

## Short Communication

# A Novel Bioactivation Pathway for 2-[2-(2,6-Dichlorophenyl)aminophenyl]ethanoic Acid (Diclofenac) Initiated by Cytochrome P450-Mediated Oxidative Decarboxylation

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### ABSTRACT:

Diclofenac (2-[2-(2,6-dichlorophenyl)aminophenyl]ethanoic acid), a nonsteroidal antiinflammatory drug, undergoes bioactivation by cytochrome P450 oxidation to chemically reactive metabolites that are capable of reacting with endogenous nucleophiles such as glutathione (GSH) and proteins and that may play a role in the idiosyncratic hepatotoxicity associated with the drug. Here, we investigated the ability of diclofenac to be metabolized to 2-(2,6-dichloro-phenyl-amino)benzyl-S-thioether glutathione (DPAB-SG) in incubations with rat liver microsomes (RLMs) and human liver microsomes (HLMs) fortified with NADPH and GSH. Thus, after incubation of diclofenac (50  $\mu$ M) with liver microsomes (1 mg protein/ml), the presence of DPAB-SG was detected in both RLM and HLM incubation extracts by liquid chromatography-tandem mass spectrometry techniques. The formation of DPAB-SG was NADPH-, concentration-, and time-de-

pendent. Coincubation of diclofenac (10  $\mu$ M) with ketoconazole (1  $\mu$ M), an inhibitor of cytochrome P450 (P450) 3A4, with HLMs led to a 75% decrease in DPAB-SG formation. However, in contrast, incubation with the P450 2C9 inhibitor sulfaphenazole (10  $\mu$ M) or the P450 2D6 inhibitor quinidine (40  $\mu$ M) led to a 1.9- and 1.6-fold increase in DPAB-SG production, respectively. From these data, we propose that P450 3A4 mediates the oxidative decarboxylation of diclofenac, resulting in the formation of a transient benzylic carbon-centered free radical intermediate that partitions between elimination (*o*-imine methide production) and recombination (alcohol formation) pathways. The benzyl alcohol intermediate, which was not analyzed for in the present studies, if formed could undergo dehydration to provide a reactive *o*-imine methide species. The *o*-imine methide intermediate then is proposed to react covalently with GSH, forming DPAB-SG.

Diclofenac (Fig. 1), a nonsteroidal antiinflammatory drug used for the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and acute muscle pain (Small, 1989), is metabolically activated by cytochrome P450 to chemically reactive species that react with endogenous nucleophiles such as glutathione (GSH) and protein (Tang, 2003). Adverse reactions to diclofenac include enteropathy (common with long-term use), acute and chronic hepatitis (15% of patients have increased plasma transaminase levels), hemolytic anemia, and fatal anaphylaxis (i.e., IgE- or IgG-mediated immune response). Reactive intermediates have been proposed to play a role in the rare, but sometimes severe, toxicity associated with use of the drug (Boelsterli, 2003). Diclofenac is metabolized by cytochromes P450 3A4 and 2C9 by oxidation of the aromatic ring to 5- and 4'-hydroxylated products, respectively, which can undergo further P450-mediated oxidation to quinone imine-type-reactive metabolites that react covalently with GSH-forming conjugates readily detected by LC-MS/MS techniques (Tang et al., 1999a). The most recent reported glutathione conjugate of diclofenac, namely 2-(2,6-dichlorophenylamino)benzyl-S-thioether glutathione (DPAB-SG), was detected from high-resolution accurate mass LTQ-Orbitrap mass spectrometric analysis of extracts from rat and human hepatocyte incubations with diclofenac and from extracts of diclofenac-dosed rat bile (Teffera et al.,

2008). In that same report, 2-(2,6-dichlorophenylamino)benzoic acid was also identified as a metabolite of diclofenac excreted in rat bile and formed in rat and human hepatocyte incubations. However, in that report, 2-(2,6-dichlorophenylamino)benzoic acid and DPAB-SG were not detected in extracts from rat and human liver microsomal preparations fortified with NADPH and GSH.

The present studies were designed to examine more closely the ability of cytochrome P450 to catalyze the NADPH- and GSH-dependent metabolism of diclofenac, leading to the formation of DPAB-SG. We propose that oxidative decarboxylation of diclofenac could be mediated by P450 catalysis. The decarboxylation of arylacetic acid- and 2-arylpropionic acid-containing drugs by cytochrome P450 has been reported (Komuro et al., 1995). Accordingly, we propose that diclofenac may undergo a similar P-450-mediated oxidative decarboxylation, leading to a transient 2-(2,6-dichlorophenylamino)benzyl-carbon-centered free radical that could either recombine with hydroxyl radical-forming 2-(2,6-dichlorophenylamino)benzyl alcohol that undergoes dehydration, leading to the *o*-imine methide, or undergo further oxidation, leading directly to the *o*-imine methide reactive intermediate (Fig. 1). Reaction of the *o*-imine methide intermediate at the benzylic position with GSH then forms DPAB-SG conjugate as proposed by Teffera et al. (2008).

### Materials and Methods

**Materials.** Diclofenac sodium, carbamazepine, ketoconazole, sulfaphenazole, quinidine, and glutathione were purchased from Sigma-Aldrich (St.

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**ABBREVIATIONS:** diclofenac, 2-[2-(2,6-dichlorophenyl)aminophenyl]ethanoic acid; GSH, glutathione; P450, cytochrome P450; LC-MS/MS, liquid chromatography-tandem mass spectrometry; DPAB-SG, 2-(2,6-dichloro-phenylamino)benzyl-S-thioether glutathione; RLM, rat liver microsome; HLM, human liver microsome; CID, collisionally induced dissociation; ACN, acetonitrile; CBZ, carbamazepine.

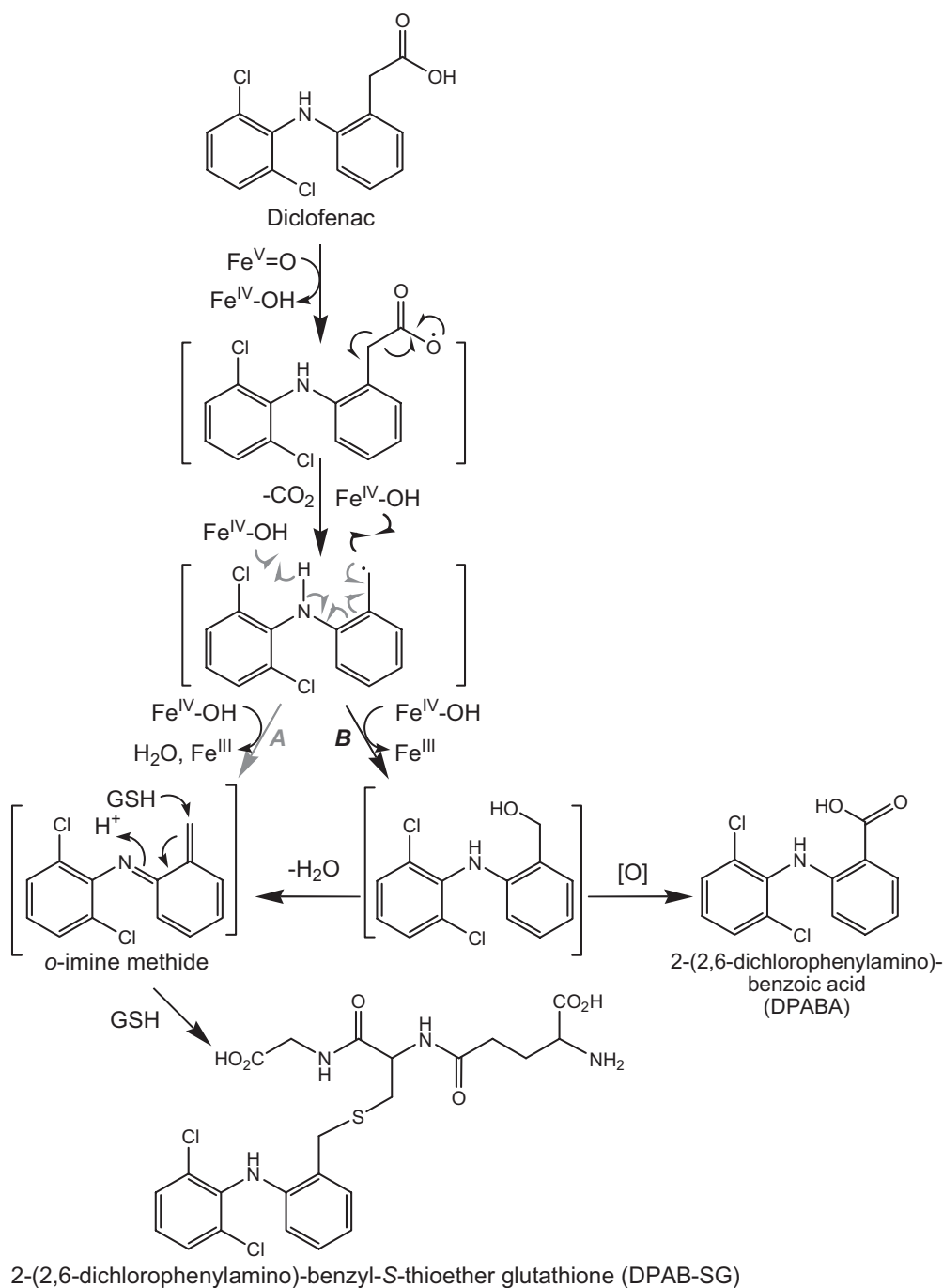


Fig. 1. Proposed scheme for the P450-mediated metabolic activation of diclofenac by P450 3A4, leading to the formation of a chemically reactive *o*-imine methide intermediate either by hydrogen atom abstraction from the intermediate benzylic-centered radical intermediate (A) or by formation of an intermediate benzylic alcohol intermediate that undergoes dehydration (B). Glutathione then is shown to react with the *o*-imine methide intermediate at the benzylic position, forming DPAB-SG.

Louis, MO). The DPAB-SG conjugate was obtained from previous work (Teffer et al., 2008). All solvents used for LC/MS were of chromatographic grade. Stock solutions of diclofenac (0.1, 1, and 10 mM) were prepared as solutions in distilled water. Stock solutions of ketoconazole, sulfaphenazole, and quinidine were prepared at 1, 10, and 40 mM, respectively, in methanol. RLMs and HLMs (pooled) were purchased from Xenotech, LLC (Lenexa, KS).

**Instrumentation and Analytical Methods.** LC-MS/MS analyses were conducted using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a binary pump, degasser, well autosampler, and column heater kept at 25°C. Chromatographic resolution was achieved with a Shiseido Capcell Pak-AQ C-18 column (4.6 × 250 mm, 3 μm; Tokyo, Japan). Mobile phases consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.08% formic acid (solvent B) run at a constant flow rate of 1 ml/min. The solvent gradient was initially held at 0% solvent B for 5 min and increased linearly to 70% solvent B over another 45 min, kept at 70% solvent B for an additional 4 min, then

immediately dropped to 0% solvent B over 0.1 min, where it was held constant at 0% solvent B for 6 min before the next sample injection (45 μl). A Thermo Fisher TSQ Quantum Discovery Max mass spectrometer (Thermo Electron Corporation, Waltham, MA), linked to a CTC HTS PAL Autosampler (Leap Technologies, Carrboro, NC), was used in the present studies. Electrospray ionization was employed with the needle potential held at 4.5 kV. MS/MS conditions used were 2 mTorr argon collision gas and 20-eV collision potential. Positive ion mode full scan (50–1200 Da) was conducted with a scan time of 0.73 s and source collision energy of 10 V. Xcalibur software (version 2.0) (Thermo Electron Corporation) was used to acquire all data.

**In Vitro Studies with Rat and Human Liver Microsomes.** To generate enough DPAB-SG conjugate for LC-MS/MS collisionally induced dissociation (CID) analysis, liver microsomes (1 mg protein/ml) were incubated with diclofenac (50 μM) in potassium phosphate buffer (0.1 M, pH 7.4) in the presence or absence of NADPH (1 mM) and GSH (5 mM). Large incubations

(8-ml total volume) were performed in 20-ml glass vials capped with plastic screw caps and in a bench-top orbital shaking (60 rpm) water bath set at 37°C. Incubations were quenched after 1 h with a solution (8 ml) of acetonitrile (ACN) containing 3% formic acid, capped, and vortex-mixed (1 min). These mixtures were transferred to microcentrifuge tubes (2 ml/tube), capped, and centrifuged (14,000 rpm, 5 min). Supernatants were combined and transferred to a 50-ml glass beaker and allowed to evaporate at room temperature and under a stream of N<sub>2</sub> gas for up to 4 h. Once dried, extracts were reconstituted with a solution (1 ml) containing 3% formic acid in ACN and potassium phosphate buffer (0.1 M, pH 7.4) [1:1 (v/v)]. The reconstituted mixtures were transferred to microcentrifuge tubes and centrifuged as described above. The resulting supernatants were transferred to high-performance liquid chromatography vials (400  $\mu$ l; Sun International, Wilmington, NC) and analyzed by LC-MS/MS as described above.

For time-dependent *in vitro* metabolism studies, diclofenac (10  $\mu$ M) was incubated with HLMs (1 mg/ml, 1-ml incubation volume) in the presence of NADPH (1 mM) and GSH (5 mM) over a 3-h time period in duplicate incubations per time point. Incubations were quenched at the 0-, 5-, 10-, 20-, 30-, 60-, 120-, and 180-min time points with a quench solution (1 ml) consisting of acetonitrile containing 3% formic acid and 0.2  $\mu$ M carbamazepine (CBZ) internal standard. The entire quenched mixture was transferred to microcentrifuge tubes (2-ml volume), capped, and the samples were centrifuged as described above. The resulting supernatants were transferred to

polypropylene autosampler vials (as above) prior to chromatographic analysis for the formation of DPAB-SG. Concentration-dependent experiments were performed with increasing concentrations of diclofenac (3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000  $\mu$ M) incubated with HLMs (1 mg/ml) and for an incubation time of 10 min, followed by processing for LC-MS/MS analysis as described above.

Inhibition experiments were performed with diclofenac (10  $\mu$ M) incubated with HLMs (1 mg protein/ml, 1-ml incubation volume) in the presence or absence of ketoconazole (1  $\mu$ M), an inhibitor of P450 3A4, sulfaphenazole (10  $\mu$ M), an inhibitor of P450 2C9, or quinidine (40  $\mu$ M), an inhibitor of P450 2D6. Incubations ( $n = 3$ ) were performed as above and quenched after 10 min of incubation (as above). For all incubations of diclofenac with liver microsomes, a preincubation period in phosphate buffer (5 min; 0.1 M, pH 7.4, 5 mM GSH) and inhibitors (where indicated) were performed prior to starting the incubation by the addition of NADPH stock solution (5 mM in 0.1 M phosphate buffer, pH 7.4).

**Quantification of DPAB-SG.** Extracts of diclofenac HLM incubations were analyzed by LC-MS/MS analysis for DPAB-SG and CBZ by using the selected ion monitoring transitions,  $m/z$  557.1 to 250.0 and 237.0 to 194.0, respectively, in the positive ion mode and using the chromatographic method described above. Authentic DPAB-SG standard eluted at the retention time of 34.5 min, whereas CBZ eluted at 31 min (data not shown). The concentration of DPAB-SG was determined from a linear standard curve generated from DPAB-SG/CBZ peak area ratios.

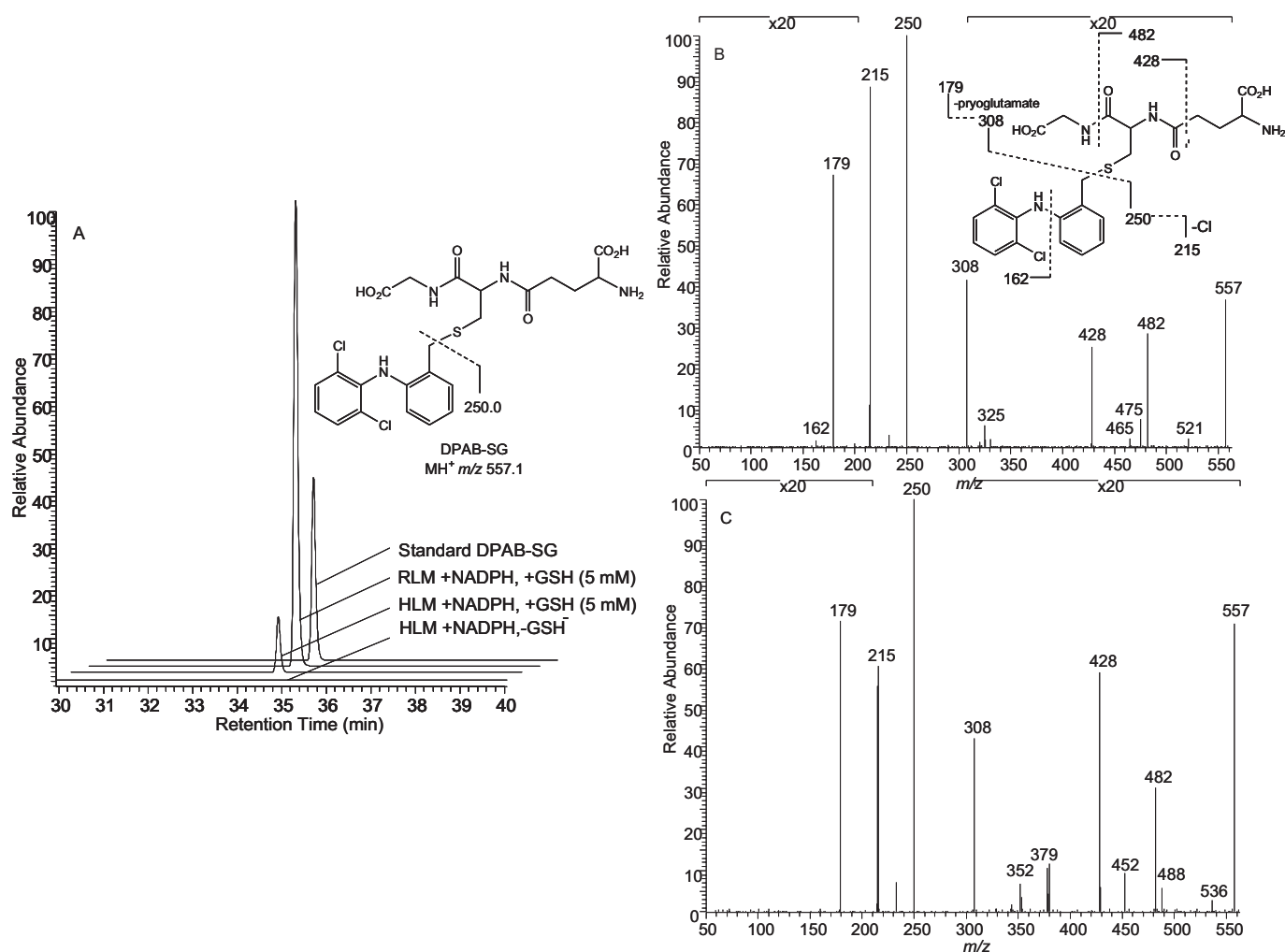


FIG. 2. A, representative reverse-phase gradient LC-MS/MS multiple reaction monitoring chromatograms of extract from human liver microsomes incubated with diclofenac (10  $\mu$ M), NADPH (1 mM), and no GSH; extract from human liver microsomes incubated with diclofenac (10  $\mu$ M), NADPH (1 mM), and added GSH (5 mM); extract from rat liver microsomes incubated with diclofenac (10  $\mu$ M), NADPH (1 mM), and added GSH (5 mM); and control human liver microsome extract with spiked authentic DPAB-SG standard (0.3  $\mu$ M). The transitions used for this LC-MS/MS analysis were  $MH^+ m/z$  557.1 to 250.0 and  $MH^+ m/z$  557.1 to 215.0 for DPAB-SG. LC-MS/MS tandem mass spectra of biologically formed DPAB-SG from incubations of diclofenac (50  $\mu$ M) with HLMs (1 mg/ml, 60 min) obtained by CID of the protonated molecular ion  $MH^+$  at  $m/z$  557.1 (B) and authentic DPAB-SG standard (C). The origins of the characteristic fragments are as shown.

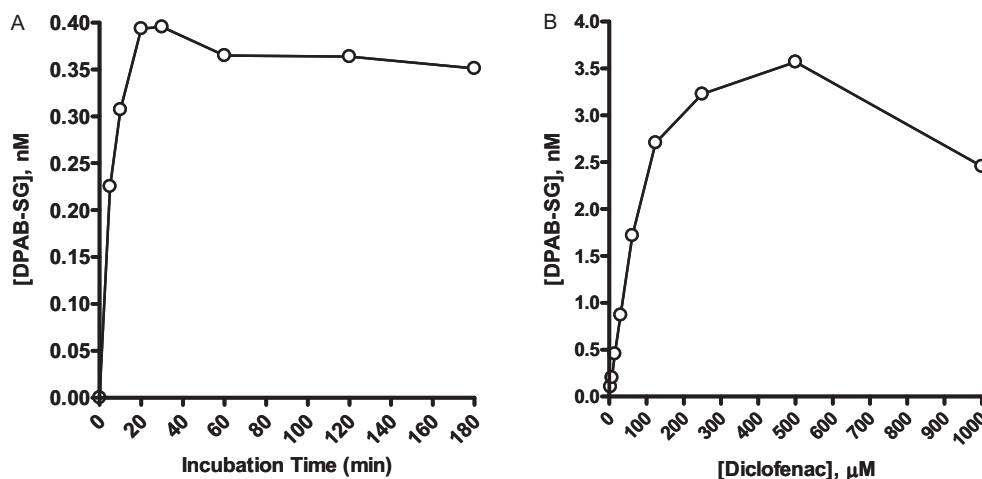


FIG. 3. Time (A)- and concentration (B)-dependent metabolism of diclofenac to DPAB-SG in incubations with HLMs (1 mg/ml). Time-dependent incubations were performed at a diclofenac concentration of 10  $\mu\text{M}$ . Concentration-dependent incubations were conducted for 10 min.

## Results

**Identification of DPAB-SG.** Tandem mass spectrometric analysis by a LC-MS/MS multiple reaction monitoring detection technique allowed for the identification of DPAB-SG formed in both RLM and HLM incubations (Fig. 2). Reverse-phase LC-MS/MS analysis showed the presence of DPAB-SG in incubations of diclofenac (50  $\mu\text{M}$ ) with RLMs and HLMs only when conducted in the presence of NADPH (data not shown) and which coeluted with authentic DPAB-SG standard at a retention time 34.5 min (Fig. 2A). No DPAB-SG was detected in extracts where GSH was not present in the incubation. LC-MS/MS CID analysis of DPAB-SG formed in incubations fortified with GSH provided a product ion spectrum that was identical to the authentic DPAB-SG standard and consistent with its chemical structure (Fig. 2, B and C). Thus, DPAB-SG product ions formed included ions at  $m/z$  482, 428, and 308, which correspond to the elimination of glycine ( $-75$  Da), pyroglutamic acid ( $-129$  Da), and glutathione (protonated product ion), respectively.

**Time- and Concentration-Dependent Formation of DPAB-SG in Incubations of Diclofenac with HLMs.** When diclofenac (10  $\mu\text{M}$ ) was incubated with HLMs (1 mg/ml), the formation of DPAB-SG was shown to reach a maximum concentration of 0.39 nM after 20 min of incubation (Fig. 3A). At the 3-h time point, the concentration of DPAB-SG in the incubation had decreased by 10%, to 0.35 nM. When incubations were performed with HLMs (1 mg/ml) in the presence of NADPH (1 mM) and GSH (5 mM) for 10 min with increasing concentrations of diclofenac (3.9–1000  $\mu\text{M}$ ), results showed a linear concentration-dependent increase in formation of DPAB-SG from 3.9 to 62.5  $\mu\text{M}$  diclofenac, and a maximal formation of 3.6 nM DPAB-SG was reached at a diclofenac concentration of 500  $\mu\text{M}$ . The concentration of DPAB-SG in incubations performed at 1 mM diclofenac was measured at 2.5 nM, which is 30% less than the maximum achieved at 500  $\mu\text{M}$  diclofenac (Fig. 3B).

**Inhibition Studies.** The effects of the P450 3A4 inhibitor, ketoconazole (1  $\mu\text{M}$ ), the P450 2C9 inhibitor, sulfaphenazole (10  $\mu\text{M}$ ), and the P450 2D6 inhibitor, quinidine (40  $\mu\text{M}$ ), on the formation of DPAB-SG from diclofenac (10  $\mu\text{M}$ ) were examined in incubations with HLMs (1 mg/ml, 10 min). Results indicated that the formation of DPAB-SG was strongly inhibited (75%) by coincubation of liver microsomes with ketoconazole (10  $\mu\text{M}$ ). However, corresponding incubations with sulfaphenazole or quinidine led to a 185 and 157% increase in DPAB-SG formation, respectively.

## Discussion

Results from the present mechanistic studies provide evidence that diclofenac undergoes a novel P450 3A4-mediated metabolic activation reaction, leading to the formation of a proposed *o*-imine methide intermediate that reacts with GSH, forming the *S*-thioether-linked glutathione adduct, DPAB-SG. The lack of detection of DPAB-SG from liver microsomal incubation extracts in prior work might have been due to inefficient extraction procedures used for this conjugate. In the present work, the quench solution employed consisted of a mixture of ACN and 3% formic acid, whereas in the prior work by Teffera et al. (2008), nonacidified ACN was used.

A potential mechanism to explain the cytochrome P450-dependent metabolism of diclofenac to DPAB-SG is shown in Fig. 1 and implements the initial formation of a carboxylate free radical via hydrogen atom abstraction from the carboxylic acid moiety by the perferryl oxygen of the heme prosthetic group. This proposal comes from a mechanism discovered by Komuro et al. (1995) for the decarboxylation of arylacetic and arylpropionic acid carboxylic acid-containing drugs. Then, the oxygen-centered free radical decomposes by loss of  $\text{CO}_2$  to give the carbon-centered diclofenac benzylic radical. This step is followed either by recombination of the carbon radical and perferric hydroxide radical to yield the benzylic alcohol or by elimination of a hydrogen atom from the carbon radical amine moiety to generate the *o*-imine methide intermediate. In the second case, the perferric hydroxide radical might function as a suitable acceptor for the second hydrogen atom that is withdrawn from the molecule.

Komuro et al. (1995) reported that  $\alpha$ -arylcarboxylic acids (e.g., phenylacetic acid, ketoprofen, and indomethacin) and  $\alpha,\alpha,\alpha$ -trisubstituted acetic acids (e.g., clofibric acid and 2,2-dimethyl-3-phenylpropionic acid) undergo a facile decarboxylation by P450, leading to the formation of the corresponding one-carbon-shortened alcohol metabolite. They used spin-trapping electron spin resonance methods, employing the radical trapping agent meso-tetrakis(pentafluorophenyl)porphyrin iron chloride, to detect the formation of an intermediate radical during the decarboxylation reaction. From these same studies, they showed that the decarboxylation could occur in rat liver microsomes incubations fortified with NADPH, that the metabolism was inhibited by treatment of liver microsome incubations with carbon monoxide or the P450 inhibitor SKF-525, and that the one-carbon reduced alcohol of ketoprofen was detected in the bile of ketoprofen-dosed rats. Based on these observations, the authors proposed it to be “highly probable that oxidative decarboxylation of carboxylic acids generally occurs as a pathway of hepatic metabolism in vivo.” The

authors also predicted that oxidative decarboxylation of susceptible carboxylic acid-containing drugs leads to the formation of carbon-centered radicals that could potentially lead to damage of macromolecules such as DNA and proteins.

In the present experiments, incubations with HLMs and diclofenac in the presence of ketoconazole, an inhibitor of P450 3A4, led to a substantial decrease in the formation of the corresponding glutathione adduct DPAB-SG, whereas incubations with the P450 2C9 inhibitor sulfaphenazole actually led to an increase in DPAB-SG formation. The ~1.9-fold increase in glutathione adduct formation is proposed to be caused by a metabolic switching from the P450 2C9-mediated 4'-hydroxydiclofenac formation to P450 3A4-mediated metabolism but was not investigated further. When HLM incubations with diclofenac included quinidine, 1.6-fold stimulation in DPAB-SG formation was observed. These data are consistent with bioactivation of diclofenac to DPAB-SG by P450 3A4 because similar studies have shown quinidine-mediated enhancement of P450 3A4-mediated bioactivation of diclofenac, leading to the formation of diclofenac GSH conjugates in vitro in incubations with HLMs (Tang et al., 1999b; Ngui et al., 2000; Masubuchi et al., 2002).

In addition to P450 3A4-mediated formation of 5-hydroxydiclofenac, which can undergo further chemical oxidation or P450-mediated oxidation to the chemically reactive diclofenac-2,5-quinone imine (Tang et al., 1999a; Fig. 1), this new mechanism involving P450 3A4-mediated decarboxylation of diclofenac represents the only other P450 3A4-mediated bioactivation route known for the drug to date. Oxidative metabolism of diclofenac has been shown to lead to time-dependent inhibition of P450 3A4, and metabolism of 5-hydroxydiclofenac to the 2,5-benzoquinone-reactive intermediate may not be relevant (Masubuchi et al., 1999). Based on the results from the present study, we propose the possibility that the *o*-imine methide-reactive intermediate may be involved in the time-dependent inactivation of the P450 3A4 caused by diclofenac, which we are currently exploring.

In summary, these findings represent the first report for oxidative decarboxylation of a carboxylic acid drug in incubations with HLMs. This reaction may proceed forward via conversion of the drug to a transient free radical intermediated, which partitions between elimination (*o*-imine methide production) and recombination (alcohol formation) pathways. In addition, these findings may have wide-ranging

implications with respect to the P450-mediated metabolism of structurally related carboxylic acid-containing drugs that may be able to undergo decarboxylation and subsequent elimination- or dehydration-type reactions leading to (for example) imine-, quinone-, or thioquinone-methide chemically reactive, and potentially toxic, intermediates.

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