H-NMR spectra, we observed signals for the aromatic proton on the purine with chemical shifts at 7.61 and 8.06 ppm and cumulative area integrations close to unity (Fig. 4).

The C-6 proton was manifested as two signals, H01/H02 (Table 1), due to two distinct magnetic environments the proton experienced which resulted from restricted rotation of R1 around the single bond attached to the amine at C-2. The net effect of this rotation caused the proton at C-6 to exist as a pair of conformers, cumulatively integrating to ~1 proton. Importantly, these proton signals were absent in the glutathione conjugate. This was consistent with a lack of a proton at C-6; a structural feature of our hypothesized metabolite (Table 1, Fig. 5).

Another indirect proof for the position of oxidation came through the observation that a methyl substitution at C-6 prevented formation of any glutathione conjugates in monkey hepatocyte incubations. The weight and nature of the evidence supported our hypothesis in establishing a thioether linkage between C-6 of the purine moiety and glutathione. A mechanism of formation is proposed in (Fig. 6).

CYP inhibition assays using a recombinant P-450 panel did not lead to any time-dependent inhibition of the metabolizing enzymes, suggesting that the bioactivation of C-6 was unlikely to result in promiscuous non-specific protein bonding. In fact, the reactivity of this intermediate was mild enough to spare the metabolizing enzyme and eventually bind with GSH. We also investigated the fate of this intermediate in absence of GSH to model for GSH depleted environments which may happen under certain circumstances [19]. When the parent compound was incubated with rCYP3A4 in absence of GSH, an oxidative metabolite was generated in a higher abundance compared to...
when GSH was present. This oxidative metabolite required NADPH and exhibited an addition of 16 amu’s onto the purine ring system. This suggested that the electrophilic center (C-6) in the activated di-imine could be hydroxylated in absence of GSH. In light of these findings, we concluded that S-glutathionylation and hydroxylation of C-6 constituted compensatory clearance mechanisms for the bioactivated di-imine intermediate.

CONCLUSION

A diaminopurine can be biotransformed via CYP3A4 to a di-imine intermediate which can subsequently undergo S-glutathionylation and hydroxylation of C-6 constituted compensatory clearance mechanisms for the bioactivated di-imine intermediate.

CONFLICT OF INTEREST

The authors have no conflict of interest and no payment has been received in the preparation of this manuscript. All authors were employees of Celgene at the time of writing this manuscript.

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REFERENCES

[1] Kulkarni, A.; Riggs, J.; Phan, C.; Bai, A.; Calabrese, A.; Moghaddam, M.F. Proposing advancement criteria for efficient DMPK triage of new chemical entities. Future Med. Chem., 2014, 6(2), 131-139.
[2] Richardson, S.; Bai, A.; Katz, J.; Kulkarni, A.; Moghaddam, M.F. Efficiency in drug discovery: using S9 fractions as a gating DMPK drug discovery screen. Drug Metab Lett., 2016, 10(2), In press.
[3] Moghaddam, M.F.; O’Brien, Z.; Tang, Y.; Bai, A.; Richardson, A.; Bacolod, M.; Kulkarni. A. A proposed screening paradigm for discovery of covalent inhibitor drugs. Drug Metab. Lett., 2014, 8(1), 19-30.
[4] Apuy, J.L.; Bai, A.; Paisner, D.; Hegde, S.G.; Moghaddam, M.F. Application of 1D and 2D 1H-NMR in the structural elucidation of a N-glucuronidated metabolite and oxidized metabolites generated in microsomal incubation. Drug. Metab. Lett., 2012, 6, 265-274.
[5] Bylund, J.; Macsari, I.; Besidski, Y.; Olofsson, S.; Petersson, C.; Arvidson P.; Bueters, T. Novel bioactivation mechanism of reactive metabolite formation from phenyl methyl-isoxazoles. Drug Metab. Dispos., 2012, 40, 2185-2191.
[6] Srivastava, A.; Lian, L-Y.; Maggs, J.L.; Chaponda, M.; Pirmohamed, M.; Williams, D.P.; Park, B.K. Quantifying the metabolic activation of nevirapine in patients by integrated applications of NMR and mass spectrometries. Drug Metab. Dispos., 2010, 38, 122-132.
[7] Hopkins, F.G. On Glutathione: A Reinvestigation. J. Biol. Chem., 1929, 84, 269-320.
[8] Jakoby, W.B. Glutathione transferases: an overview. Methods Enzymol., 1985, 113, 495-499.
[9] Abbott, W.A.; Meister, A. Intrahepatic transport and utilization of biliary glutathione and its metabolites. Proc. Natl. Acad. Sci., 1986, 83(5), 1246-1250.
[10] Anders, M.W.; Dekant, W.; Vamvakas, S. Glutathione-dependent toxicity. Xenobiotica, 1992, 22(9-10), 1135-1145.

Table 1. Relevant aromatic proton chemical shift assignments for the parent molecule and the glutathione conjugated metabolite.

| Proton Number | Parent Molecule | Glutathione Conj Metabolite |
|---------------|----------------|-----------------------------|
| δ (ppm) | No. of Protons | Type | δ (ppm) | No. of Protons |
| H01 | 7.61 | 0.5 | s | - |
| H02 | 8.06 | 0.4 | s | - |

*a*Number of protons integrated relative to each other.

![Image](image_url)

Fig. (6). A proposed mechanism for glutathione conjugate formation on diaminopurine moiety.
[11] Hu, Y.; Yang, S.; Shilliday, B.; Heyde, B.R.; Mandrell, K.M.; Robins, R.H.; Xie, J.; Reding, M.T.; Lai, Y.; Thompson, D.C. Novel metabolic bioactivation mechanism for a series of anti-inflammatory agents (2,5-Diaminothiophene Derivatives) mediated by cytochrome P450 enzymes. *Drug Metab. Dispos.*, 2010, 38, 1522-1531.

[12] Gunduz, M.; Argikar, U.; Kamel, A.; Colizza, K.; Bushee, J.; Cirillo, A.; Lombardo, F.; Harriman, S. Oxidative ipso substitution of 2,4-Difluoro-benzylphthalazines: identification of a rare stable quinone methide and subsequent GSH conjugate. *Drug Metab. Dispos.*, 2012, 40, 2074-2080.

[13] Jakoby, W.B.; Habig, W.H. Glutathione Transferases. In: *Enzymatic Basis of Detoxification*; Jakoby, W.B. Ed.; Academic Press: New York, 1980, Vol. 2, pp. 63-70.

[14] Okada, R.; Maeda, K.; Nishiyama, T.; Aoyama, S.; Tozuka, Z.; Hiratsu, A.; Ikeda, T.; Kusuhara, H.; Sugiya, Y. Involvement of different human glutathione transferase isoforms in the glutathione conjugation of reactive metabolites of troglitazone. *Drug Metab. Dispos.*, 2011, 39, 2290-2297.

[15] Kang, P.; Dalvie, D.; Smith, E.; Zhou, S.; Deese, A. Identification of a novel glutathione conjugate of flutamide in incubations with human liver microsomes. *Drug Metab. Dispos.*, 2007, 35, 1081-1088.

[16] Hall, W.W.; Krenitsky, T.A. Aldehyde oxidase from rabbit liver: specificity towards purines and their analogs. *Arch. Biochem. Biophys.*, 1986, 251, 36-46.

[17] Kwienty, H.; Gershon, L.; Bergmann, F. Mechanism of enzymatic oxidation of purines. *Science*, 1959, 130 (3377), 711-712.

[18] Bergmann, F.; Kwienty, H.; Levin, G.; Engelberg, H. Studies on the enzymatic oxidation of aminopurines. *Biochim. Biophys. Acta.*, 1960, 37, 433-441.

[19] Rinaldi, R.; Eliasson, E.; Swedmark, S.; Morgenstern, R. Reactive intermediates and the dynamics of glutathione transferases. *Drug Metab. Dispos.*, 2002, 30, 1053-1058.