Mechanistic Studies on the Bioactivation of Diclofenac: Identification of Diclofenac− S −acyl−glutathione in Vitro in Incubations with Rat and Human Hepatocytes †

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · DECEMBER 2003

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Mechanistic Studies on the Bioactivation of Diclofenac: Identification of Diclofenac-*S*-acyl-glutathione in Vitro in Incubations with Rat and Human Hepatocytes†

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Received March 3, 2003

Diclofenac, a nonsteroidal anti-inflammatory drug, is metabolized to diclofenac-1-*O*-acyl glucuronide (D-1-*O*-G), a chemically reactive conjugate that has been implicated as playing a role in the idiosyncratic hepatoxicity associated with its use. The present studies investigated the ability of diclofenac to be metabolized to diclofenac-*S*-acyl-glutathione thioester (D-SG) in vitro in incubations with rat and human hepatocytes and whether its formation is dependent on a transacylation-type reaction between D-1-*O*-G and glutathione. When diclofenac (100 μM) was incubated with hepatocytes, D-SG was detected in both rat and human incubation extracts by a sensitive LC-MS/MS technique. The initial formation rate of D-SG in rat and human hepatocyte incubations was rapid and reached maximum concentrations of 1 and 0.8 nM, respectively, after 4 min of incubation. By contrast, during incubations with rat hepatoctyes, the formation of D-1-*O*-G increased over 30 min of incubation, reaching a maximum concentration of 14.6 μM. Co-incubation of diclofenac (50 μM) with (−)-borneol (400 μM), an inhibitor of glucuronidation, led to a 94% decrease in D-1-*O*-G formation, although no significant decrease in D-SG production was observed. Together, these results indicate that diclofenac becomes metabolically activated in vitro in rat and human hepatocytes to reactive acylating derivatives that transacylate glutathione forming D-SG, but which is not solely dependent on transacylation by the D-1-*O*-G metabolite. From these results, it is proposed that reactive acylating metabolites of diclofenac, besides D-1-*O*-G, may be significant in the protein acylation that occurs in vivo and therefore also be important with regard to the mechanism(s) of diclofenac-mediated idiosyncratic hepatotoxicity.

Introduction

Diclofenac (*o*-[(2,6-dichlorophenyl)amino]phenylacetic acid, Figure 1) is a nonsteroidal anti-inflammatory drug used for the treatment of patients with osteoarthritis, rheumatoid arthritis, or ankylosing spondylitis (*1*, *2*). Chemically reactive metabolites of diclofenac have been proposed to mediate the idiosyncratic hepatotoxicity associated with the clinical use of the drug (*2*, *3*). Diclofenac is metabolized to diclofenac-1-*O*-acyl glucuronide (D-1-*O*-G;[1] Figure 1; *4*), an unstable and reactive acyl glucuronide metabolite (*5*) that has been implicated as playing a role in the mechanism(s) of diclofenac-induced hepatotoxicity. Acyl glucuronide metabolites of acidic drugs are proposed to bind covalently to protein by two different mechanisms. These include transacylation-type reactions with protein nucleophiles by the 1-*O*-acyl glucuronide isomer and by a glycation mechanism that involves the reaction of open-chain aldehyde forms of the acyl migration glucuronide isomers with protein amino groups (Figure 1; *6*, *7*). Then it is proposed that the drug−protein adducts are recognized by the immune system as foreign, resulting in an immune response and thereby leading to the associated idiosyncratic hepatoxicity (*2*, *8*).

Recently, we showed D-1-*O*-G to be chemically reactive in a transacylation-type reaction with glutathione (GSH) in vitro in buffer at pH 7.4 and 37 °C, leading to the

† A preliminary account of this work was presented at the XIVth World Congress of Pharmacology (IUPHAR), San Francisco, CA, July 2002.

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[1] Abbreviations: D-1-*O*-G, diclofenac-1-*O*-acyl glucuronide; GSH, glutathione; D-SG, diclofenac-*S*-acyl-glutathione thioester; D-SCoA, diclofenac-*S*-acyl-CoA thioester; CA-SG, clofibryl-*S*-acyl-glutathione thioester; ACN, acetonitrile; CID, collisionally induced dissociation; MRM, multiple reaction monitoring; SRM, selected reaction monitoring; LC-MS/MS, liquid chromatography−tandem mass spectrometry; GST, glutathione *S*-transferase.

10.1021/tx034038b CCC: $25.00
Published on Web 10/22/2003

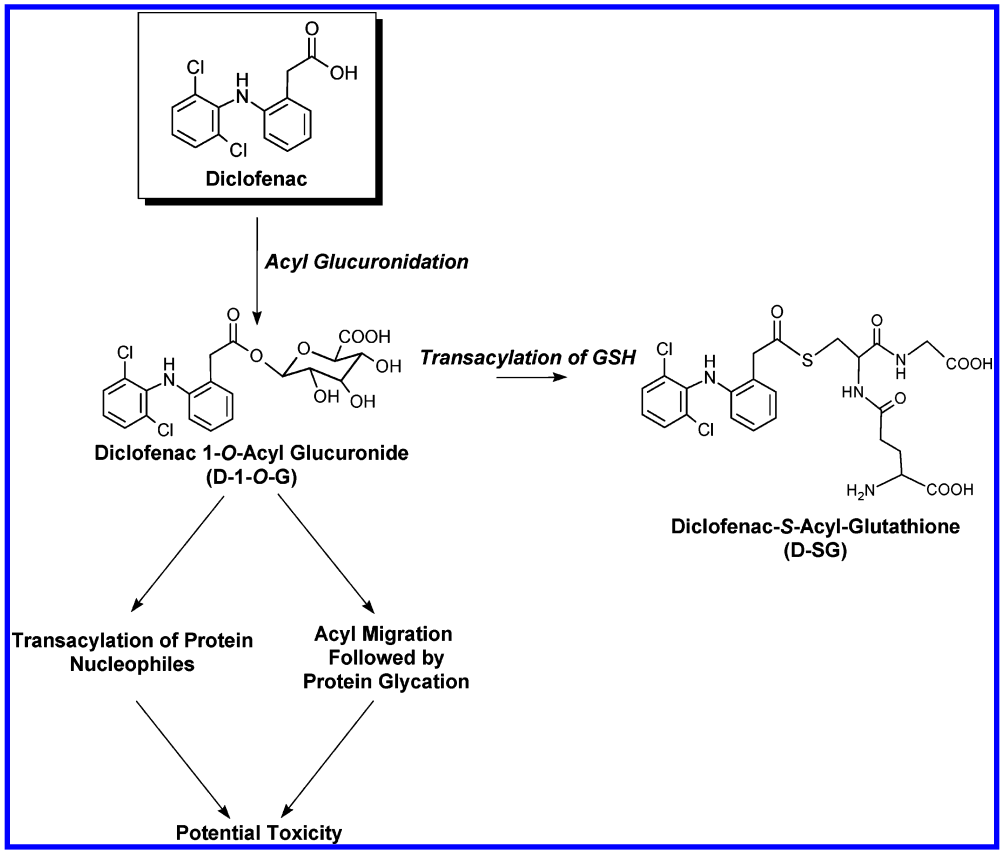

Figure 1. Proposed scheme for the metabolic activation of diclofenac by acyl glucuronidation leading to covalent binding to protein and the acylation of glutathione.

formation of diclofenac-*S*-acyl-glutathione (D-SG; *9*). In addition to these data, it was shown that D-SG is formed in vivo in diclofenac-dosed rats and excreted into bile (*9*). Other types of GSH adducts of diclofenac have been detected in rat bile and in incubations with human hepatoctyes (*10*). P450-mediated aromatic hydroxylation of diclofenac, leading to 4′- and 5-hydroxydiclofenac, followed by further oxidation to reactive benzoquinone imine intermediates (*10*) and subsequent reactions with GSH, is proposed to have been the mechanism of formation of the thioether-linked GSH conjugates that were detected. In addition to the detection of GSH adducts of diclofenac in hepatocytes, it was found that the incubation of diclofenac in cultured rat hepatocytes led to a time- and concentration-dependent covalent binding of the drug to hepatocyte protein (*4*), which was proposed to occur, at least in part, from reactions with diclofenac glucuronide. Hepatic protein adducts of diclofenac (50, 70, 110, and 140 kDa) detected in the livers of diclofenac-treated mice (*11*) provided additional evidence for chemically reactive metabolites of diclofenac formed in vivo.

We suspect that diclofenac may form a glutathione thioester derivative (D-SG) in vitro in incubations with freshly isolated hepatoctyes. The conjugate then could serve as a marker derivative for mechanistic studies on the bioactivation and hepatotoxicity of diclofenac. To date, no *S*-acyl-linked GSH derivatives of acidic drugs have been detected in vitro in incubations with hepatocytes. In the present experiments, we utilized rat and human hepatocytes as an in vitro model to characterize the time- and concentration-dependent formation of D-SG and to determine if its formation is dependent on the metabolism of diclofenac to D-1-*O*-G. Results from the present in vitro studies provide evidence that diclofenac does indeed undergo bioactivation to reactive acylating metabolite(s) that transacylate GSH. The data also indicate that reactive acylating species, besides D-1-*O*-G, may contribute significantly to the acylation of GSH, and presumably cellular protein nucleophiles, in vitro and in vivo.

Experimental Section

Materials. Diclofenac sodium salt, carbamazepine, collagenase (for hepatocyte isolation), and (−)-borneol were purchased from Sigma Chemical Co. (St. Louis, MO). D-SG and D-1-*O*-G were synthesized as described below. All solvents used for HPLC and LC-MS were of chromatographic grade. Stock solutions of diclofenac (10 mM), D-SG (10 mM), and D-1-*O*-G (1.3 mM) were prepared as solutions in 50:50 acetonitrile (ACN)/water (3% formic acid).

Instrumentation and Analytical Methods. HPLC was carried out on a Hewlett-Packard 1090 series II HPLC equipped with diode array detection. All analytical HPLC analyses were performed on a reverse-phase column (Zorbax, 3 μm, C18, 150 × 2.1 mm, Agilent Technologies). HPLC (with UV detection at 275 nm) and LC-MS analyses were performed using a gradient system of 0.1% formic acid with elution from 5% ACN to 100% over 13 min at a flow rate of 0.3 mL/min. LC-MS and LC-MS/MS were performed on a Finnigan TSQ-7000 equipped with API2 and running Excalibur version 1.2 (San Jose, CA). Electrospray ionization was employed with the needle potential held at ∼4.5 kV. Tandem LC-MS/MS analysis conditions used were ∼2 mTorr argon collision gas and 25 eV collision potential.

Synthesis of D-SG Thioester. D-SG thioester was synthesized as described previously (*12*) and provided D-SG as a white solid (13% yield). D-SG thioester was characterized by tandem mass spectrometry on a Finnigan-MAT TSQ-7000 tandem mass spectrometer, and LC-MS/MS analysis was performed by gradient elution as described above. Synthetic D-SG standard eluted

at a retention time of 9.2 min and showed no detectable impurities when analyzed by both positive and negative ion LC-MS scan modes (data not shown) via reverse-phase gradient elution (described above). Tandem LC-MS/MS analysis of D-SG (CID of MH^+ ion [^{35}Cl] at *m/z* 585): *m/z* (%) 510 ([M + H − Gly]$^+$, 10%), 438 ([M + H − pyroglutamic acid − water]$^+$, 100%), 308 ([glutathione + H]$^+$, 8%), 278 ([2-([2,6-dichlorophenyl]-amino)benzene − CHCO]$^+$, 23%), 250 ([2-([2,6-dichlorophenyl]-amino)benzene − CH_2]$^+$, 25%). The analysis for D-SG from incubations of diclofenac with hepatocytes was conducted by LC-MS/MS with multiple reaction monitoring (MRM) detection. The MRM transitions used were from *m/z* 585 to *m/z* 438 for D-SG and from *m/z* 237 to *m/z* 194 for the internal standard carbamazepine.

Biosynthesis of D-1-*O*-G. D-1-*O*-G was obtained by preparative reverse-phase HPLC purification of rat bile (6 h collection, bile acidified to pH 3) ethyl acetate extracts from a rat dosed with diclofenac (50 mg/kg). Tandem LC-MS/MS analysis of D-1-*O*-G (CID of MH^+ ion [^{35}Cl] at *m/z* 472): *m/z* (%) 454 ([M + H − H_2O]$^+$, 7%), 296 ([diclofenac + H]$^+$, 100%), 178 ([glucuronic acid]$^+$, 2%). A stock solution of D-1-*O*-G (1.3 mM) was kept in ACN and stored frozen (∼-20 °C) until use. Quantification of D-1-*O*-G in these studies was performed by HPLC analysis (UV 275 nm, as above) or by LC-MS in positive ion scan mode and utilizing a linear standard curve generated from peak areas measurements.

In Vitro Studies with Rat and Human Hepatocytes. Freshly isolated rat (250−300 g, male Sprague−Dawley, $N = 2$ experiments) or human (male, $N = 2$ experiments) hepatocytes were prepared according to the method of Moldéus et al. (*13*). Recent studies have shown that this method allows for the retention of cell viability and GSH concentration for hepatocytes in suspension over 3 h of incubation (*14*). The viabilities of hepatocytes achieved for the two rat hepatocyte isolations performed in the present studies were 89 and 95%, as assessed by trypan blue exclusion. Encapsulated human liver samples (200−400 g) were obtained from Tissue Transformation Technologies (Edison, NJ), where an agreement was formed between the company and Pharmacia Corp. for the use of the liver samples for research purposes. **Caution:** *Human liver tissue may contain infectious agents. This product should be handled carefully.* In general, hepatocytes isolated from an encapsulated human liver section were prepared using the same buffers and reagents as used in the rat hepatocyte isolation and as follows: Briefly, the liver section was first perfused with Hank-I EDTA-containing buffer (pH 7.4, 37 °C) with two perfusion lines (connected to perfusion needles having 5 mm outer diameters, 4 mm inner diameters, and 5 cm in length) and perfused at ∼100 mL/min (for each line) for 12 min. The perfusion needles were placed in large (∼5 mm diameter) vasculature openings on the face of the cross section such that the flow from one perfusion line did not impede the flow from the second line. During the perfusion, the liver section was located in the same beaker containing the perfusion buffer, which was continuously recirculated. After the first perfusion step had been completed, the Hank-I buffer was aspirated off and replaced with Hank-II collagenase-containing (1 mg/mL, collagenase for hepatocyte isolation, Sigma Chemical Co.) buffer and perfused again at 100 mL/min at 37 °C for 45 min. Subsequently, this perfusion was stopped and the digested liver taken out of the collagenase-containing Hank-II buffer mixture and placed in a 2 L glass beaker containing 1 L of gassed (95% O_2/5% CO_2) Krebs−Henseleit buffer at room temperature and pH 7.4. The digested liver was cut into small pieces (∼2 in.) and gently shaken (∼5 min) to disperse the hepatocytes. Once most of the cells were dispersed from the liver section, additional Krebs−Henseleit buffer was added to the hepatocyte solution to give a final volume of ∼2 L. The entire hepatocyte solution then was filtered through cotton gauze into four 500 mL plastic Nalgene (screw cap) bottles. The hepatocyte solutions then were centrifuged (while capped) at 1000 rpm for 10 min at 4 °C to pellet the hepatocytes. The resultant supernatants were aspirated off, and fresh Krebs−Henseleit buffer (∼400 mL for each bottle) was added to the Nalgene bottles containing the hepatocyte pellets, followed by gentle mixing to suspend the hepatocytes, and once again centrifuged as above. This washing procedure, performed to remove cell debris and collagenase enzyme, was repeated twice more. The washed hepatocyte pellets then were suspended again in Krebs−Henseleit buffer (as above), gently mixed, and filtered through cotton gauze to remove cell aggregates. The resulting hepatocyte solution then was assessed for cell concentration and percent viability by trypan blue exclusion as above. Hepatocyte viabilities for the two experiments performed in theses studies were 87 and 93%. A final cell concentration of 2 million viable cells/mL was obtained by adding an appropriate volume of Krebs−Henseleit buffer. The yield of cells obtained from this method was ∼1 billion viable cells/100 g of liver tissue.

Incubations of hepatocytes (2 million viable cells/mL) with diclofenac were performed in Krebs−Henseleit buffer (pH 7.4) in 20 mL glass vials capped with a plastic screw cap containing a small hole ($^1/_8$ in. diameter) to reduce evaporation. Incubations (5 mL total volume for all experiments, $N = 3$ incubations per experiment) were performed with continuous rotation and under an atmosphere of 5% CO_2 at 37 °C in a VWR model 1927 incubator (Willard, OH). All procedures in this study have been approved and conducted in compliance with the Animal Welfare Act Regulations (9 CFR Parts 1, 2 and 3) and the *Guide for the Care and Use of Laboratory Animals* (ILAR, 1996), as well as with all internal company policies and guidelines.

For time-dependent studies, freshly isolated hepatocytes were incubated with diclofenac (100 μM) and aliquots of the incubation mixture were analyzed for D-1-*O*-G and D-SG formation over a 60 min period. Aliquots (200 μL) of the incubation mixture were taken at 0.2, 1, 2, 4, 6, 8, 10, 20, 30, and 60 min of incubation and added directly to microcentrifuge tubes (1.5 mL) containing a quench solution (200 μL) consisting of methanol, 3% formic acid, and 10 μM carbamazepine internal standard. Samples were centrifuged (14 000 rpm, 2 min), and the supernatant was transferred to 0.25 mL polypropylene autosampler vials (Sun International, Wilmington, NC) prior to chromatographic analysis. These extracts were analyzed for both D-SG and D-1-*O*-G derivatives by LC-MS/MS and HPLC, respectively, as described above. The same extracts were also analyzed for thioether-linked glutathione (GSH) conjugates of diclofenac benzoquinone imine reactive intermediates. For this analysis, extracts were assayed by LC-MS by positive ion scan (from *m/z* 50 to 1000) and by the same chromatography as described above. Authentic standards of the thioether-linked GSH adducts were not available in the present studies; therefore, the relative amounts formed over time were represented as the peak area ratio of the diclofenac GSH adducts (summed peak areas for three thioether-linked GSH adducts detected between retention times of 6 and 8 min) and carbamazepine (extracted ion at MH^+ *m/z* 237).

Concentration-dependent experiments were performed with increasing concentrations of diclofenac (7.8, 15.6, 31.3, 62.5, 100, 125, 250, 500, 1000, and 2000 μM) with a rat hepatocyte concentration of 2 million viable cells/mL and an incubation time of 4 min. Hepatocyte concentration-dependent experiments, ranging from 0.125 to 2 million viable rat hepatocytes/mL, were performed with a diclofenac concentration of 100 μM and an incubation time of 4 min. Samples from these studies were processed as described above.

Inhibition experiments were performed with diclofenac (50 μM) incubated with rat hepatocytes (2 million viable cells/mL) in the presence of increasing concentrations of (−)-borneol (0, 50, 100, and 400 μM) for the inhibition of D-1-*O*-G formation. Incubations ($N = 3$ for each inhibitor concentration) were performed as above and quenched after 4 min of incubation (as above). Stock solutions of (−)-borneol were prepared in absolute ethanol, and all incubations included the same final concentration of ethanol (0.1%, v/v).

Incubations performed to assess the metabolic stability of D-SG thioester were conducted with a rat hepatocyte concentra-

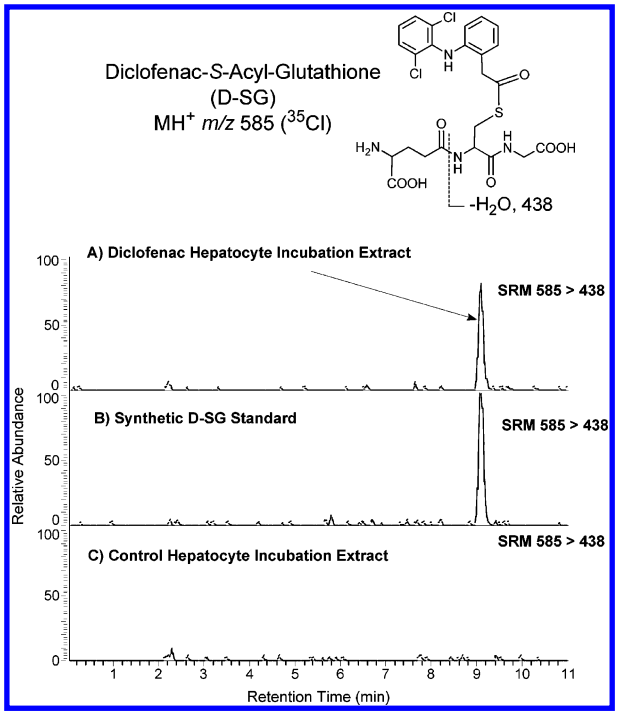

Figure 2. Representative reverse-phase gradient LC-MS/MS SRM chromatograms of (A) extract from rat hepatocytes incubated with 100 μM diclofenac, (B) extract from control rat hepatocytes spiked with D-SG authentic standard, and (C) extract of control hepatocytes not treated with diclofenac or D-SG standard. The transition used for this LC-MS/MS SRM analysis was from *m*/*z* 585 to *m*/*z* 438.

tion of 2 million viable cells/mL and an initial D-SG concentration of 100 μM. Aliquots (200 μL) of the incubation were taken at 0.2, 1, 2, 3, and 5 min, added to quench solution, and further processed as described above. Analysis of the amount of D-SG remaining and the amount of diclofenac formed in the incubation was conducted by HPLC with UV detection at 275 nm and quantified by linear standard curves of D-SG and diclofenac peak areas.

Results

Identification of D-SG. Analysis by a sensitive and selective LC-MS/MS MRM detection technique allowed for the identification of D-SG formed in rat and human hepatocytes (Figure 2). The transition used for this analysis was from *m*/*z* 585 to *m*/*z* 438, which was chosen because of its being the major fragmentation pathway for D-SG as assessed by positive ion LC-MS/MS collisionally induced dissociation (CID) of the MH^+ ion at *m*/*z* 585 of authentic D-SG (Figure 3B). Reverse-phase LC-MS/MS analysis showed the presence of D-SG in incubations of diclofenac (100 μM) with freshly isolated rat hepatocytes, which coeluted with authentic D-SG standard at a retention time of 9.2 min (Figure 2). LC-MS/MS analysis of biologically formed D-SG provided a product ion spectrum that was identical to that of the authentic D-SG standard and consistent with its chemical structure (Figure 3; see ref *15* for a review of characteristic glutathione conjugate product ions formed from analysis by electrospray LC-MS/MS).

Time Course of D-SG and D-1-*O*-G Formation in Freshly Isolated Rat and Human Hepatocytes. When freshly isolated hepatocytes were incubated with diclofenac (100 μM), the formation of D-SG was shown to reach maximum concentrations of ~1 and 0.8 nM after

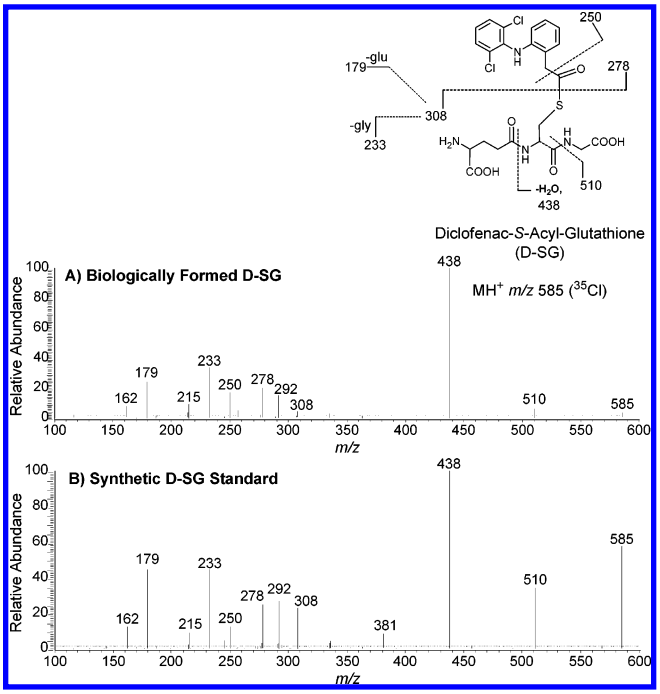

Figure 3. LC-MS/MS tandem mass spectra of (A) biologically formed D-SG and (B) synthetic standard D-SG, obtained by CID of the protonated molecular ion MH^+ at *m*/*z* 585. The origins of the characteristic fragments are shown.

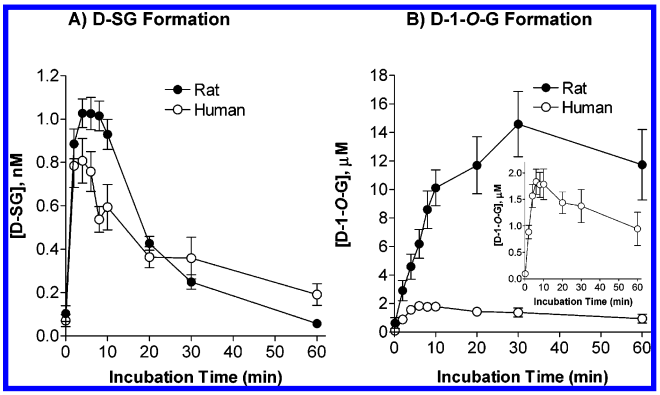

Figure 4. Time course for the formation of (A) D-SG and (B) D-1-*O*-G in freshly isolated rat and human hepatocytes (2 million viable cells/mL) incubated with 100 μM diclofenac. Values are the mean ± SE of six incubations (three incubations per hepatocyte experiment). (Inset) Expanded time course plot for the formation of D-1-*O*-G from incubations of diclofenac with human hepatocytes.

4 min in incubations with rat and human hepatocytes, respectively (Figure 4A). By contrast, the formation of D-1-*O*-G in incubations with rat hepatocytes increased over a 30 min incubation period, reaching a maximum of 14.6 μM (Figure 4B). Incubations in human hepatocytes showed that the amount of D-1-*O*-G formed over time reached a maximum concentration of ~1.8 μM at 4 min and then decreased in a nearly linear fashion to ~1 μM at 60 min. No D-SG or D-1-*O*-G formation was detected in incubations with heat-denatured rat hepatocytes (data not shown), which indicated the need for viable hepatocytes and functional enzymes in order to bioactivate diclofenac. Results from these experiments indicate that the time course of D-1-*O*-G formation did not correlate with the time course of D-SG formation in incubations with rat hepatoctyes. In addition, the time course for the degradation of authentic D-SG (100 μM)

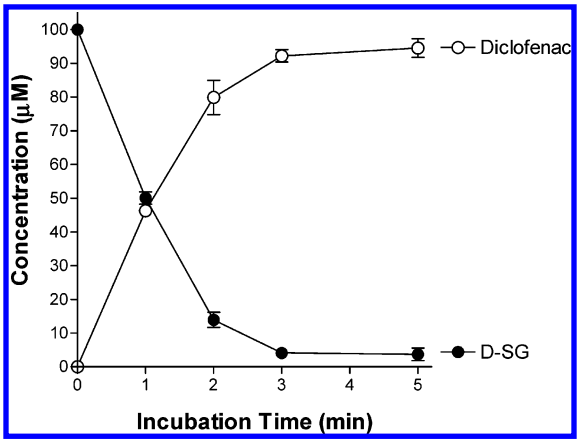

Figure 5. Time-dependent degradation of D-SG (1 μM) in incubations with freshly isolated rat hepatocytes (2 million viable cells/mL) leading to the rapid formation of diclofenac free acid. Values are the mean ± SE of triplicate incubations.

when incubated with rat hepatocytes revealed the derivative to be metabolically unstable, resulting in its rapid hydrolysis (Figure 5, apparent half-life of ~1 min). In these experiments, no evidence for γ-glutamyltranspeptidase (γ-GT)-mediated degradation product, diclofenac-*N*-acylcysteinylglycine (data not shown), was detected in extracts from incubations of D-SG with rat hepatocytes, which is consistent with results showing the lack of sufficient γ-GT activity in rat liver tissue (*16*).

In addition to the detection of the thioester-linked D-SG adduct in these hepatocyte extracts, three thioether-linked GSH adducts were detected by reverse-phase LC-MS positive ion scan detection. The three GSH adducts were detected at an MH^+ of *m/z* 617 (^{35}Cl) and eluted at retention times of 6.3, 6.9, and 7.4 min (Figure 6A). Tandem mass spectrometric analysis of each of these adducts, by CID of the MH^+ *m/z* 617 ion, provided product ion spectra that were consistent with their chemical structures being glutathione adducts of diclofenac benzoquinone imine reactive intermediates (*10*; data not shown). On the basis of the work by Tang et al. (*10*), who incubated diclofenac with cultured human hepatocytes and detected three thioether-linked adducts with MH^+ *m/z* 617, we propose that the adducts with MH^+ of *m/z* 617 found in the present studies similarly are 5-hydroxy-4-(glutathion-*S*-yl)diclofenac, 4′-hydroxy-3′-(glutathion-*S*-yl)diclofenac, and 5-hydroxy-6-(glutathion-*S*-yl)diclofenac, although we are not sure of their exact elution order during the chromatography performed in the present work. Figure 6B shows the time-dependent formation of the three thioether-linked adducts (sum of the three peak areas then divided by the peak area of the internal standard carbamazepine) over 60 min of incubation of diclofenac with rat hepatocytes. Results show that the formation of these adducts increased in a fairly linear fashion over 30 min of incubation and did not decrease throughout the remainder of the incubation.

Diclofenac Concentration-Dependent Formation of D-SG and D-1-*O*-G in Freshly Isolated Rat Hepatocytes. When rat hepatocytes were incubated for 4 min with increasing concentrations of diclofenac (from 7.8 μM to 2 mM), results showed a linear concentration-dependent formation of D-SG reaching ~1.25 nM D-SG at a concentration of 125 μM diclofenac and then decreased D-SG formation with increasing diclofenac concentration, where no D-SG could be detected at the 2 mM diclofenac concentration (Figure 7A). The concentration-dependent formation of D-1-*O*-G in the same incubations was shown to increase to ~3 μM at a concentration of 125 μM diclofenac, and at the highest diclofenac concentration used (2 mM), the amount of D-1-*O*-G in the incubation was ~2 μM (Figure 7B). These data show that D-SG is not formed in the incubations at the 2 mM diclofenac concentration, even though 2 μM D-1-*O*-G was present in the incubation. Therefore, as with the time-dependent results (above), the concentration-dependent formation of D-SG did not correlate with the concentration-dependent formation of D-1-*O*-G.

Hepatocyte Concentration-Dependent Formation of D-SG and D-1-*O*-G. When freshly isolated rat hepatocytes (0.125−2 million viable cells/mL) were incubated with diclofenac (100 μM), the formation of both D-SG and D-1-*O*-G increased with increasing rat hepatocyte concentration up to 1 million viable cells/mL, but, above that concentration, no further significant increase in either analyte was detected (Figure 8).

Inhibition Studies. To determine the importance of diclofenac acyl glucuronidation on the formation of D-SG in rat hepatocytes, the effect of the glucuronidation inhibitor, (−)-borneol (*17*), was examined. Inhibition experiments were performed with diclofenac (50 μM) in incubations with rat hepatocytes (2 million viable cells/mL, 4 min) in the presence of increasing concentrations of (−)-borneol (0−400 μM final concentration) for the inhibition of D-1-*O*-G formation and effect on D-SG production. Figure 9A shows that the formation of D-1-*O*-G decreased with increasing (−)-borneol concentration and that a 94% decrease in D-1-*O*-G formation in hepa-

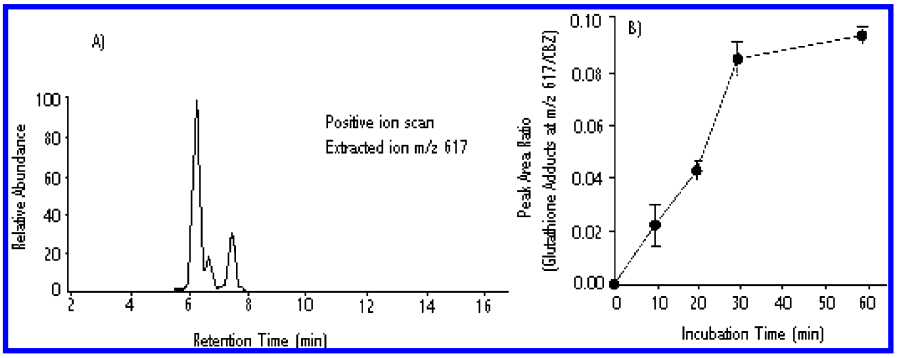

Figure 6. Analysis of thioether-linked diclofenac glutathione adducts formed in diclofenac-treated (100 μM) rat hepatocytes. The data show (A) a representative reverse-phase gradient LC-MS extracted ion chromatogram for MH^+ *m/z* 617 from the 30 min hepatocyte extract, and (B) the time course for the formation of three thioether-linked glutathione adducts [shown in (A)] as summed peak area ratios (relative to the internal standard carbamazepine). Values are the mean ± SE of triplicate incubations.

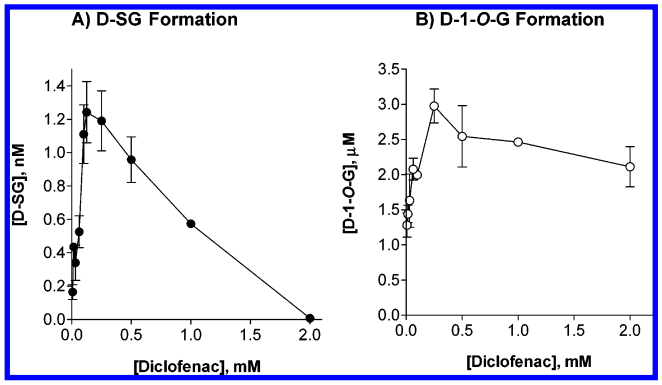

Figure 7. Concentration-dependent formation of (A) D-SG and (B) D-1-*O*-G in freshly isolated rat hepatocytes (2 million viable cells/mL) incubated with various concentrations of diclofenac for 4 min. Values are the mean ± SE of triplicate incubations.

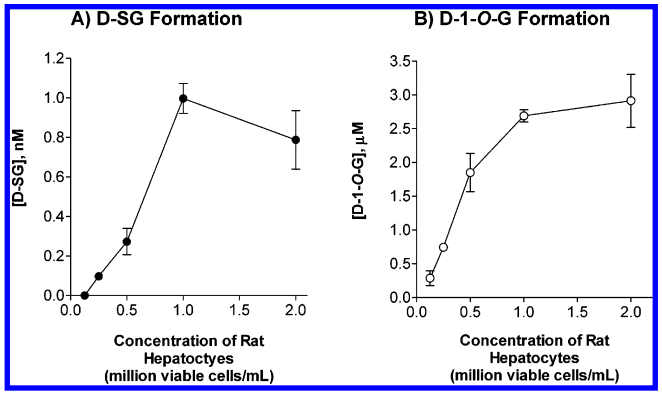

Figure 8. Hepatocyte concentration-dependent formation of (A) D-SG and (B) D-1-*O*-G in freshly isolated rat hepatocytes incubated with 100 μM diclofenac for 4 min. Values are the mean ± SE of triplicate incubations.

tocytes treated with 400 μM (−)-borneol had no significant effect on the formation of D-SG (Figure 9B). These results are consistent with the time- and diclofenac concentration-dependent data reported above, where the amount of D-1-*O*-G formed in incubations of diclofenac with rat hepatocytes did not correlate with the extent of D-SG formation. Hence, from these data, we propose that reactive acylating metabolites, besides D-1-*O*-G, may be functioning to acylate GSH, and presumably other cellular protein nucleophiles, in vitro in hepatocytes.

Discussion

For many carboxylic acid-containing drugs frequent types of adverse reactions leading to idiosyncratic toxicities, such as liver damage, skin reactions, and renal toxicity, have been documented (*2*, *18*, *19*). The biochemical mechanisms responsible for the production of these side effects are not well understood. One mechanism put forward to explain some of this idiosyncratic toxicity proposes that covalent modification of cellular proteins by chemically reactive species formed during the metabolism of carboxylic acid-containing drugs leads to immunotoxic reactions (*6*, *7*). For most acidic drugs, acyl glucuronidation is a common metabolic pathway that leads to the generation of chemically reactive metabolites (Figure 1). Irreversible binding of acidic drugs to plasma proteins via acyl glucuronide metabolites has been well documented (*20*). In addition, it has been shown that within a range of acyl glucuronides of carboxylic acid-containing drugs having increasing chemical instability

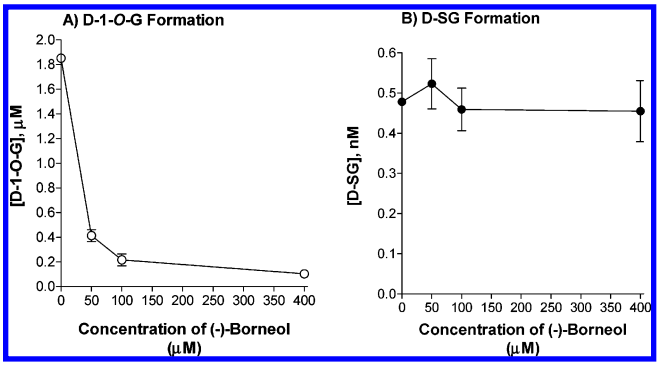

Figure 9. Concentration-dependent inhibitory effects of (−)-borneol on (A) diclofenac acyl glucuronidation and (B) D-SG formation in incubations of 50 μM diclofenac with freshly isolated rat hepatocytes (2 million viable cells/mL, 4 min) and increasing concentrations of (−)-borneol. Values are the mean ± SE from three incubations for each inhibitor concentration.

there exists a correlation with increased covalent binding to protein in vitro and in vivo (*21*).

Results from the present in vitro studies provide evidence that D-1-*O*-G may not be the sole reactive metabolite of diclofenac that is able to acylate the nucleophilic cysteinylthiol of glutathione forming D-SG and, we propose, not the sole reactive metabolite of diclofenac that acylates protein nucleophiles in liver tissue in vivo. Because results from these studies showed that the formation of D-SG in hepatocytes did not seem to correlate with the formation of D-1-*O*-G, we propose that some other acylating metabolites of diclofenac may be formed in hepatocytes that are also able to transacylate cellular nucleophiles.

As shown in Figure 4, the formation of D-SG reaches a maximum concentration after 4 min of incubation of diclofenac with rat hepatocytes but steadily decreases for the remainder of the incubation. Therefore, a reactive metabolite(s) of diclofenac is proposed to be formed in the early part of the incubation but over time ceases to be formed at the same initial rate, resulting in decreased formation of the D-SG adduct. Because, in incubations with rat hepatocytes, the D-1-*O*-G derivative increases over 30 min of incubation, it does not correlate with the rapid decrease in D-SG formation seen after 8 min.

Recent studies have shown that the transacylation reaction of the 1-*O*-acyl glucuronide of 2-phenylpropionic acid (2-PPA) with GSH occurred by a second-order reaction mechanism that was first order with respect to the concentrations of both 2-PPA-1-*O*-G and GSH (*22*). Therefore, we would assume that the transacylation reaction between D-1-*O*-G and GSH also occurs by a second-order reaction and that a significant increase in D-1-*O*-G concentration would lead to a respective increase in the acylation of GSH forming of D-SG. The measurement of diclofenac acyl glucuronidation in the present studies was restricted to the D-1-*O*-G isomer, but, at later time points during the hepatocyte incubations (30 and 60 min), one minor acyl migration isomer was detected, which eluted slightly later than D-1-*O*-G (data not shown). We assume that the acyl migration isomers of D-1-*O*-G would not contribute significantly to the acylation of GSH and the formation of D-SG in hepatocytes, due to the finding that the acyl migration isomers are much less reactive than the 1-*O*-acyl isomer with GSH in buffer at pH 7.4 and 37 °C (*9*). Acyl migration isomers

of acyl glucuronide derivatives of clofibric acid (*23*) and 2-PPA (*22*) have also been shown to be nonreactive or much less reactive with GSH, respectively.

One explanation for the decrease in D-SG formation in hepatocyte incubations at later time points could be that the concentration of GSH in the hepatocyte incubation decreases upon incubation with diclofenac, therefore resulting in less GSH being available to be transacylated by the reactive metabolite(s). Evidence against this assumption comes from studies showing that diclofenac (150−325 μM), in incubations with cultured rat hepatocytes, actually led to a significant increase, rather than a depletion, in the amount of GSH in the cells per milligram of protein (*24*). We did not measure the effect of diclofenac on the concentration of GSH in the present studies, but we did measure the formation of thioether-linked glutathione adduct formed from the reaction of quinone-imine reactive metabolites of diclofenac with GSH in rat hepatocytes (Figure 6). Unlike the time course of D-SG concentration in rat hepatocyte incubations with diclofenac, which showed a 75% decrease from 8 min (1 nM D-SG) to 30 min (0.25 nM D-SG), the formation of three thioether-linked adducts (summed peak areas) increased in a roughly linear fashion over the same incubation period (Figure 6B). Therefore, from these data, we propose that sufficient GSH is present in the diclofenac rat hepatocyte incubations to support glutathione adduct formation. Further evidence against the importance of the D-1-*O*-G metabolite functioning in the formation of D-SG thioester is shown by the similarity in the amount of D-SG formed in rat and human hepatocytes incubations versus the marked species difference in the overall formation of D-1-*O*-G (Figure 4). The difference in the amount D-1-*O*-G formed in incubations with rat and human hepatocytes is consistent with results in vivo showing 50 and 10−20% of the drug metabolized by acyl glucuronidation, respectively (*25*). The most convincing data that precludes a major role of the D-1-*O*-G metabolite in the acylation of GSH comes from inhibition studies with (−)-borneol (Figure 8), where a 94% inhibition of D-1-*O*-G formation did not lead to a significant decrease in the amount of D-SG formed. Once again, we propose that if D-1-*O*-G is important in the acylation of GSH, that a significant inhibition of D-1-*O*-G formation should lead to a significant decrease in D-SG formation, because the formation rate of the acyl-glutathione conjugate is probably dependent on the concentration of the acyl glucuronide (*22*).

The D-SG thioester derivative was shown to be unstable in incubations with rat hepatocytes, where it underwent a rapid hydrolysis (apparent $t_{1/2}$ of ∼1 min) leading to the formation of diclofenac free acid (Figure 5). We believe that D-SG may be hydrolyzed by thioesterases present in the incubation mixture extracellularly, or intracellularly if the conjugate is able to enter the cell. If species differences exist in thioesterase enzyme activity, which was not characterized in the present studies, it may have influenced the relative amount of D-SG detected in the rat and human hepatocyte incubations. Another route by which D-SG could be cleaved is by glutathione *S*-transferase (GST)-mediated hydrolysis (*26*), where the ability of GST A1-1 to hydrolyze D-SG thioester was shown in vitro with purified enzyme.

D-SG thioester is reactive in its own right (*12*), where it was shown to be reactive with *N*-acetylcysteine in a transacylation-type reaction, in buffer, leading to the quantitative formation of the respective mercapturate. In addition, D-SG was shown to undergo an intramolecular transacylation reaction in vitro in buffer (pH 7.4, 37 °C) forming an indolinone-type derivative (*9*). Hence, D-SG thioester is a reactive intermediate that might also contribute to the covalent binding to protein in the liver.

We propose that diclofenac may be bioactivated to a reactive acyl-CoA-linked thioester derivative, diclofenac-*S*-acyl-CoA (D-SCoA), that could transacylate endogenous nucleophiles including GSH. Indirect evidence for the formation of D-SCoA in vivo comes from studies in dogs showing that a major metabolite of diclofenac is the taurine amide, which is formed via an acyl-CoA intermediate (*25*, *27*).

An example of an acidic drug shown to form an *S*-acyl-glutathione conjugate is clofibric acid, forming clofibryl-*S*-acyl-glutathione thioester (CA-SG; *23*). The authors from that study detected CA-SG in the bile of clofibric acid-dosed rats. They also showed that clofibryl-1-*O*-acyl glucuronide reacted with GSH in vitro in buffer to form the GSH thioester product but that the major reaction that occurred was acyl migration to isomers that were not able react with GSH. A possible alternative reactive acylating metabolite was suggested (*12*) when it was shown that clofibryl-*S*-acyl-CoA, a known intermediary metabolite of the drug, was 40-fold more reactive with glutathione, forming CA-SG, compared to similar studies with the clofibryl-1-*O*-acyl glucuronide (*23*). Related studies have shown that the acyl-CoA thioester of 2-phenylpropionic acid is ∼70-fold more reactive with glutathione, forming 2-phenylpropionyl-*S*-acyl-glutathione, than the respective 1-*O*-acyl glucuronide in vitro in buffer under physiological conditions (*22*).

The focus of the present studies was to determine the importance of the D-1-*O*-G conjugate in the acylation of biological nucleophiles, such as GSH, in vitro in rat and human hepatocytes. We do not discount the proposed importance of the ability of the respective acyl migration isomers of D-1-*O*-G to contribute to the covalent binding to protein in vivo (*28*), where it was shown that hepatic microsomes incubated with [^{14}C]UDP-glucuronic acid and non-radiolabeled diclofenac resulted in similar covalent binding of radiolabeled drug to microsomal proteins. Because it is not yet known what type of protein adducts may be causing the idiosyncratic toxicity (*29*), we at least presume, from the present data, that D-1-*O*-G probably does not contribute to the covalent binding to protein via transacylation-type reactions as much as the acyl migration isomers do via the glycation mechanism.

Studies with diclofenac that are ongoing in our laboratory include (1) analytical experiments to examine the formation of D-SCoA in vitro in rat and human hepatocytes and (2) experiments with inhibitors of acyl-CoA formation to examine the effect of decreased D-SCoA formation on the production of D-SG. Together, results from these experiments should provide insight into the mechanism(s) involved in the idiosyncratic allergic toxicity related to the use diclofenac and other carboxylic acid-containing drugs.

Acknowledgment. We thank Terri L. VandeGiessen, Lori R. Norris, and Brian W. Jones from the Preclinical Research Laboratory, Pharmacokinetics, Dy-

namics, and Metabolism, Pfizer, Inc. (Kalamazoo, MI), for assistance in the preparation of freshly isolated rat hepatocytes. We acknowledge Dr. Chungang Gu (Pharmacokinetics, Dynamics, and Metabolism, Pfizer, Inc., Kalamazoo, MI) for support in the mass spectrometric analysis of D-SG and D-1-*O*-G derivatives. We also thank Professor Leslie Z. Benet from the Department of Biopharmaceutical Sciences at the University of California, San Francisco, for valuable discussions related to this work. The present studies were partially supported by National Institutes of Health Grant GM36633.

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TX034038B