Bioactivation of Glafenine by Human Liver Microsomes and Peroxidases:
Identification of Electrophilic Iminoquinone Species and Glutathione Conjugates

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ABBREVIATIONS: P450, cytochrome P450; HLM, human liver microsomes; HRP, horseradish peroxidase; MPO, myeloperoxidase; GSH, glutathione; PDA, photodiode array; CID, collision-induced dissociation; MS/MS, tandem mass spectrometry; COSY, homonuclear correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy.
Abstract

Glafenine (Privadol; 2,3-dihydroxypropyl 2-[(7-chloro-4-quinolinyl)amino]benzoate) is a non-narcotic analgesic agent widely used for the treatment of pains of various origins. Severe liver toxicity and a high incidence of anaphylaxis were reported in patients treated with glafenine, eventually leading to its withdrawal from market in most countries. It is proposed that bioactivation of glafenine and subsequent binding of reactive metabolite(s) to critical cellular proteins play a causative role. The study described herein aimed at characterizing pathways of glafenine bioactivation and the metabolic enzymes involved. Two glutathione (GSH) conjugates of glafenine were detected in human liver microsomal incubations using liquid chromatography-tandem mass spectrometry (LC/MS/MS). The structures of detected conjugates were determined as GSH adducts of 5-hydroxyglafenine (M3) and 5-hydroxy glafenic acid (M4), respectively. GSH conjugation took place with a strong preference at C-6 of the benzene ring of glafenine, ortho to the carbonyl moiety. These findings are consistent with a bioactivation sequence involving initial P450-catalyzed 5-hydroxylation of the benzene ring of glafenine, followed by two electron oxidations of M3 and M4 to form corresponding para-quinone imine intermediates that react with glutathione to form GSH adducts M1 and M2, respectively. Formation of M1 and M2 was primarily catalyzed by heterologously expressed recombinant CYP3A4, and to a less extent, CYP2C19 and CYP2D6. We demonstrated that M3 can also be bioactivated by peroxidases, such as horseradish peroxidase and myeloperoxidase. In summary, these findings are of significance in understanding of the bioactivation pathways of glafenine and their potential link to mechanisms of toxicity of glafenine.
Introduction

Glafenine (2,3-dihydroxypropyl 2-[(7-chloro-4-quinolinyl)amino]benzoate, Scheme 1) is a non-narcotic analgesic agent widely used in the treatment of pains of various origins. Despite its therapeutic benefits, treatment with glafenine has been overshadowed by rare, but severe incidences of hepatic injury (Ypma et al., 1978; Boyer et al., 1984; Stricker et al., 1986; Verhamme et al., 1984; Brissot et al., 1982; Caballería Rovíra et al., 1992) as well as a high incidence of anaphylaxis (van der Klauw et al., 1996; van der Klauw et al., 1993; Stricker et al., 1990), which eventually led to its withdrawal from market in most countries. Although the mechanism of glafenine hepatotoxicity is not clearly understood, a probable causal link between glafenine use and the onset of hepatic injury has been established (Stricker et al., 1986).

In humans, glafenine is rapidly absorbed after oral administration and undergoes extensive hepatic first-pass metabolism mainly by hydrolysis, aromatic hydroxylation, and N-oxidation (Pottier et al., 1979; Vermerie et al., 1992). One of the primary biotransformation routes of glafenine is the carboxyesterase-catalyzed hydrolysis to the active metabolite glafenic acid (Scheme 1), which is excreted in the urine potentially as an acyl glucuronide conjugate. Glafenic acid was also detected as a major metabolite in plasma, with the ratio of $C_{\text{max}}$, glafenic acid to $C_{\text{max}}$, glafenine equal to 18.9 in healthy subjects. A dramatic decrease of first-pass effects was observed in cirrhotic subjects (Vermerie et al., 1992). In addition to hydrolysis metabolism, other major clearance pathways include hydroxylation of the benzene ring para to the amino-substituent and N-oxidation of the 7-chloroquinoline ring (Pottier et al., 1979).
Of particular interest in the biotransformation pathways of glafenine in humans is the detection of 5-hydroxy metabolites of glafenine and glafenic acid (Pottier et al., 1979). As depicted in Scheme 1, glafenine is an anthranilic acid ester derivative and contains a 7-chloroquinoline ring system. 5-hydroxyglafenine or 5-hydroxy glafenic acid can undergo P450-mediated two-electron oxidations to form electrophilic quinone imine intermediates, which are capable of reacting with cellular proteins and other nucleophiles such as glutathione. However, to date, no such reactive intermediates of glafenine and/or their corresponding GSH conjugates has been reported, and the mechanism of bioactivation of glafenine remains unknown.

In the present study, we report the detection and identification of two GSH conjugates of glafenine, derived from the addition of the sulphydryl nucleophile to 5-hydroxyglafenine and 5-hydroxy glafenic acid, respectively. GSH conjugation is found to occur specifically at C-6 of the benzene ring of glafenine, ortho to the carbonyl moiety. In addition, efforts are made to evaluate the relative contributions from individual P450 isoforms to formation of these GSH conjugates. Metabolic activation of 5-hydroxyglafenine by peroxidases, such as horseradish peroxidase (HRP) and myeloperoxidase (MPO), is demonstrated. These findings are of significance for understanding the relationship between metabolic activation and hepatotoxicity of glafenine.
Materials and Methods

Materials. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): glafenine, HRP, MPO, H2O2 (30 wt % in H2O), L-ascorbic acid, GSH, trichloroacetic acid and NADPH. Pooled human liver microsomes and Supersomes™ containing cDNA-baculovirus-insect cell-expressed P450s (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) were obtained from BD Gentest (Woburn, MA). Formic acid, methanol, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). All other commercially available reagents and solvents were of either analytical or HPLC grade.

Microsomal Metabolism. All incubations were performed at 37 °C in a water bath. Pooled human liver microsomes and the human cDNA-expressed P450 isozymes were carefully thawed on ice prior to the experiment. Glafenine (10 μM and 50 μM) was mixed with human liver microsomal proteins (1 mg/ml) in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 1 mM GSH. The total incubation volume was 1 mL. After 3 min pre-incubation at 37 °C, the incubation reactions were initiated by the addition of 1 mM NADPH. Reactions were terminated by the addition of 150 μL of trichloroacetic acid (10%) after 60 min incubation. Incubations with the recombinant cDNA-expressed P450 isozymes were performed similarly except that liver microsomes were substituted by Supersomes™, including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (100 nM). Control samples containing no NADPH or substrates were included. Samples were centrifuged at 10,000 g.
for 15 min at 4 °C to pellet the precipitated proteins, and supernatants were subjected to LC/MS/MS analysis of GSH adducts. For human liver microsomal incubations, supernatants were concentrated by solid phase extraction as described below, prior to LC/MS\textsuperscript{n} analyses.

**Solid-Phase Extraction.** Samples resulting from HLM incubations were desalted and concentrated by solid-phase extraction (SPE), prior to the LC-MS/MS analyses. SPE was performed using Oasis\textsuperscript{®} solid-phase extraction cartridges packed with 60 mg of sorbent C\textsubscript{18} (Waters, Milford, MA). Cartridges were first washed with 2 mL methanol and then conditioned with 2 mL of water. Supernatants resulting from centrifugation were loaded onto the cartridges, and cartridges were washed with 2 mL of water and then eluted with 2 mL of methanol. The methanol fractions were dried by nitrogen gas and reconstituted with 100 \(\mu\)L of a water-methanol (70:30) mixture. Aliquots (20 \(\mu\)L) of the reconstituted solutions were subjected to LC/MS/MS analysis.

**Instrumentation.** Metabolite profiling was performed on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) as described previously with modifications (Wen et al., 2008a). Separation was achieved using a Polaris\textsuperscript{®} C18 column (5 \(\mu\)m, 250 x 2.1 mm, Varian Inc., Palo Alto, CA) at a flow rate of 0.3 mL/min. A gradient of solvent A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) was as follows: 5% solvent B for 5 min, followed by 5-70% B in 30 min and 70-90% B in 2 min. Formation of metabolites was estimated based on the PDA total scan
chromatograms using an Agilent 8453 Diode Array UV-Visible Spectrophotometer (Agilent Technologies, Palo Alto, CA). Major operating parameters for the ion trap ESI-MS method were set as follows: capillary temperature, 300 °C; spray voltage, 5.0 kV; capillary voltage, 15 V; sheath gas flow rate, 90 (arbitrary value); and auxiliary gas flow rate, 30 (arbitrary value). For a full scan, the automatic gain control was set at 5.0 x 10^8, maximum ion time was 100 ms, and the number of microscans was set at 3. For MS^n scanning, the automatic gain control was set at 1.0 x 10^8, maximum ion time was 400 ms, and the number of microscans was set at 2. For data-dependent scanning, the default charge state was 1, default isolation width was 2.0, and normalized collision energy was 35. Polarity switching was applied to acquire full scan and data-dependent MS^n spectra in both positive and negative ion mode. Fourier transform MS (FTMS) was set up to acquire high resolution full scan MS in the positive ion mode.

Complete profiling of reactive metabolites was also carried out using the precursor ion (PI)-enhanced product ion (EPI) method previously described (Wen et al, 2008b). Briefly, the PI scan of m/z 272 was run in the negative mode with 0.2 Da step size, 5 ms pause between mass ranges and 2 s scan rate or 50 ms dwell. The TurboIonSpray® ion source conditions were optimized and set as follows: curtain gas = 35, collision gas = medium, ionspray voltage = –4500, temperature = 500. Information dependent acquisition was used to trigger acquisition of EPI spectra. The EPI scans were run in the positive mode at a scan range for daughter ions from m/z 100 to 1000. Data were processed using Analyst 4.1 software (Applied Biosystems, Foster City, CA). The Shimadzu HPLC system was coupled with an Agilent Eclipse XDB-Phenyl C18 column (3.0 x 150 mm, 3.5 μm, Agilent Technologies, Palo Alto, CA). The HPLC mobile phase
A was 10 mM ammonium acetate in water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. A Shimadzu LC-20AD solvent delivery module (Shimadzu Scientific Instruments, Columbia, MD) was used to produce the following gradient elution profile: 5% solvent B for 2 min, followed by 5-70% B in 20 min and 70-90% B in 2 min. The HPLC flow rate was 0.3 mL/min. At 26 min, the column was flushed with 90% acetonitrile for 3 min before re-equilibration at initial conditions. LC/MS/MS analyses were performed on 20 μL aliquots of cleaned samples. For relative comparison of GSH adduct levels, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions were simultaneously monitored for detecting glafenine GSH conjugate (M1): m/z 694→565 and 694→421; for glafenic acid GSH conjugate (M2): m/z 620→491 and 620→422; for 5-hydroxyglafenine (M3): m/z 389→297 and 389→315. Data were analyzed using Analyst 4.1 version software (Applied Biosystems, Foster City, CA).

**Isolation of Glafenine Metabolites and NMR Characterization.** Human liver microsomal incubations with glafenine (50 μM) were performed as described above on a 50 mL scale. Trichloroacetic acid (10%) was added to terminate the reaction after 60 min incubation. Samples were centrifuged at 10,000 g for 15 min at 4 °C to pellet the precipitated proteins, and supernatants were concentrated by solid phase extraction as described above. The major metabolites M1 and M3 were therefore isolated from the cleaned and concentrated reaction mixture. Separation was achieved using a Polaris® C18 column (5 μm, 250 x 4.6 mm, Varian Inc., Palo Alto, CA) at a flow rate of 1 mL/min. A gradient of solvent A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid).
acid) was as follows: 10% solvent B for 5 min, followed by 10-50% B in 30 min and 50-90% B in 2 min.

All NMR spectra were acquired on a Bruker Avance 500 Ultrashield NMR spectrometer equipped with a 5 mm CPQNP z-gradient cryoprobe (Bruker, Rheinstetten, Germany) operating at ¹H frequency of 500.13 MHz and ¹³C frequency of 125.76 MHz. Each NMR sample was dissolved in 180 μL of methanol-d₄ with tetramethylsilane (TMS) as an internal reference standard. Water suppression was carried out on the samples as needed. All assignments were proven by conventional NMR experiments including the two-dimensional COSY and NOESY.

**Incubations of M3 with Peroxidases.** The isolated metabolite M3 (final concentration, 50 μM) was added in an incubation mixture containing phosphate buffer (100 mM, pH 7.4), GSH (5 mM), and HRP (5 units) in a final volume of 1 mL. The reaction was initiated by adding H₂O₂ (final concentration, 200 μM). After 30 min of incubation at 37 °C, the reaction was terminated by adding ascorbic acid (final concentration, 2 mM), and an aliquot (20 μL) was injected for LC-MS/MS analysis using the PI-EPI experiments described above. Similar incubations were performed for MPO except that KCl (150 mM) and MPO (5 units/mL) were used in the incubation mixture. Control experiments were also performed without enzymes or cofactors.

**P450 Inhibition by Chemical Inhibitors.** The effect of specific inhibitors of individual P450 enzymes on the formation of reactive metabolites was examined using
pooled human liver microsomes as described previously (Wen et al, 2008c). Incubation mixtures consisted of glafenine (10 μM) individual chemical inhibitors, GSH (1 mM) and HLMs (1 mg/mL). The P450 specific inhibitors α-naphthoflavone (1 μM), sulfaphenazole (5 μM), tranylcypromine (15 μM), quinidine (2 μM) and ketoconazole (1 μM) were used to investigate the involvement of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively. Incubations containing glafenine were started with the addition of 1 mM NADPH, and reactions were terminated by trichloroacetic acid. Controls containing no chemical inhibitors were included. Each incubation was performed in triplicate. The effectiveness of individual P450 inhibitors was also evaluated using P450 marker substrates 50 μM phenacetin (CYP1A2), 150 μM tolbutamide (CYP2C9), 100 μM (S)-mephenytoin (CYP2C19), 10 μM dextromethorphan (CYP2D6), and 100 μM testosterone (CYP3A4) in HLMs. Individual marker substrates were pre-incubated for 5 min at 37 °C in the presence and absence of P450 specific inhibitors. Reactions were started with the addition of 1 mM NADPH, and terminated after 20 min. Formation of metabolites from individual P450 marker substrate were analyzed by LC/MS/MS as previously described (Wen et al, 2008c). A comparison was made relative to the controls without inhibitors, and P450 activity was expressed as the percentage of control activity.
Results

**Glafenine Metabolites in Human Liver Microsomes.** Incubation of glafenine in human liver microsomes with GSH and NADPH generated several products. The major *in vitro* metabolite was M5 based on the UV chromatogram (Fig. 1). The MS spectrum of M5 showed an [M + H]$^+$ ion at \( m/z \) 299. Upon further fragmentation of an ion at \( m/z \) 281, the [M + H – H$_2$O]$^+$ ion lost CO (–28), and Cl (–35) to yield ions at \( m/z \) 253, and 246, respectively (Supplemental Figure S1). Cleavage of the carbon-nitrogen linkage afforded the product ion at \( m/z \) 162, corresponding to the element of 7-chloroquinoline moiety. M5 was assigned to be glafenic acid based on the MS fragmentation pattern, and the previous observations that glafenic acid was the predominant metabolite of glafenine *in vivo* (Pottier et al., 1979; Vermerie et al., 1992).

The MS spectrum of M3 revealed an [M + H]$^+$ ion at \( m/z \) 389 with a chlorine isotope peak at \( m/z \) 391 (~35% of the [M + H]$^+$ ion, Fig. 2A), suggesting that M3 is a mono-oxygenated metabolite of glafenine. Fragmentation of the ion at \( m/z \) 389 generated product ions at \( m/z \) 371, 315, 297 (Fig. 2B). MS$^3$ mass spectrum of the product ion of M3 at \( m/z \) 297 afforded several fragment ions at \( m/z \) 269, 262 and 162 (Fig. 2C). The product ion at \( m/z \) 162 suggested that hydroxylation occurred at the benzene ring of glafenine, instead of 7-chloroquinoline ring. In an attempt to identify the absolute structure of M3, this metabolite was isolated for further spectroscopic analysis. $^1$H NMR of M3 showed eight aromatic proton signals at 8.41 ppm (C-2’), 8.28 ppm (C-5’), 7.89 ppm (C-8’), 7.60 ppm (C-6’), 7.57 ppm (C-6), 7.50 ppm (C-3), 7.13 ppm (C-4), and 6.93 ppm (C-3’), respectively (Table 1). The dihydroxypropyl proton signals appeared in the region from 3.65 to 4.50 ppm. The COSY spectrum of M3 revealed cross-peaks between aromatic...
protons at C-3 and C-4, between C-5′ and C-6′, and between C-2′ and C-3′ (Supplemental Figure S3). It also revealed a weak cross-peak between aromatic protons at C-4 and C-6. No other couplings to the aromatic proton at C-6 were exhibited (Supplemental Figure S3). These NMR data suggested that the hydroxylation occurred at the C-5 position of glafenine, para to the amino substituent. On the basis of the spectroscopic data of MS and NMR, M3 was identified as 5-hydroxyglafenine. Similar to that of M3, the MS spectrum of M4 revealed an [M + H]^+ ion at m/z 315 with a chlorine isotope peak at m/z 317, suggesting that M4 is a mono-oxygenated metabolite of glafenic acid (Fig. 3). Further fragmentation of the [M + H – H_2O]^+ ion of M4 at m/z 297 generated product ions at m/z 269, 262 and 162 (Fig. 3C), which is the same fragmentation pattern observed in the MS^3 mass spectrum of the product ion of M3 at m/z 297 (Fig. 2C). The fragment ion at m/z 162 suggested that mono-hydroxylation occurred on the benzene ring, instead of the 7-chloroquinoline ring system. Thus, M4 was tentatively assigned as a 5-hydroxy glafenic acid.

The MS/MS spectrum of M6 afforded product ions at m/z 371, 354, 315, 297 and 280 (Supplemental Figure S2). Further fragmentation of the product ion at m/z 297 generated a predominant fragment ion at m/z 280 (Supplemental Figure S2). It was observed that M6 eluted (R_t = 16.5 min) after the parent drug glafenine (R_t = 14.9 min), suggesting that M6 is an N-oxide metabolite of glafenine. An N-oxide metabolite of glafenic acid was previously reported (Pottier et al., 1979). This assignment was supported by its facile reduction back to the parent drug in the presence of TiCl_3 (data not shown). Taken together, M6 was assigned as an N-oxide metabolite of glafenine, with N-
oxidation of the 7-chloroquinoline ring nitrogen. M6 was not reported in previous in vivo studies (Pottier et al., 1979).

**Formation of Glafenine GSH Conjugates in Human Liver Microsomes.** For the LC/MS/MS analysis of GSH adducts, samples generated from incubations with human liver microsomes were desalted and concentrated by solid-phase extractions, and resulting samples were subjected to both data-dependent LC-MS^n scanning and PI-EPI experiments described above. The metabolic profile of glafenine in human liver microsomes showed two GSH conjugates M1 and M2 eluted at 12.7 and 12.4 min, respectively (Fig. 1).

The major adduct M1 displayed a molecular ion [M + H]^+ of \( m/z \) 694, suggesting that this component was a GSH conjugate of mono-oxygenated glafenine (Fig. 4A). Fragmentation of M1 molecular ions resulted in neutral loss of 129 and 75, corresponding to elimination of the pyroglutamate and glycine of GSH, respectively (Fig. 4B). The ion at \( m/z \) 421 was formed via cleavage of sulfur-carbon bond of the glutathionyl moiety, and its occurrence suggested the presence of an aromatic thioether motif in this GSH adduct (Baillie and Davis, 1993). Further fragmentation of the ion at \( m/z \) 421 afforded several fragment ions including ions at \( m/z \) 403, 347, 329, 295 and 256 (Fig. 4C). These data suggested that M1 is a GSH adduct with attachment of the glutathionyl moiety to the hydroxylated benzene ring instead of the 7-chloroquinoline ring of glafenine. This was supported by the negative MS spectra of M1 (Fig. 5). Fragmentation of the [M – H]^− ions of M1 at \( m/z \) 692 afforded product ions at \( m/z \) 530, 419, 327, 294 and 272. The anion at \( m/z \) 272 corresponds to deprotonated \( \gamma \)-glutamyl-dehydroalanyl-glycine originated from
the glutathionyl moiety (Dieckhaus et al., 2005). The presence of the product ion at \( m/z \) 530 supported the structural assignment that the glutathionyl moiety is attached to the hydroxylated benzene ring instead of the 7-chloroquinoline ring of glafenine. To identify the absolute structure of M1, this metabolite was purified and subjected to NMR analysis. Seven aromatic proton signals at 8.46 ppm (C-5’), 8.33 ppm (C-2’), 7.87 ppm (C-8’), 7.60 ppm (C-6’), 7.27 ppm (C-3), 7.10 ppm (C-4), and 6.36 ppm (C-3’) were present in \(^1\)H NMR spectrum of M1, respectively (Table 1), revealing the absence of aromatic proton signals at C-6. Glutathionyl proton signals appeared in the region from 2.0 to 4.8 ppm. The COSY spectrum of M1 revealed cross-peaks between aromatic protons at C-3 and C-4, between C-5’ and C-6’, and between C-2’ and C-3’ (Fig. 7). It also revealed a weak cross-peak between aromatic protons at C-6’ and C-8’. For the aromatic proton at C-4, there is only one single cross peak between aromatic protons at C-3 and C-4 in the COSY spectrum of M1, in contrast to the two cross peaks between aromatic protons at C-3 and C-4, between C-6 and C-4, observed in the COSY spectrum of M3 (Supplemental Figure S3). Full assignment of the glutathionyl and dihydroxypropyl protons of M1 was achieved with the aid of the COSY experiment. Additionally, \(^1\)H NOSEY spectrum of M1 showed NOE connectivity between aromatic protons at C-3 and C-3’ (Fig. 8). This NOE connectivity between aromatic protons at C-3 and C-3’ ruled out the possibility of any conjugation or hydroxylation at the C-3 position, and further confirmed the structural assignment of M1. Taken together, the MS and NMR data identified M1 as a glafenine GSH conjugate with the glutathionyl moiety attached at C-6 position of 5-hydroxyglafenine.
The MS spectrum of M2 revealed an $[M - H]^-$ ion at $m/z$ 618 with a chlorine isotope peak at $m/z$ 620 (Fig. 6A), suggesting that M2 is a GSH conjugate of mono-oxygenated glafenic acid. Fragmentation of the $[M - H]^-$ ions of M2 at $m/z$ 618 afforded product ions at $m/z$ 456, 345, 301, and 272 (Fig. 6B). The anion at $m/z$ 272 corresponds to deprotonated $\gamma$-glutamyl-dehydroalanyl-glycine originated from the glutathionyl moiety (Dieckhaus et al., 2005). The ion at $m/z$ 345 was formed via cleavage of sulfur-carbon bond of the glutathionyl moiety, suggesting the presence of an aromatic thioether motif in this GSH adduct (Baillie and Davis, 1993). Further fragmentation of the ion at $m/z$ 345 afforded fragment ions at $m/z$ 327 and 301, resulting from loss of water and elements of CO$_2$, respectively (Fig. 6C). The presence of the product ion at $m/z$ 456 supported the structural assignment that the glutathionyl moiety is attached to the 5-hydroxybenzene ring instead of the 7-chloroquinoline ring of glafenic acid.

**Bioactivation of M3 by Peroxidases.** Because a synthetic standard of M3 was not available, this metabolite was generated via human liver microsomal incubations of glafenine. After isolation by HPLC, the purified M3 ($\sim$50 $\mu$M) was subjected to oxidation by HRP/H$_2$O$_2$ in the presence of 5 mM GSH. The PI-EPI analyses of reaction mixtures revealed the formation of the GSH conjugate M1 which was identified in human liver microsomal incubations (Fig. 9B). The GSH conjugate M2 was not detected by the PI-EPI experiments or data-dependent scanning. Control experiments without HRP or H$_2$O$_2$ did not show turnover of M3 or formation of any GSH conjugate (Fig. 9A). In addition, glafenine itself was not oxidized by HRP/H$_2$O$_2$ (data not shown). M3 was also oxidized and bioactivated by MPO. Incubations of M3 with MPO/H$_2$O$_2$/Cl$^-$ resulted in the
formation of M1, but not M2 (Fig. 9C). Similar to HRP/H₂O₂, glafenine itself was not oxidized or bioactivated by the MPO/H₂O₂/Cl⁻ reaction system.

**Formation of M1, M2 and M3 with Recombinant P450s.** To investigate the roles of individual human P450 isozymes in the bioactivation of glafenine to the GSH adducts, incubations were carried out with insect cell-expressed recombinant P450s. The rates of metabolite formation obtained from individual incubations with recombinant P450 enzymes were multiplied by the mean specific content of the corresponding P450 enzyme in human liver microsomes to obtain the ‘normalized’ reaction rates for each P450 enzyme (Rodrigues, 1999). After normalization for the relative hepatic abundance of P450 isozymes, CYP3A4 was the predominant enzyme in the formation of the major GSH conjugate M1 in incubations of glafenine (Fig. 10). CYP2C19 and CYP2D6 also catalyzed M1 formation, and the levels were approximately 12% and 5% of those formed by CYP3A4, respectively. Only trace amounts or no M1 were detected in incubations with other P450 enzymes including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP2E1. Similarly, formation of M2 and M3 was predominantly carried out by CYP3A4 (Fig. 10). These results suggested that CYP3A4 is the major P450 enzyme involved in the formation of M1 and M2 presumably via an para-quinone imine intermediate upon 5-hydroxylation followed by two-electron oxidations (Scheme 2).

**Chemical Inhibition of Glafenine Bioactivation.** The inhibitory effects of P450 isozyme specific inhibitors on the formation of M1, M2 and M3 were examined using pooled human liver microsomes. Inhibitory activity was confirmed using P450 marker
substrates. In the incubations of glafenine, formation of M1, M2 and M3 was greatly inhibited by 86, 88, and 80% respectively in the presence of CYP3A4 selective inhibitor ketoconazole (Table 2). Tranylcypromine, a selective CYP2C19 inhibitor, inhibited M1 and M2 formation by 12 and 15%, respectively. It also inhibited M3 formation approximately by 10%. The inhibitory effects on the formation of M1, M2 and M3 were minimal (<10%) for other P450 specific inhibitors including α-naphthoflavone (CYP1A2), sulfaphenazole (CYP2C9) and quinidine (CYP2D6) (Table 2). These data are consistent with the predominant role of CYP3A4 for the formation of M1, M2 and M3 in the incubations of glafenine with recombinant P450s.
Discussion

The results from the current investigation constitute the first report on the cytochrome P450- and peroxidase-catalyzed bioactivation of the non-narcotic analgesic glafenine. Two novel GSH conjugates of glafenine M1 and M2 were formed in the human liver microsomal incubations and characterized by LC/MS/MS and/or NMR experiments. Formation of M1 and M2 was mediated primarily by CYP3A4, relative to other major P450 isoforms. It was found that the 5-hydroxyglafenine metabolite, but not glafenine itself, was oxidized and bioactivated by peroxidases, such as HRP and MPO. These findings are of importance to understand the bioactivation pathways of glafenine and potential links to its mechanism of toxicity.

Bioactivation of glafenine was found to be mediated via its 5-hydroxy metabolites by human liver microsomes. The current study demonstrated that one of the in vitro biotransformation pathways of glafenine was the hydroxylation of the benzene ring on the C-5 position para to the amino-substituent (Scheme 2). This was in parallel with a previous in vivo study that 5-hydroxy metabolites of glafenine (M3) and glafenic acid (M4) were detected in human urine (Pottier et al., 1979). Further two-electron oxidations of these metabolites M3 and M4 can presumably lead to formation of reactive quinone imine intermediates. A proposed mechanism for the formation of the GSH conjugates M1 and M2 is depicted in Scheme 2. Upon 5-hydroxylation of glafenine and glafenic acid, M3 and M4 undergo an overall two-electron oxidation by P450 enzymes or peroxidases to yield their respective quinone imine species, which can be trapped by GSH via a nucleophilic addition to generate the GSH conjugates M1 and M2, respectively. Such a two-step oxidation mechanism has been proposed for the bioactivation of several
diphenylamine-containing compounds, such as diclofenac (Shen et al., 1999; Yu et al., 2005; Tang et al., 1999; Poon et al., 2001), and more recently, lumiracoxib (Li et al., 2008; Kang et al., 2009). Formation of \( p \)-aminophenol metabolites followed by further two-electron oxidations lead to electrophilic quinone imine species and their corresponding GSH conjugates. Based on the results from the current investigation, bioactivation of glafenine requires initial 5-hydroxylation by P450 enzymes. Although the 5-hydroxyglafenine metabolite M3 is bioactivated by both P450 enzymes and peroxidases, such as HRP and MPO, the current study demonstrated that peroxidases alone were not able to oxidize/bioactivate glafenine, supporting that 5-hydroxylation of the benzene ring is the initial step for glafenine bioactivation. Apart from these oxidative activation pathways, it is noteworthy that the role of phase II metabolism (e.g. acyl glucuronidation) of the hydrolyzed metabolites of glafenine, e.g. glafenic acid, remains to be elucidated.

GSH conjugation occurred specifically at C-6 of the benzene ring of glafenine, \textit{ortho} to the carbonyl moiety presumably due to the electronic effects. By LC/MS\(^a\) and NMR analysis of the isolated conjugate, M1 was unambiguously identified as 6-glutathionyl 5-hydroxyglafenine with the glutathionyl addition \textit{ortho} to the carbonyl moiety. This finding is particularly of interest because one would expect that the glutathionyl conjugation should take place at positions with less steric hindrance, such as C-4. Based on the electronic effects, however, it is likely that the carbonyl group serves as an electron withdrawing group and draws electrons from the reaction center at C-6, thus making it more electron deficient and susceptible to the glutathione attack (Scheme 2). This proposed electronic specificity is supported by the lack of any positional isomer
of M1 in HLM incubations of glafenine. M2 was formed presumably via a quinone imine intermediate of M4 mediated by P450 enzymes and/or peroxidases. Based on the similarity of MS fragmentation, it was likely that the GSH conjugate M2 shared the same glutathionyl addition at C-6, next to the carboxylic acid moiety (Scheme 2). Several attempts were made to isolate M2, but failed to obtain enough of the conjugate in the desired purity for NMR analysis, presumably due to its low abundance in the incubations.

The NADPH-dependent formation of GSH conjugates indicated that one or more P450 enzymes were involved in the generation of reactive intermediates of glafenine by human liver microsomes. Experiments with recombinant P450 enzymes revealed that formation of GSH conjugates M1 and M2 was predominantly mediated by CYP3A4. It was noteworthy that formation of 5-hydroxyglafenine M3 was also mediated primarily by CYP3A4. In addition, the CYP3A4-mediated formation of M1, M2 and M3 was demonstrated in human liver microsomes where ketoconazole, a specific CYP3A4 inhibitor, strongly inhibited formation of these metabolites. These observations were in line with the proposed mechanism that glafenine was bioactivated via 5-hydroxylation by human liver microsomes.

In conclusion, we found that glafenine undergoes P450-mediated 5-hydroxylation which is subsequently involved in bioactivation and formation of glafenine GSH conjugates in human liver microsomes. GSH addition occurred specifically at C-6 ortho to the carbonyl moiety presumably due to electronic effects. Upon 5-hydroxylation, M3 was oxidized/bioactivated by both P450 enzymes and peroxidases, such as HRP and MPO. In parallel with CYP3A4-mediated 5-hydroxylation of glafenine, formation of GSH conjugates M1 and M2 was found to be predominately mediated by CYP3A4. It is
our hypothesis that formation of GSH conjugates M1 and M2 from the \textit{p}-aminophenol ring moiety is mediated by a common quinone imine species. In summary, findings from the current study are of significance in understanding of the bioactivation pathways of glafenine and their potential link to mechanisms of toxicity of glafenine.

**Supplemental Data:** MS\textsuperscript{n} spectra of M5 at \textit{m/z} 299 ([M+H]\textsuperscript{+}) (Fig. S1); MS\textsuperscript{n} spectra of M6 at \textit{m/z} 389 ([M+H]\textsuperscript{+}) (Fig. S2); \textsuperscript{1}H-\textsuperscript{1}H-COSY spectrum of the isolated M3 (Fig. S3).

**Authorship Contributions:** Conducted experiments and performed data analysis: Wen. Wrote or contributed to the writing of the manuscript: Wen, Moore.
References


Scheme Legends

**Scheme 1.** Structures of glafenine and its metabolites glafenic acid, 5-hydroxyglafenine, glafenine N-oxide.

**Scheme 2.** Proposed mechanisms for the formation of GSH conjugates of glafenine by human liver microsomes and peroxidases.

Figure Legends

**Fig. 1.** UV chromatogram (PDA total scan) of metabolites in human liver microsomal incubations of glafenine in the presence of GSH and NADPH.

**Fig. 2.** Mass spectra of 5-hydroxyglafenine (M3) obtained in the positive ion mode. (A) full scan mass spectrum of M3; (B) MS/MS spectrum of M3 at m/z 389 ([M + H]⁺); (C) MS³ mass spectrum of the fragment ion of M3 at m/z 297.

**Fig. 3.** Mass spectra of 5-hydroxy glafenic acid (M4) obtained in the positive ion mode. (A) full scan mass spectrum of M4; (B) MS/MS spectrum of M4 at m/z 315 ([M + H]⁺); (C) MS³ mass spectrum of the fragment ion of M4 at m/z 297.

**Fig. 4.** Mass spectra of glafenine GSH conjugate (M1) obtained in the positive ion mode. (A) full scan mass spectrum of M1; (B) MS/MS spectrum of M1 at m/z 694 ([M + H]⁺); (C) MS³ mass spectrum of the fragment ion of M1 at m/z 421.

**Fig. 5.** Mass spectra of glafenine GSH conjugate (M1) obtained in the negative ion mode. (A) full scan mass spectrum of M1; (B) MS/MS spectrum of M1 at m/z 692 ([M – H]⁻); (C) MS³ mass spectrum of the fragment ion of M1 at m/z 419.
Fig. 6. Mass spectra of glafenic acid GSH conjugate (M2) obtained in the negative ion mode. (A) full scan mass spectrum of M2; (B) MS/MS spectrum of M2 at m/z 618 ([M – H]−); (C) MS3 mass spectrum of the fragment ion of M2 at m/z 345.

Fig. 7. 1H-1H COSY spectrum of M1 showing the coupling between aromatic protons at C-3 and C-4.

Fig. 8. 1H NOSEY spectrum of M1 showing NOE connectivity between aromatic protons at C-3 and C-3’.

Fig. 9. PI-EPI analyses of GSH conjugates formed in peroxidase incubations of the isolated 5-hydroxyglafenine M3 in the presence of 5 mM GSH. (A) HRP without H2O2; (B) HRP with H2O2; (C) MPO with H2O2.

Fig. 10. Formation of M1, M2 and M3 in incubations with cDNA-expressed recombinant P450 isozymes. The enzyme activities were expressed as the percentage of CYP3A4 activity and shown as an average of three measurements.
Table 1. $^1$H-NMR Data of Glafenine, 5-Hydroxyglafenine (M3) and Its GSH Conjugate (M1) ($\delta$ in CD$_3$OD)$^a$

<table>
<thead>
<tr>
<th>Proton signals</th>
<th>Glafenine</th>
<th>M3</th>
<th>M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-3</td>
<td>7.74 (1H, bd, $J = 8.2$ Hz)</td>
<td>7.50 (1H, d, $J = 8.8$ Hz)</td>
<td>7.27 (1H, d, $J = 8.6$ Hz)</td>
</tr>
<tr>
<td>C-4</td>
<td>7.62 (1H, ddd, $J = 8.2$, 7.9 and 1.8 Hz)</td>
<td>7.13 (1H, dd, $J = 8.8$ and 2.9 Hz)</td>
<td>7.10 (1H, d, $J = 8.6$ Hz)</td>
</tr>
<tr>
<td>C-2'</td>
<td>8.57 (1H, d, $J = 5.4$ Hz)</td>
<td>8.41 (1H, d, $J = 5.8$ Hz)</td>
<td>8.33 (1H, d, $J = 6.2$ Hz)</td>
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<tr>
<td>C-3'</td>
<td>7.41 (1H, d, $J = 5.4$ Hz)</td>
<td>6.93 (1H, d, $J = 5.8$ Hz)</td>
<td>6.36 (1H, d, $J = 6.2$ Hz)</td>
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<tr>
<td>C-5'</td>
<td>8.20 (1H, d, $J = 9.0$ Hz)</td>
<td>8.28 (1H, d, $J = 9.0$ Hz)</td>
<td>8.46 (1H, d, $J = 9.0$ Hz)</td>
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<tr>
<td>C-6'</td>
<td>7.60 (1H, dd, $J = 9.0$ and 2.1 Hz)</td>
<td>7.60 (1H, dd, $J = 9.0$ and 1.9 Hz)</td>
<td>7.60 (1H, dd, $J = 9.0$ and 2.0 Hz)</td>
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<tr>
<td>C-8'</td>
<td>7.94 (1H, d, $J = 2.1$ Hz)</td>
<td>7.89 (1H, d, $J = 1.9$ Hz)</td>
<td>7.87 (1H, d, $J = 2.0$ Hz)</td>
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<tr>
<td>C-6</td>
<td>8.19 (1H, dd, $J = 8.1$ and 1.8 Hz)</td>
<td>7.57 (1H, d, $J = 2.9$ Hz)</td>
<td></td>
</tr>
<tr>
<td>C-5</td>
<td>7.16 (1H, bdd, $J = 8.1$ and 7.9 Hz)</td>
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</table>

$^a$ Refer to Figure 7 for signal assignments of the thioether moiety; cysteine 1''--H: $\delta$ 3.3/3.4, cysteine 2''--H: $\delta$ 4.4, glutamate 3''--H: $\delta$ 2.5, glutamate 4''--H: $\delta$ 2.1/2.3, glutamate 5''--H: $\delta$ 3.6, glycine 6''--H: $\delta$ 3.7.
Table 2. Effects of P450 Isoform-specific Inhibitors on the Formation of M1, M2 and M3 in HLM Incubations of Glafenine

<table>
<thead>
<tr>
<th>P450 inhibitor</th>
<th>M1 formation</th>
<th>M2 formation</th>
<th>M3 formation</th>
<th>P450 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoflavone (CYP1A2)</td>
<td>97.4</td>
<td>95.8</td>
<td>92.4</td>
<td>15.8</td>
</tr>
<tr>
<td>Sulfaphenazole (CYP2C9)</td>
<td>95.8</td>
<td>93.6</td>
<td>95.6</td>
<td>28.2</td>
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<tr>
<td>Tranylcypromine (CYP2C19)</td>
<td>87.6</td>
<td>84.9</td>
<td>89.5</td>
<td>24.2</td>
</tr>
<tr>
<td>Quinidine (CYP2D6)</td>
<td>92.2</td>
<td>95.1</td>
<td>91.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Ketoconazole (CYP3A4)</td>
<td>14.5</td>
<td>12.2</td>
<td>20.2</td>
<td>16.4</td>
</tr>
</tbody>
</table>

*Values are an average of duplicated measurements and expressed as percentage relative to the control without inhibitors.*

*P450 activities were determined using known P450 substrates.*
Scheme 1

- **Glafenine**

- **Glafenic acid**

- **5-Hydroxyglafenine**

- **Glafenine N-oxide**
Scheme 2
Figure 1

Relative Absorbance vs Time (min)
Figure 2
Figure 4
Figure 6

(A) 

(B) 

(C)
Figure 9
Figure 10