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ABSTRACT

The current study examined the bioactivation potential of ghrelin receptor inverse agonists, 1-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]nonan-7-yl)-2-(imidazol[2,1-b]thiazol-6-yl)ethanone (1) and 1-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]nonan-7-yl)-2-(2-methylimidazo[2,1-b]thiazol-6-yl)ethanone (2), containing a fused imidazo[2,1-b]thiazole motif in the core structure. Both compounds underwent oxidative metabolism in NADPH- and glutathione-supplemented human liver microsomes to yield glutathione conjugates, which was consistent with their bioactivation to reactive species. Mass spectral fragmentation and NMR analysis indicated that the site of attachment of the glutathionyl moiety in the thiol conjugates was on the thiazole ring within the bicyclic core. Two glutathione conjugates were discerned with the imidazo[2,1-b]thiazole derivative 1. One adduct was derived from the Michael addition of glutathione to a putative S-oxide metabolite of 1, whereas, the second adduct was formed via the reaction of a second glutathione molecule with the initial glutathione-S-oxide adduct. In the case of the 2-methylimidazo[2,1-b]thiazole analog 2, glutathione conjugation occurred via an oxidative desulfation mechanism, possibly involving thiazole ring epoxidation as the rate-limiting step. Additional insights into the mechanism were obtained via 18O exchange and trapping studies with potassium cyanide. The mechanistic insights into the bioactivation pathways of 1 and 2 allowed the deployment of a rational chemical intervention strategy that involved replacement of the thiazole ring with a 1,2,4-thiadiazole group to yield 2-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]nonan-7-yl)-2-(2-methylimidazo[2,1-b]thiazol-6-yl)ethanone (3). These structural changes not only abrogated the bioactivation liability but also retained the attractive pharmacological attributes of the prototype agents.

Introduction

Ghrelin is a 28-amino acid hunger-stimulating peptide that is produced in the stomach, pancreas, and hypothalamus (Kojima et al., 1999; Inui et al., 2004). Ghrelin is a ligand for the growth hormone secretagogue receptor type 1a, GHSR-1α, which is primarily expressed in the pituitary gland, brain, and to a lesser extent in the periphery. Through its action on GHSR-1α, ghrelin exerts a variety of metabolic functions including stimulation of growth hormone release, stimulation of appetite and weight gain, and suppression of insulin secretion in rodents and humans (Nakazato et al., 2001; Wren et al., 2001; Dezaki et al., 2008; Cardona Cano et al., 2012; Heppner et al., 2012). Thus, antagonizing growth hormone secretagogue receptor (GHSR)-1α with small molecule antagonists or inverse agonists is anticipated to improve glucose homeostasis and insulin sensitivity, while eliciting beneficial effects on body weight (Serby et al., 2006; Esler et al., 2007; Rudolph et al., 2007; Soares et al., 2008; Costantino and Barlocco, 2009). A recent report from our laboratory disclosed a proprietary series of spirocyclic piperidine-azetidine derivatives as potent and orally active inverse agonists of GHSR-1α (Kung et al., 2012). Structure-activity relationship studies revealed that analogs with distributed polarity in the form of the fused imidazo[2,1-b]thiazole acetamide and phenyl triazole functionalities (e.g., 1-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]nonan-7-yl)-2-(imidazo[2,1-b]thiazol-6-yl)ethanone (1) and 1-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]nonan-7-yl)-2-(2-methylimidazo[2,1-b]thiazol-6-yl)ethanone (2) depicted in Fig. 1) demonstrated significant improvements in binding affinity (IC50 < 10 nM) to the ghrelin receptor, while consistently displaying inverse agonism in a ghrelin functional assay. In addition, these structural changes also led to an improved pharmacokinetic profile (1 clearance; ↑ oral bioavailability) in animals, which...
warranted a further examination of relevant preclinical safety endpoints as a prelude to candidate nomination.

From a drug metabolism perspective, the presence of the imidazo[2,1-b]thiazole scaffold in 1 and 2 raised a significant cause for concern, given the possibility of a cytochrome P450 (P450)-mediated S-oxidation on the thiazole sulfur (within the imidazo[2,1-b]thiazole framework) to yield an electrophilic S-oxide metabolite capable of reacting with proteins and/or the endogenous antioxidant glutathione (GSH). Indeed, the characterization of a GSH conjugate derived from Michael addition to an S-oxide metabolite of the imidazo[2,1-b]thiazole derivative, 5-[4-(methylsulfonyl)phenyl]-6-phenylimidazo[2,1-b]thiazole inhibitor (L-766,112) (Fig. 1) provides precedence for this bioactivation pathway in liver microsomes (Thérien et al., 1997; Trimble et al., 1997). Furthermore, a successful medicinal chemistry strategy involving internalization of the thiazole motif, resulting in the elimination of reactive metabolite liability of L-766,112, while retaining the primary pharmacology (selective inhibition of cyclooxygenase-2) has been presented in subsequent work (Fig. 1) (Roy et al., 1997).

Other than thiazole-S-oxidation, an additional bioactivation pathway can arise through thiazole ring scission (via the C4–C5 epoxidation → diol pathway) (Chatfield and Hunter, 1973; Mizutani et al., 1993; Yabuki et al., 1997; Yoon et al., 1998; Obach et al., 2008). The corresponding thioamide metabolites, which are the by-products of thiazole ring cleavage, can undergo additional S-oxidation to electrophilic sulfenic acid derivatives (Dansette et al., 2005; Mansuy and Dansette, 2011) capable of oxidizing or forming mixed disulfide adducts with proteins or GSH and eliciting toxicity (Ziegler-Skylakakis et al., 1998; Hajovsky et al., 2012).

In this report, we summarize our findings on the in vitro bioactivation of 1 and 2 in human liver microsomes (HLM) utilizing exogenously added nucleophiles to trap reactive species. In the case of the imidazo[2,1-b]thiazole derivative 1, two GSH adducts were formed. One adduct was derived from the addition of the thiol to a putative S-oxide metabolite of 1, whereas the formation of the second adduct involved the attachment of a second GSH molecule to the initial GSH-S-oxide adduct with concomitant liberation of the oxidized thiazole sulfur. With respect to the corresponding 2-methylimidazo[2,1-b]thiazole analog 2, GSH conjugation occurred via an oxidative desulfation mechanism, possibly involving thiazole ring epoxidation as the rate-limiting step. Characterization of the GSH conjugate structure and additional trapping studies with cyanide ion proved to be useful in deciphering the overall bioactivation mechanism associated with 2. The in vitro metabolism information was used to design the corresponding thiaadiazole analog, 1-{2-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]nonan-7-yl}-2-(2-methylimidazo[2,1-b][1,3,4]thiadiazol-6-yl)ethanone analog 3 (see Fig. 1) that would be devoid of reactive metabolite formation. Indeed, 3 was resistant to bioactivation in HLM (inferred from the lack of GSH conjugate formation) while retaining inverse agonist potency for the ghrelin receptor and HLM stability noted with 1 and 2.

Materials and Methods

Materials. NADPH, GSH, and potassium cyanide were obtained from Sigma-Aldrich (St. Louis, MO). HLM were prepared from a mixed sex pool of 50 donors (provided by BD Biosciences, Woburn, MA). H218O (97%) and dimethyl sulfoxide (DMSO)-d6 “100%” were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). All other commercially available reagents and solvents were of either analytical or high-performance liquid chromatography (HPLC) grade. The preparation of title compounds 1–3 is provided in the Supplemental Methods and Supplemental Fig. 1.

Metabolite Identification and Reactive Metabolite Trapping Studies in HLM. Liver microsomal incubations were conducted at 37°C for 60 minutes in a shaking water bath. The incubation volume was 1.0 ml and consisted of 0.1 M potassium phosphate buffer (pH 7.4), MgCl2 (3.3 mM), HLM (protein concentration = 1.0 mg/ml), P450 concentration = 0.5 μM), test compound (10 μM), and NADPH (1.3 mM). Additional microsomal incubations also included GSH.
to dryness. In the case of M3-1, it was difficult to obtain a pure isolate because of poor chromatographic resolution between M3-1 and M2-1. Hence, a crude isolate of M3-1 was used in NMR characterization. The residues were evacuated in a dry box for ~2 hours prior to reconstitution in DMSO-d6 for NMR analysis.

**NMR Analysis of Metabolites.** NMR spectra for 1 and M3-1 were recorded on aBruker Avance 600 MHz instrument (Bruker BioSpin Corporation, Billerica, MA) controlled by TOPSPIN V3.0 and equipped with a 1.7 mm cryo-triple resonance probe (TCI). NMR spectra for 2 and M5-2 were recorded on a Bruker Avance 600 MHz instrument (Bruker BioSpin Corporation) controlled by TOPSPIN V2.1 and equipped with a 5 mm cryo-TCI probe. Synthetic samples and isolated materials were dissolved in 0.15 ml (5 mm TCI probe) or 0.05 ml (1.7 mm TCI probe) of DMSO-d6. All spectra were referenced using residual DMSO-d6 (δ = 2.49 ppm relative to tetramethylsilane, δ = 0.00 for 1H and δ = 39.5 ppm relative to tetramethylsilane, δ = 0.00 for 13C). One-dimensional spectra were typically recorded using a sweep width of 8000 Hz and a total recycle time of approximately 7 seconds. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal-to-noise ratio. The two-dimensional data [correlation spectroscopy, total correlation spectroscopy (TOCSY), multiplicity edited heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation] were recorded using the standard pulse sequences provided by Bruker. A 1 K × 128 data matrix was acquired using a minimum of 4 scans and 16 dummy scans. The data were zero-filled to a size of 1 K × 1 K. A mixing time of 80 milliseconds was used in the TOCSY experiments.

**Results**

**In Vitro Metabolism of Imidazo[2,1-b]thiazole Derivative 1 in HLM.** In HLM supplemented with NADPH and GSH, three metabolites of 1 (denoted as M1-1, M2-1, and M3-1) were observed (Fig. 2A). Collision-induced dissociation (CID) spectra of 1 and its metabolites M1-1, M2-1, and M3-1 are shown in Figs. 3–5. In the case of 1 (tR = 18.82 minutes; MH+ = 482.1524), the major fragment ions present at mass-to-charge ratio (m/z) 318.1476, 301.1210, 262.1004, 192.0318, and 165.0112 were consistent with the structure (see CID spectrum in Fig. 3A). Metabolite M1-1 eluted at tR = 17.70 minutes and possessed MH+ = 516.1579, which is an addition of 34.0055 Da to the molecular mass of 1 (Fig. 3B). The presence of the fragment ions at m/z 318.1476 and 192.0318 in the CID spectrum of M1-1 indicated that the 2-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3,5]-nonane portion was unaltered. A proposed structure of M1-1 that is consistent with the observed molecular weight and mass spectrum is shown in Fig. 3B.

Metabolites M2-1 (tR = 17.08 minutes) and M3-1 (tR = 16.47 minutes) possessed MH+ at 1096.3200 and 805.2311 m/z, respectively. The CID spectra of M2-1 and M3-1 are shown in Figs. 4 and 5, respectively. M2-1 contained two molecules of GSH in its structure as evident from the facile loss of two pyroglutamic acid components (m/z 967.2753 and m/z 838.2335) in its CID spectrum. Furthermore, the presence of the fragment ion at m/z 318.1468 implied that the 2-[2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3,5]-nonane portion in M2-1 was unchanged. A structure of M2-1 that is compatible with the observed molecular weight and fragmentation pattern is shown in Fig. 4, and a mechanistic hypothesis that rationalizes its formation is provided in Fig. 15 in the Discussion.

The molecular mass of M3-1 (MH+ = 805.2312) suggested that the metabolite was derived from the addition of GSH to an oxidized metabolite of 1. The fragment ions at m/z 730.1973 and m/z 676.1872 in the CID spectrum of M3-1 were derived from the characteristic losses of the glycine (75 Da) and glutamic acid components (129 Da) of GSH (Baillie and Davis, 1993). The diagnostic fragment ions at m/z 488.0904, 359.0471, and 318.1476 indicated that the 2-[2-chloro-4-(2H-1,2-
2,3-triazol-2-yl)benzyl-2,7-diazaspiro[3,5]-nonane] scaffold in 1 was unmodified. On the basis of the fragmentation pattern, we inferred that oxidation and subsequent GSH conjugation had occurred on the imidazo[2,1-b]thiazole ring. There are two obvious pathways through which this can arise, either via a P450-mediated epoxidation at the C4-C5 position of the thiazole ring with addition of GSH or through a P450-mediated S-oxidation of the thiazole sulfur followed by addition of GSH to the S-oxide metabolite (structure depicted in Fig. 5), similar to one discerned with the imidazo[2,1-b]-thiazole derivative L-766,112 (Trimble et al., 1997). To distinguish between the two possibilities, a

Fig. 2. Extracted ion chromatograms of incubation mixtures of 1 (A) and 2 (B) in NADPH- and GSH-supplemented HLM conducted at 37°C for 60 minutes.

Fig. 3. CID spectra of 1 (A) and M1-1 (B).
crude isolate of M3-1 was obtained from a large-scale HLM incubation of 1 in the presence of NADPH and GSH and subsequently characterized by NMR spectroscopy.

Observed in the $^1$H-$^1$C HSQC spectrum of the M3-1 isolate (Fig. 6B) is a methine cross-peak with a $^1$H chemical shift of $\delta$ 6.05 ppm that correlates to a $^1$C atom with a chemical shift of $\delta$ 58.6 ppm. Furthermore, there is also set of cross-peaks that indicate a pair of inequivalent methylene resonances with $^1$H shifts of $\delta$ 3.56/4.65 ppm and a correlation to a $^1$C at $\delta$ 62.9 ppm in the HSQC data. If the bioactivation of 1 is mediated via the epoxidation pathway, the resulting molecule would have two adjacent methine protons with chemical shifts in the range of $\delta$ 5.0-6.0 ppm with corresponding $^1$C...
shifts in the range $\delta$ 80 ppm (for HCOH) and $\delta$ 60 ppm (for HCSG). Alternatively, if the bioactivation of 1 is mediated via the S-oxidation pathway, the resulting molecule would have one methine $^1$H adjacent to a methylene, again each with $^1$H chemical shifts in the range of $\delta$ 5.0–6.0 ppm. Unlike the GSH-epoxide addition product, the $^{13}$C chemical shifts of the GSH-S-oxide conjugate would have $^{13}$C shifts for both carbons in the range of $\delta$ 50–60 ppm. The chemical shifts observed in the $^1$H-$^{13}$C HSQC spectrum of M3-1 are more consistent with the structure resulting from the S-oxidation pathway and are also closely aligned with the NMR data reported for the GSH conjugate of L-766,112 S-oxide (Trimble et al., 1997). Furthermore, the TOCSY data set (Fig. 6A) for the M3-1 isolate contains cross-peaks between the $\delta$ 6.05 ppm and the $\delta$ 3.56/4.65 ppm resonances, indicating a direct coupling between all of these protons. On the basis of these data the M3-1 isolate is tentatively assigned as the thiazole-S-oxide with a GSH molecule attached at the C3 position of the thiazole ring. This structure strongly infers that the route of formation of M3-1 is through a biotransformation step involving S-oxidation.

The potential for S-oxide formation was then specifically examined in NADPH-supplemented HLM incubations of 1 (10 $\mu$M) in the absence and presence of GSH (10 mM) (Fig. 7, A and B, respectively). Two monohydroxylated metabolites (MH$^+$ = 498.1473) designated as M4-1 (minor) and M5-1 (major) were observed in HLM incubations in the absence of GSH. The CID spectrum of the minor metabolite M4-1 is shown in Supplemental Fig. 2. The detection of a fragment ion at $m/z$ 334.1420 (addition of oxygen to $m/z$ 318.1473) indicated that M4-1 was derived from a monohydroxylation on the spirocyclic-azetidine ring. In contrast, the CID spectrum of the major metabolite M5-1 (Fig. 8) contained fragment ions that suggested that the imidazo[2,1-b]thiazole motif was the site of oxidation. Furthermore, the fragment ion at $m/z$ 452.1401 (MH$^+$-SO) implied that M5-1 was the S-oxide metabolite of 1. Addition of GSH to the NADPH-supplemented HLM incubation of 1 significantly diminished the levels of M5-1 (Fig. 7B), which is consistent with the adduction of M5-1 to GSH resulting in the formation of M3-1.

In Vitro Metabolism of 2-Methylimidazo[2,1-b]thiazole Derivative 2 in HLM. Figure 2B depicts an extracted ion chromatogram of an incubation mixture comprised of NADPH, GSH, and 2 conducted at 37°C for 60 minutes. A total of five metabolites was observed in a NADPH-dependent fashion. The CID spectrum of 2 ($t_r = 19.47$ minutes, MH$^+$ = 496.1680) and proposed structures of diagnostic fragment ions ($m/z$ 318.1473, 276.1159, and 192.0317) is shown in Fig. 9A. Metabolites M1-2 ($t_r = 19.0$ minutes) and M2-2 ($t_r = 21.46$ minutes) possessed MH$^+$ at 512.1630, suggesting that they were isomers derived from a monohydroxylation in 2. The CID spectra of both metabolites (Supplemental Figs. 3 and 4) revealed fragment ions at $m/z$ 494.1510 (loss of a water molecule) and $m/z$ 334.1420 indicative of monohydroxylation on the spirocyclic-azetidine ring. Postulated structures of M1-2 and M2-2 are depicted in Supplemental Figs. 3 and 4, respectively. Considering that

![Fig. 6. Abbreviated $^1$H-$^1$H TOCSY (A) and $^1$H-$^{13}$C HSQC spectrum (B) of M3-1. Red cross peaks in (B) indicate methine, whereas the blue cross peaks indicate the methylene resonances.](imageURL)
M2-2 elutes after 2 under the reversed phase HPLC conditions, we speculate that M2-2 is an \( N \)-oxide metabolite of 2 by inference to related tertiary basic amine drugs, such as loperamide, that undergo \( N \)-oxidation (Kalgutkar and Nguyen, 2004).

Metabolite M3-2 (\( t_R = 18.0 \) minutes) displayed a MH\(^+\) at 530.1735. The addition of 34.0055 Da to the MH\(^+\) of 2 indicated that M3-2 was the corresponding diol metabolite derived from hydrolysis of an intermediate thiazole ring epoxide. The fragment ions at \( m/z \) 318.1473, 213.0322, and 192.0317 were consistent with the proposed structure (Fig. 9B).

Metabolite M4-2 (\( t_R = 17.1 \) minutes) also possessed a MH\(^+\) at 512.1630, consistent with a monohydroxylation in 2. In contrast with M1-2 and M2-2, metabolite M4-2 appeared to be derived from an...
oxidation on the 2-methylimidazo[2,1-b]thiazole ring system as indicated by the fragment ion \( m/z \) 318.1473 in the CID spectrum of M4-2. A proposed structure for M4-2 that is compatible with the observed mass spectrum is shown in Supplemental Fig. 5.

Metabolite M5-2 (\( t_R = 15.89 \) minutes) possessed a MH+ at 787.2747 and demonstrated a fragment ion at \( m/z \) 658.2308, which was consistent with the loss of a glutamic acid component (129 Da) of GSH (Fig. 10A). This observation suggested that M5-2 was a GSH conjugate of 2. The presence of the fragment ion at \( m/z \) 318.1474 implied that the site of attachment of GSH was on the 2-methylimidazo[2,1-b]thiazole ring system. Furthermore, the fragment ion at \( m/z \) 514.1776 was assigned as a cleavage adjacent to the cysteinyl thioether moiety with charge retention on the imidazole residue. The occurrence of the fragment ion at \( m/z \) 514.1776 is consistent with the presence of an aromatic thioether motif in M5-2 (Baillie and Davis, 1993). A proposed structure for M5-2 that is consistent with its mass spectrum is shown in Fig. 10A.

Replacement of GSH with N-acetylcysteine as a trapping agent in NADPH-supplemented HLM incubations of 2 led to the formation of M6-2 (\( t_R = 18.2 \) minutes, MH′ = 643.2212), an analogous conjugate of M5-2. The CID spectrum of M6-2 and interpretation of key fragment ions is depicted in Fig. 10B. As such, elucidation of the structure of the GSH conjugate (M5-2) was largely aided by the isolation of the thiol adduct from large-scale HLM incubations of 2 in the presence of NADPH and GSH, and subsequent characterization by NMR spectroscopy.

**NMR Characteristics of GSH Conjugate M5-2.** The \(^1\)H spectrum M5-2 contains several critical differences from that of a similarly acquired spectrum of 2. Most notably the change in both the \(^1\)H and \(^13\)C chemical shifts of the methyl of the imidathiazole (\( \delta = 2.37 \) ppm for \(^1\)H and \( \delta = 13.1 \) ppm for 2 and \( \delta = 2.15 \) ppm for \(^1\)H and \( \delta = 26.7 \) ppm for M5-2) and the appearance of a new methylene resonance in the \(^1\)H spectrum of M5-2 at \( \delta = 4.93 \) ppm (Fig. 11, top panel). In the HSQC data set this new \(^1\)H resonance correlates with a \(^13\)C resonance at \( \delta = 55.1 \) ppm (unpublished data). Additionally, the \(^1\)H resonances for GSH as well as those from the remaining 1-[2-2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl]-2,7-diazaspiro[3.5]non-7-yl]-2-(2-methylimidazo[2,1-b][1,3]thiazol-6-yl)ethanone can be rationalized. The critical data for the structural determination of M5-2 is the \(^1\)H-\(^13\)C heteronuclear multiple bond correlation data (Fig. 12). In this data set there are correlations from both the new methylene resonance (\( \delta = 4.93 \) ppm) and the methyl resonance (\( \delta = 2.37 \) ppm) to a \(^13\)C with a chemical shift of \( \delta = 201.7 \) ppm. A \(^13\)C chemical shift in the range of 190–220 ppm is unique and indicates a carbonyl with no adjacent hetero-atom, either a ketone or aldehyde. A structure that satisfies all these data is shown in Fig. 10A, the biosynthetic pathway of which will be revealed in the Discussion.

\textbf{\(^18\)O Incorporation Studies.} The source of the carbonyl oxygen in GSH conjugate M5-2 was investigated in NADPH- and GSH-supplemented HLM incubations of 2 conducted in H\(_2\)\(^{18}\)O. As expected, the diol metabolite M3-2 incorporated \(^18\)O (MH′ = 532.1778) consistent with epoxide ring opening by H\(_2\)\(^{18}\)O (Supplemental Fig. 6). MS/MS analysis of the molecular ions of \(^16\)O- and \(^18\)O-incorporated M3-2 (MH′ = 530.1725 and MH′ = 532.1778, respectively) clearly indicated the 2-atomic mass unit shift in the parent ion and in the acylion fragment \( m/z \) 213.0322 (\(^16\)O M3-2) to \( m/z \) 215.0369 (\(^18\)O M3-2) (compare Fig. 13 with Fig. 9B). In contrast, \(^18\)O incorporation
was not observed in the GSH adduct M5-2. This observation suggested that the source of the carbonyl oxygen in M5-2 is derived from molecular oxygen.

**Trapping Studies with Potassium Cyanide.** Attempts to trap the putative iminium ion intermediate involved in the formation of GSH conjugate M5-2 (see Discussion and Fig. 16 on the proposed mechanism for the formation of M5-2) were initiated in NADPH-supplemented HLM incubations of 2 in the presence of potassium cyanide (5 mM). Figure 14 indicates the CID spectrum of an adduct M7-2 ($t_R = 18.4$ minutes, $MH^+ = 539.1739$) formed in these incubations in a NADPH- and cyanide-dependent fashion. The proposed structure of the cyano adduct M7-2 and corresponding fragment ions that led to the structural elucidation are also shown in Fig. 14. A mechanism for the formation of M7-2 is shown in Fig. 16 (see Discussion).

**In Vitro Bioactivation of 2-Methylimidazo[2,1-b]thiazole Derivative 3 in Human Liver Microsomes.** LC-MS/MS analysis of an incubation mixture of 3 in NADPH- and GSH-supplemented HLM did not reveal the presence of any GSH conjugates of 3, suggesting that 3 is devoid of the bioactivation liability associated with 1 and 2.

**Comparison of the In Vitro Ghrelin Receptor Pharmacology and HLM Stability of Compounds 1–3.** Potency against the ghrelin receptor was determined in the previously described GHSR-1a binding assay using $[^{125}]$Ighrelin (Kung et al., 2012). As shown in Fig. 1, the IC$_{50}$ values for 1, 2, and 3 were 8.0, 4.7, and 2.6 nM, respectively. The microsomal stability of compounds 1–3 (final concentration = 1 μM) was assessed by monitoring substrate consumption after incubation with HLM in the presence of NADPH cofactor for 30 minutes at 37°C. Microsomal half-lives, reflecting depletion of test compounds, were scaled to the corresponding intrinsic clearance values using the well-stirred model (Obach, 1999). Under these experimental conditions, the half-lives of 1, 2, and 3 in HLM were 19.1, 28.6, and 38.8 minutes, which translated into intrinsic clearance values (ml/min/kg) of 48, 32, and 24, respectively.

**Discussion**

The otherwise attractive pharmacological and pharmacokinetic attributes of ghrelin inverse agonists 1 and 2 were offset by the presence of the imidazo[2,1-b]thiazole bicycle as part of the core structure, especially in light of previous studies by Trimble et al. (1997) who demonstrated the microsomal S-oxidation of the thiazole portion (within the bicycle) to an electrophilic sulfoxide metabolite that underwent a 1,4-Michael addition reaction with GSH. In addition, evidence linking thiazole ring bioactivation with preclinical (or clinical) toxicity has been presented for several thiazole-based xenobiotics and drugs (Kalgutkar et al., 2005). For example, the clinical hepatotoxicity noted with the nonsteroidal anti-inflammatory drug sudoxicam (Supplemental Fig. 6) has been attributed to a metabolic process involving thiazole ring scission to a toxic thiourea metabolite (Obach et al., 2008). The structurally related anti-inflammatory agent (and marketed drug) meloxicam does not possess the hepatotoxic liability associated with sudoxicam. Although introduction of a methyl group at the C5 position of the thiazole ring in meloxicam is the only structural difference, the change dramatically alters the metabolic profile such that oxidation of the C5 methyl group to the alcohol metabolite (see Supplemental Fig. 7) constitutes the principal metabolic
fate of meloxicam in humans with virtually no detectable thiazole ring opening (Chesné et al., 1998; Obach et al., 2008).

Taking into consideration the above mentioned structure-toxicity relationship and the growing evidence linking immune-mediated drug toxicity with reactive metabolite formation (Park et al., 2011; Stepan et al., 2011; Sakatis et al., 2012; Thompson et al., 2012), we decided to examine the bioactivation potential of 1 and 2 in HLM. Incubation of 1 and 2 in NADPH- and GSH-supplemented HLM led to the formation of reactive species that were trapped with GSH. Metabolism (including bioactivation) of both 1 and 2 was blocked when the HLM incubations were conducted in the presence of ketoconazole (a selective CYP3A4 inhibitor), which implicated a role for CYP3A4 in the metabolism of the two imidazo[2,1-b]thiazole derivatives. LC-MS/MS characterization of the GSH conjugates suggested that the site of bioactivation in both compounds was on the imidazo[2,1-b]thiazole bicycle. In the case of the imidazo[2,1-b]thiazole derivative 1, the formation of the diol metabolite M1-1 was consistent with a bioactivation pathway involving thiazole ring oxidation by P450 enzyme(s) at the

![Fig. 11. Full $^1$H spectrum of M5-2 and 2.](image1)

![Fig. 12. Abbreviated $^1$H-$^{13}$C heteronuclear multiple bond correlation (HMBC) spectrum of M5-2 (A and B represent specific $^1$H correlations highlighted in the text).](image2)
C2–C3 position to yield the electrophilic epoxide intermediate 12, which is hydrolyzed to the diol M1-1 or can potentially react with GSH to yield the sulfydryl conjugate 13 (Fig. 15, pathway A). An alternate pathway that leads to M3-1 involves P450-mediated oxidation on the thiazole sulfur to the S-oxide metabolite M5-1, followed by the Michael addition of GSH to yield adduct M3-1 (see Fig. 15, pathway B) as outlined previously with the imidazo[2,1-b]thiazole analog L-766,112 (Trimble et al., 1997). NMR analysis of the crude M3-1 isolate indicated that the GSH adduct was derived from addition of the thiol nucleophile to the electrophilic S-oxide M5-1 and not from the ring opening of epoxide 12. The hypothesis is further strengthened by the observation that addition of GSH to NADPH-supplemented HLM incubations of 1 significantly diminished the levels of M5-1 formed in the incubations. Reason(s) for the lack of formation of the GSH adduct 13 shown in Fig. 15, pathway A, are not apparent from this analysis. It is possible that hydrolytic ring

Fig. 13. MS/MS data reflecting 18O incorporation in the diol metabolite M3-2 generated in an NADPH-and H218O-supplemented HLM incubation of 2.

Fig. 14. CID spectrum of the cyanide conjugate M7-2 derived from NADPH- and potassium cyanide-supplemented HLM incubations of 2.
opening of 12 proceeds at a faster rate than the corresponding reaction with GSH.

Apart from the mono-GSH adduct M3-1, we also noted the formation of a novel bis-GSH conjugate M2-1 with two molecules of GSH attached to the imidazo[2,1-b]thiazole framework. A plausible mechanism outlining the formation of M2-1 is presented in Fig. 15 (pathway C) and involves the initial addition of a GSH molecule to the putative S-oxide metabolite of 1 (i.e., M5-1) to generate M3-1 followed by addition of a second molecule of GSH across the electron-deficient C5 position on the imidazole ring system in M3-1, which results in imidazo[2,1-b]thiazole-S-oxide ring scission. The outlined mechanism is certainly not beyond the realm of possibilities, considering the well-known chemical (and glutathione transferase-mediated) displacement reaction of GSH with electron-deficient heteroaromatic rings (e.g., pyridine, pyrimidine, etc.) that contain alkylsulfoxide and/or alkylsulfone as leaving groups (Clapp, 1956; Colucci and Buyske, 1965; Conroy et al., 1984; Graham et al., 1989; Yang et al., 2012). A literature example that bears much commonality to the present situation is evident with the proton pump inhibitor pantoprazole wherein the benzimidazole-2-sulfoxide motif undergoes nucleophilic attack by GSH at the electron-deficient C2 position on the benzimidazole ring (Zhong et al., 2005). Certainly, one can view M5-1 (in Fig. 15, pathway C) as an electron-deficient heterocycle (imidazole ring) with an excellent leaving group (alkylsulfoxide) at the C5 position, which is poised for nucleophilic displacement by GSH.

In the case of 2, LC-MS/MS data on the GSH and N-acetylcysteinyl conjugates M5-2 and M6-2, respectively, were also consistent with thiazole ring bioactivation. From a structure-activity relationship standpoint, the C-2 methyl group on the imidazo[2,1-b]thiazole ring in 2 did not prevent thiazole ring bioactivation to reactive metabolite(s), which contrasts the structure-toxicity relationship noted for sudoxicam and meloxicam. From a mechanistic perspective, we speculate that the rate-limiting step in the formation of M5-2 and M6-2 proceeds via a P450-mediated oxidation on the thiazole ring in 2 to generate epoxide 14, which upon hydrolysis would yield the diol metabolite M3-2 or undergo ring scission to an iminium species 15 (Fig. 16). A two-electron reduction of the iminium bond in 15, possibly mediated by GSH, would lead to the 2-mercaptoimidazole 16, which may be trapped by GSH or N-acetylcysteine via a developing ring-opened isothiocyanate 17 to afford sulfydryl conjugates M5-2 and M6-2, respectively. Overall the mechanistic possibility is compatible with the results of the 18O labeling studies, wherein lack of incorporation of 18O in M5-2 indicates that the source of the carbonyl oxygen in M5-2 is from molecular oxygen (i.e., from a P450-mediated oxidation).

To examine whether iminium species 15 is indeed formed as an intermediate in the pathway leading to M5-2, 2 was incubated in HLM.
in the presence of NADPH and excess potassium cyanide, which is typically used to trap electrophilic iminium ions generated via a two-electron oxidation of amines (Rose and Castagnoli, 1983; Argoti et al., 2005). The accurate mass of the metabolite M7-2 detected in these incubations was consistent with the addition of cyanide across 15, which provided additional support for our overall mechanistic hypothesis. One can rationalize the formation of M7-2 to occur via the initial addition of cyanide across the iminium bond in 15 to yield the cyano adduct 18, which upon cyclization would lead to M7-2 (see Fig. 16).

With reference to the step involving the reduction of the iminium bond in 15 (to yield 2-mercaptoimidazole 16), we invoked the involvement of GSH as a reducing agent, because two-electron reduction of imines by GSH is a well-precedented reaction. For example, N-acetyl-p-benzoquinone imine, the oxidation product of acetaminophen, can react with GSH in a 1,4-Michael fashion to yield a stable GSH adduct or undergo a two-electron reduction to the parent drug in the presence of the thiol (Rosen et al., 1984; Potter and Hinson, 1986). In the present situation, we anticipate that the thioaminal conjugation product obtained through addition of GSH across the imine double bond in 15 will be highly unstable and spontaneously decompose back to 15. Clearly, a more rigorous experimental analysis of these individual mechanistic steps will be needed to fully validate our hypothesis on the oxidative metabolism/bioactivation of these unique bicyclo-heterocyclic compounds. Toward this end, the imidazo[2,1-b]thiazole intermediates used in the synthesis of the title compounds 1 and 2 (see Supplemental Methods) could provide much utility in testing certain mechanistic hypotheses (e.g., the use of deuterium exchange to distinguish S-oxidation from mono-hydroxylation).

Overall, given this information, it appeared reasonable to attempt to eliminate bioactivation liability in 1 and 2 via small structural modifications of the thiazole ring that would preserve the physicochemical properties (e.g., molecular weight, lipophilicity) largely responsible for pharmacologic potency and HLM stability. Consequently, the 2-methylimidazo[2,1-b][1,3,4]thiadiazole analog 3 was synthesized to prevent thiazole ring epoxidation and/or addition of GSH to an S-oxide metabolite. An identical medicinal chemistry strategy had been successfully used to abrogate thiazole ring bioactivation with nonpeptidyl thrombopoietin receptor agonists containing the 2-aminothiazole motif (Kalgentkar et al., 2007). As such, the absence of the GSH conjugate formation in NADPH- and GSH-supplemented HLM incubations with 3 indicates that the thiadiazole derivative is devoid of the reactive metabolite liability associated with 1 and 2 and demonstrates the success of this medicinal chemistry tactic. Compound 3 also retained the potent in vitro ghrelin inverse agonism and HLM stability characteristics discerned with lead compounds 1 and 2. In summary, we demonstrated the human liver microsomal bioactivation of the imidazo[2,1-b]thiazole functionality present as part of the core structure in novel ghrelin inverse agonists. Characterization of the GSH adducts using LC-MS/MS and NMR techniques provided indirect information on the structures of the reactive species, thereby providing insight into the bioactivation mechanism. The information gained from these studies was used to direct medicinal chemistry efforts in the design of a compound with the attractive pharmacology and disposition characteristics observed with the prototypes while avoiding potential safety concerns associated with the bioactivation of the imidazo[2,1-b]thiazole functionality. Additional lead optimization

**Fig. 16.** Proposed bioactivation pathway for 2-methylimidazo[2,1-b]thiazole derivative 2.
efforts on 3, which culminated in the discovery of the clinical candidate, will be reported in due course.

Authorship Contributions
Participated in research design: Kalugtak, Goosen, Lapham, Ryder, Walker, Eng.

Conducted in vitro experiments: Ryder, Walker, Eng.

Contributed new reagents or analytic tools: Orr, Cabral.

Performed data analysis: Kalugtak, Ryder, Walker, Orr, Eng, Goosen.

Wrote or contributed to the writing of the manuscript: Kalugtak, Ryder, Walker, Orr, Eng.

References


Colucci DF and Buyske DA (1965) The biotransformation of a sulfonamide to a mercaptan and to a 2,4-diaminopyrimidine derivative. J Med Chem 8:1537–1540.

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References
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Drug Metabolism and Disposition
Supplemental Methods

Materials. All chemicals, reagents and solvents used in organic synthesis were purchased from commercial sources, and used without further purification. The imidazoazole acetic acid or ethyl acetate derivatives were purchased from commercial sources or prepared according to literature procedures (Kung et al., 2012; Abignente et al., 1976). $^1$H NMR were recorded on a Varian Unity 400 MHz spectrometer (Varian Inc., Palo Alto, CA) at room temperature. Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual solvent signal as an internal reference (i.e., for deuterated chloroform (CDCl$_3$) δ $^1$H = 7.27 ppm, for DMSO-$d_6$ δ $^1$H = 2.50 ppm, and for deuterated methanol (CD$_3$OD) δ $^1$H = 4.78 and 3.31 ppm). The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. $^1$H NMR coupling constant (J) is expressed in Hertz (Hz). Atmospheric pressure chemical ionization mass spectra were obtained on a Waters™ Spectrometer (Waters Corp., Milford, MA) with a flow rate of 0.3 ml/min and utilizing a 50:50 water/acetonitrile eluent system. Electrospray ionization mass spectra were obtained on a liquid chromatography mass spectrometer from Waters™ (Micromass ZQ or ZMD instrument, Waters Corp., Milford, MA) utilizing a gradient of 95:5–0:100 water in acetonitrile with 0.1% formic acid added to each solvent. These instruments utilized a Varian Polaris 5 C18-A 20 x 2.0 mm column (Varian Inc., Palo Alto, CA) at flow rates of 1 ml/min for 3.75 min or 2 ml/min for 1.95 min. Nitrogen was used as a carrier gas. Column chromatography was performed using silica gel with either Flash 40 Biotage columns (ISC, Inc., Shelton, CT) or Biotage SNAP cartridge KPsil or Redisep Rf silica (Teledyne Isco Inc., Lincoln, NE) under nitrogen pressure.
Synthesis of 2-Chloro-4-(2H-1,2,3-triazol-2-yl)benzaldehyde (5). To a flame-dried one liter round bottom flask containing 2H-1,2,3-triazole (16.3 g, 236.5 mmol) in dimethyl formamide (240 ml) was added sodium hydride (60% dispersion in mineral oil, 8.52 g, 355 mmol) in small portions at room temperature. The mixture was stirred for 30 min, or until the gas evolution ceased, and 2-chloro-4-fluorobenzaldehyde (4) (30 g, 189.2 mmol) in dimethyl formamide (240 ml) was added via an addition funnel. This mixture was heated at 80 °C for 3 h. The reaction mixture was cooled to room temperature and poured into a flask containing water (1 liter). The aqueous solution was extracted with ethyl acetate (3 x 500 ml), and the combined organic layers were washed with water and brine, dried (magnesium sulfate), filtered and concentrated under reduced pressure to give an off-white solid. This material was dissolved in boiling heptane (4 liter), and cooled to room temperature. The resulting precipitate was collected by filtration (mostly the undesired N-1-regioisomer), and the filtrate was concentrated under reduced pressure to give an off-white residue (mostly the desired N-2 regioisomer). This residue was purified via silica gel column chromatography, eluting with dichloromethane to afford a solid, which was re-dissolved in dichloromethane and washed with 1N sodium hydroxide, brine, dried (magnesium sulfate), and concentrated under reduced pressure to give 14.3 g (36%) of the title compound 5 as a white solid. 1H NMR (CDCl₃) δ 10.46 (s, 1 H), 8.06–8.02 (m, 1 H), 8.14–8.10 (m, 1 H), 8.23 (d, J = 1.6 Hz, 1 H), 7.88 (s, 2 H).

Synthesis of tert-Butyl 2-(2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl)-2,7-diazaspiro[3.5]nonane-7-carboxylate (7). To 50 ml flask containing 5 (1.197 g, 5.76 mmol) and tert-butyl 2,7-diazaspiro[3.5]nonane-7-carboxylate (6, 1.5 g, 5.71 mmol) in methylene chloride (23 ml) was added triethylamine (3.2 ml, 23 mmol), and the mixture was stirred at room temperature for 1 h. Sodium triacetoxyborohydride (1.8 g, 8.61 mmol) was added to this mixture
and stirring was continued for an additional 18 h. A saturated solution of aqueous sodium bicarbonate (10 ml) was added and the mixture was stirred further for 1 h. Dichloromethane was added and the organic solution was washed with 1N sodium hydroxide, water and brine, dried (sodium sulfate), filtered and concentrated under reduced pressure to afford 2.13 g (89%) of the title compound 7 as a yellow solid. $^1$H NMR (CD$_3$OD) δ 8.10 (d, $J = 2.5$ Hz, 1 H), 8.00 (dd, $J = 8.7$, 2.5 Hz, 1 H), 7.92 (s, 2 H), 7.55 (d, $J = 8.7$ Hz, 1 H), 3.84 (s, 2 H), 3.35–3.31 (m, 4 H), 3.22 (s, 4 H), 1.74–1.70 (m, 4 H), 1.43 (s, 9 H).

Synthesis of 2-(2-Chloro-4-(2H-1,2,3-triazol-2-yl)benzyl)-2,7-diazaspiro[3.5]nonane hydrochloride (8). To compound 7 (495 mg, 1.18 mmol) was added 4M HCl in dioxane (5 ml) at room temperature, followed by methanol (1 ml). The mixture was stirred at room temperature for 2 h, followed by the addition of diethyl ether (10 ml). The resultant precipitate was collected by filtration and dried under reduced pressure to give 420 mg (>99%) of the title compound 8 as a off-white solid. $^1$H NMR (CD$_3$OD) δ 8.28 (d, $J = 2.1$ Hz, 1 H), 8.19–8.11 (m, 1 H), 7.98 (s, 2 H), 7.83 (d, $J = 8.3$ Hz, 1 H), 4.71 (s, 1 H), 4.24–4.18 (m, 4 H), 3.64 (s, 2 H), 3.24– 3.12 (m, 4 H), 2.20–2.16 (m, 4 H); LCMS (atmospheric pressure chemical ionization) m/z 318.1 (MH)$^+$.

Synthesis of 1-(2-(2-chloro-4-(2H-1,2,3-triazol-2-yl)benzyl)-2,7-diazaspiro[3.5]nonan-7-yl)-2-(imidazo[2,1-b]thiazol-6-yl)ethanone (1). 2-(Imidazo[2,1-b]thiazol-6-yl)acetic acid (9, 30.3 mg, 0.128 mmol) was dissolved in dichloromethane (2 ml) followed by the addition of triethylamine (54 µl, 0.384 mmol) and carbonyldiimidazole (26.3 mg, 0.16 mmol). After 5 minutes of stirring, compound 8 (50 mg, 0.13 mmol) was added and the mixture was stirred for 18 h. The reaction mixture was diluted with dichloromethane (3 ml) and washed with 1N sodium hydroxide (5 ml), water (5 ml) and brine (5 ml). The organic layer was dried (sodium
sulfate), concentrated under reduced pressure, and the crude material was purified by silica gel chromatography (eluting with 0-20% gradient of methanol in dichloromethane) to give 9 mg (10%) of the title compound 1 as a clear film. $^1$H NMR (CD$_3$OD) $\delta$ 8.10 (d, $J = 2.1$ Hz, 1 H), 8.00 (dd, $J = 8.4$, 2.1 Hz, 1 H) 7.90 (s, 2 H), 7.68 (d, $J = 4.5$ Hz, 1 H) 7.59–7.50 (m, 2 H) 7.08 (d, $J = 4.5$ Hz, 1 H) 3.85 (s, 2 H), 3.75 (s, 2 H), 3.62–3.48 (m, 4 H), 3.21 (s, 4 H), 1.83–1.67 (m, 4 H); LCMS (electrospray ionization) $m/z$ 482.4 (MH)$^+$.  

**Synthesis of 1-(2-(2-Chloro-4-(2H-1,2,3-triazol-2-yl)benzyl)-2,7-diazaspiro[3.5]nonan-7-yl)-2-(2-methylimidazo[2,1-b]thiazol-6-yl)ethanone (2).** Compound 2 was prepared in a similar fashion as 1 using 2-(2-methylimidazo[2,1-b]thiazol-6-yl)acetic acid (10) as the starting material. $^1$H NMR (CD$_3$OD) $\delta$ 8.09 (d, $J = 2.1$ Hz, 1 H), 7.99 (dd, $J = 8.3$, 2.1 Hz, 1 H), 7.91 (s, 2 H), 7.54 (d, $J = 8.3$ Hz, 1 H), 7.41–7.39 (m, 2 H), 3.83 (s, 2 H), 3.75 (s, 2 H), 3.54–3.51 (m, 4 H), 3.22 (s, 4 H), 2.39 (s, 3 H), 1.80–1.68 (m, 4 H); LCMS (electrospray ionization) $m/z$ 496.0 (MH)$^+$.  

**Synthesis of 1-(2-(2-Chloro-4-(2H-1,2,3-triazol-2-yl)benzyl)-2,7-diazaspiro[3.5]nonan-7-yl)-2-(2-methylimidazo[2,1-b][1,3,4]thiadiazol-6-yl)ethanone (3).** To a solution of compound 8 (429 mg, 1.10 mmol) in dichloromethane (9 ml) was added triethylamine (310 $\mu$l, 2.19 mmol). The solution was cooled to 0 °C, and trimethylaluminum solution (2.0 M in hexane, 1.1 ml, 2.2 mmol) was added in a dropwise fashion. The mixture was stirred at room temperature for 2 h, followed by the addition of ethyl 2-(2-methylimidazo[2,1-b][1,3,4]thiadiazol-6-yl)acetate (11, 65 mg, 0.29 mmol) in dichloromethane (630 $\mu$l). The reaction mixture was stirred at 40 °C for 16 hours and slowly quenched with saturated aqueous potassium sodium tartrate (20 ml). The aqueous layer was extracted with ethyl acetate (3 x 20 ml), and the combined organic layers were dried (sodium sulfate), filtered and concentrated to afford a yellow oil. The crude product was purified by silica gel chromatography (eluting with 0-20% ethyl acetate in methanol) to give 17.2
mg (12%) of the title compound 3 as a clear glass. $^1$H NMR (CD$_3$OD) δ 8.08 (d, $J = 2.1$ Hz, 1 H), 7.98 (dd, $J = 8.5, 2.1$ Hz, 1 H), 7.90 (s, 2 H), 7.71 (s, 1 H), 7.53 (d, $J = 8.5$ Hz, 1 H), 3.85 (s, 2 H), 3.72 (s, 2 H), 3.56–3.52 (m, 4 H), 3.24–3.21 (m, 4 H), 2.68 (s, 3 H), 1.80–1.67 (m, 4 H); LCMS (atmospheric pressure chemical ionization) m/z 496.6 (M)$^+$. 

References


Supplemental Figure 1. Synthesis of the title compounds 1–3 used in the study.

1: $R_1 = H, A = S, B = CH$
2: $R_1 = CH_3, A = S, B = CH$
3: $R_1 = CH_3, A = S, B = N$
4: $R_1 = H, A = S, B = CH$
5: $R_1 = CH_3, A = S, B = CH$
6: $R_1 = CH_3, A = S, B = N$

Chemical structures and reactions:

4. $\text{F} \xrightarrow{\text{HNN}} \xrightarrow{\text{NaH}} 5$

6. $\text{Na(OAc)}_3BH \xrightarrow{\text{Et}_3\text{N}} 7$

8. $\xrightarrow{\text{CDI, Et}_3\text{N}} \xrightarrow{\text{CH}_3\text{Al, Et}_3\text{N}} 9, 10, 11$

9: $R_1 = R_2 = H, A = S, B = CH$
10: $R_1 = CH_3, R_2 = H, A = S, B = CH$
11: $R_1 = CH_3, R_2 = CH_2CH_3, A = S, B = N$

HCl
Supplemental Figure 2. CID spectra of metabolite M4-1.
Supplemental Figure 3. CID spectra of metabolite M1-2.
Supplemental Figure 4. CID spectra of metabolite M2-2.
Supplemental Figure 5. CID spectra of metabolite M4-2.
Supplemental Figure 6. CID spectra of the $^{18}$O-labeled diol M3-2.
Supplemental Figure 7. Structure-toxicity relationship for thiazole analogs sudoxicam and meloxicam.