IDENTIFICATION OF ZOMEPIRAC-S-ACYL-GLUTATHIONE IN VITRO IN INCUBATIONS WITH RAT HEPATOCYTES AND IN VIVO IN RAT BILE

MARK P. GRILLO AND FENGMEI HUA
Pharmacokinetics, Dynamics, and Metabolism, Pfizer, Inc., Kalamazoo, Michigan

(Received August 13, 2003; accepted August 19, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Zomepirac (ZP), a nonsteroidal anti-inflammatory drug that was withdrawn from use, is metabolized to zomepirac-1-O-acyl-glucurononide (ZP-1-O-G), a chemically reactive conjugate that has been implicated in the toxicity of the drug. In the present studies, we investigated the ability of ZP to become bioactivated to reactive metabolites that transacylate glutathione (GSH) forming ZP-S-acyl-glutathione thioester (ZP-SG) in vitro and in vivo in rat. When ZP (100 μM) was incubated with rat hepatocytes, ZP-SG was detected in incubation extracts by a sensitive selected reaction monitoring liquid chromatography/tandem mass spectrometry (LC/MS-MS) technique. The initial formation rate of ZP-SG was rapid and reached a maximum concentration of 0.24 ± 0.03 nM after 4 min of incubation, then decreased, in a fairly linear fashion, to 0.07 ± 0.03 nM after 60 min of incubation. The product ZP-SG (1 μM) was shown to be unstable by undergoing rapid hydrolysis (apparent half-life ~0.8 min) in incubations with rat hepatocytes. After administration of ZP to a male Sprague-Dawley rat (100 mg/kg IP), bile was collected and analyzed for ZP-SG by LC/MS-MS. Results indicated the presence of ZP-SG in bile (6.7 μg excreted after 6 h of collection), which was confirmed by coelution with synthetic standard and by its tandem mass spectrum. Together, these results demonstrate that ZP becomes metabolically activated in vitro in rat hepatocytes and in vivo in rat to reactive acylating derivative(s), such as ZP-1-O-G, that transacylate GSH forming ZP-SG. Finally, we propose that ZP-SG thioester could be used as a marker derivative for mechanistic studies on the bioactivation of the drug.

Zomepirac (ZP1; 5-[p-chlorobenzoyl]-1,4-dimethylpyrrole-2-acetic acid; Fig. 1) is a carboxylic acid-containing nonsteroidal anti-inflammatory drug that was withdrawn from clinical use in March 1983 due to unexplained severe, and sometimes fatal, adverse allergic reactions (Kiani and Kushner; 1983; Levy and Vasilomanolakis, 1984). ZP is metabolized primarily to an unstable and chemically reactive acyl glucuronide, ZP-1-O-acyl-glucuronide (ZP-1-O-G; Hasegawa et al., 1982; O’Neill et al., 1982; Smith et al., 1985, 1990) that has been implicated in the toxicity of the drug (Fig. 1). In general, 1-O-acylglucuronides of acidic drugs are proposed to covalently bind to protein by two different mechanisms: transacylation-type reactions with protein nucleophiles by the 1-O-acylglucuronide isomer, and a glycation mechanism involving the reaction of protein amino-groups with open-chain aldehyde forms of the acyl migration isomers of the acyl glucuronide (Fig. 1; Faed, 1984; Spahn-Langguth and Benet, 1992). These drug-protein adducts then are proposed to be recognized by the immune system as foreign, thereby eliciting an immune response leading to the associated toxic side-effects (Zia-Amirhosseini et al., 1995).

Studies with ZP have shown that the drug becomes covalently bound to protein in vivo in human plasma (Smith et al., 1986) and in vivo in rat liver (Wang and Dickinson, 2000). Dipeptidyl peptidase IV, a cell membrane glycoprotein, was shown to be a target for covalent adduct formation via reactions with ZP-1-O-G in vivo in rats (Wang et al., 2001). We propose that glutathione (GSH), an endogenous cysteine-containing cytoprotective tripeptide, should also be able to form covalent adducts with reactive acylating metabolites of the drug, such as ZP-1-O-G, forming ZP-S-acyl-glutathione thioester (ZP-SG) from transacylation-type reactions. However, the ZP-SG derivative has not yet been identified. Evidence toward the proposal for ZP-SG formation comes from studies with clofibric acid, a carboxylic acid-containing lipid-lowering drug, which showed it to be metabolized to a reactive acylating glucurononide conjugate that transacylates GSH, forming clofibryl-S-acyl-glutathione thioester in vivo and in vitro (Stogniew and Fenselau, 1982; Shore et al., 1995). Therefore, we suspect that ZP may also form a glutathione thioester conjugate that could be used as a marker derivative for mechanistic studies on the bioactivation of the drug. In the present experiments, we examined the formation of ZP-SG in vitro in incubations of ZP with rat hepatocytes and in vivo in the bile of a ZP-dosed rat.

Materials and Methods

Materials. ZP sodium salt, carbamazepine, and bovine kidney γ-glutamyltranspeptidase (γ-GT, EC 2.3.2.2) were purchased from Sigma-Aldrich (St. Louis, MO). ZP-SG was synthesized as described below. All solvents used for HPLC and LC/MS were of chromatographic grade. Stock solutions of ZP and ZP-SG were prepared as 10 mM solutions in distilled water.
Instrumentation and Analytical Methods. HPLC was carried out on an Agilent 1100 series HPLC apparatus (Agilent Technologies, Palo Alto, CA) with diode array detection. HPLC analyses were performed on a reverse-phase column (Phenomenex Synergi, 4 μm, MAX-RP, 150 × 2.0 mm; Phenomenex, Torrance, CA) for the analysis of rat hepatocyte incubation extracts, or on a Zorbax (3 μm, C18, 150 × 2.1 mm; Agilent Technologies) reverse-phase column for the analysis of rat bile extracts. HPLC (with UV detection at 313 nm), LC/MS, and LC/MS-MS analyses were conducted using a gradient system of 0.1% formic acid with elution from 5% to 100% acetonitrile 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 (Thermo Finnigan, San Jose, CA). Electrospray ionization was employed with the needle potential held at about 4.5 kV. MS-MS conditions used were 2 mTorr argon collision gas and 25 eV collision potential.

Synthesis of ZP-SG. ZP-SG thioester was synthesized as described previously (Grillo and Benet, 2002) and provided the derivative as a white solid (11% yield). LC/MS-MS analysis of ZP-SG was performed by gradient elution as described above. Synthetic ZP-SG eluted at a retention time of ~9.1 min (analysis using the Phenomenex Synergi HPLC column as described above) and showed no detectable impurities when analyzed by both positive and negative ion LC/MS scan modes via reverse-phase gradient elution (described above). Tandem LC/MS-MS analysis of ZP-SG: [collision-induced dissociation (CID) of MH⁺ ion [13Cl] at m/z 581], m/z (％): m/z 506 ([M + H – Gly]⁺, 1%), m/z 452 ([M + H – pyrogulmatatic acid]⁺, 1%), m/z 434 ([M + H – pyrogulmatatic acid – water]⁺, 2%), m/z 308 (glutathione + H)⁺, 4%), m/z 274 [5-(p-chlorobenzyl)-1,4-dimethylpyrrole-CH₂-CO]⁺, 50%], m/z 246 [5-(p-chlorobenzyl)-1,4-dimethylpyrrole-CH₃]⁺, 1%], m/z 136 [(1,4-dimethylpyrrole-CH₂-CO)², 5%], m/z 108 [(1,3-dimethylpyridinium)⁺, 0.8%]. The analysis for ZP-SG formation in both rat hepatocyte and rat bile extracts was conducted by LC/MS-MS with multiple reaction monitoring (MRM) detection. The MRM transitions used were m/z 581 to m/z 274 for ZP-SG, and m/z 237 to m/z 194 for the internal standard carbamazepine.

In Vitro Studies with Rat Hepatocytes. Freshly isolated rat hepatocytes were prepared from male Sprague-Dawley rats (250–300 g) according to the method of Moldeus et al. (1978), and greater than 85% viability was achieved routinely, as assessed by trypan blue exclusion. Incubations of hepatocytes (2 million viable cells/ml in suspension) with ZP 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 inch diameter) to avoid evaporation. Incubations (5-ml total volume for all experiments) were conducted with continuous rotation and under an atmosphere of 95% O₂ and 5% CO₂ at 37°C in a VWR model 1927 incubator (VWR, West Chester, PA). 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” (Institute for Laboratory Animal Research, 1996), as well as with all internal company policies and guidelines.

Fig. 1. Proposed scheme for the metabolic activation of ZP by acyl glucuronidation leading to covalent binding to protein and the acylation of glutathione.
The solution was centrifuged and the supernatant transferred to a 20-ml glass screw cap vial and stored frozen (−20°C) until further analysis. Forty microliters of thawed solution was added to 1 ml of phosphate buffer (0.1 M, pH 7.4) containing 100 μl of a stock solution of γ-glutamyltranspeptidase (1 unit/ml in 0.1 M potassium phosphate buffer, pH 7.4), and the mixture was incubated at 37°C for 30 min. During this time, another incubation containing 1 μM standard ZP-SG was performed with γ-glutamyltranspeptidase in a similar fashion. The incubations then were quenched with methanol containing 3% formic acid (1 ml), and the resulting solutions were analyzed for the loss of ZP-SG and the formation of the γ-glutamyltranspeptidase ZP-SG degradation product, zomepirac-N-acyl-cysteinylglycine (ZP-NCG), by MRM LC/MS-MS analysis in the positive ion mode and using the transitions m/z 581 to m/z 274, and m/z 452 to m/z 274, respectively.

In Vivo Rat Bile Study. A male Sprague-Dawley rat (230 g) was given a dose of ZP (100 mg/kg i.p.), and bile, including predose bile (0.5 h), was collected (over glacial acetic acid and on ice) over a 6-h time period at the 0-, 1-, 2-, 3-, 4-, 5-, and 6-h time points after administration. Collected bile (200 μl) was added to 200 μl of a quench solution (the same solution used for the rat hepatocyte incubation quench), followed by sonication (1 min) at room temperature. The resulting extracts then were centrifuged (14,000 rpm), and the supernatants were analyzed by LC/MS-MS. Quantification of ZP-SG in the bile was performed by MRM analysis and from a linear standard curve of authentic ZP-SG as described above.

Results

Identification of ZP-SG. Using a sensitive and selective electrospray LC/MS-MS SRM technique facilitated the identification of ZP-SG excreted in ZP-dosed rat bile, and which was formed in vitro in ZP-treated rat hepatocytes. Reverse-phase LC/MS-MS analysis showed the presence of ZP-SG in the bile of a ZP-dosed rat (230 g, 100 mg/kg), which coeluted with authentic ZP-SG at a retention time of 9.7 min (Fig. 2). The transition used for the analysis of ZP-SG was m/z 581 to m/z 274, which was chosen because it is the major fragmentation pathway for ZP-SG as assessed by positive ion LC/MS-MS analysis of authentic ZP-SG standard (B).
MS-MS CID of the MH⁺ ion at m/z 581 of authentic ZP-SG (Fig. 3B). The identity of the substance eluting at retention time ~10 min is unknown (Fig. 2A) but was also detected in pre-ZP-dosed bile (data not shown). Tandem mass spectrometric analysis of the ZP-SG metabolite by CID of the MH⁺ ion at m/z 581 provided a product ion spectrum that was consistent with the structure of the GSH adduct (Fig. 3). Analysis of the time-dependent elimination of ZP-SG into bile showed that ~6.7 µg (less than 0.01% of the dose) of the derivative had been excreted 6 h after administration of the drug (Fig. 4). Reverse-phase LC/MS-MS analysis also showed the presence of ZP-SG in incubations of ZP (100 µM) with freshly isolated rat hepatocytes, which coeluted with authentic ZP-SG standard at a retention time of 9.1 min (Fig. 5). The small (~0.6-min) difference in LC/MS-MS retention times of ZP-SG during the analysis of rat bile and rat hepatocyte extracts is due to the different reverse-phase columns that were used (see Materials and Methods). LC/MS-MS analysis of ZP-SG formed in hepatocytes did not provide enough sensitivity to obtain a product ion spectrum; therefore, techniques utilizing γ-GT were performed to confirm its identity. The analysis of γ-GT-mediated degradation of authentic ZP-SG, showed the major cleavage to be m/z 452 to m/z 274, occurring from fragmentation of the amide bond (Fig. 6). Thus, when rat hepatocyte extracts were treated with γ-GT (0.1 unit/ml, pH 7.4, 37°C, 30 min) and the extracts analyzed for ZP-SG and for the formation of ZP-NCG, results showed the complete degradation of ZP-SG (data not shown) and the respective production of ZP-NCG, which coeluted with the γ-GT degradation product of authentic ZP-SG (Fig. 7). As shown in Figs. 6 and 7, the γ-GT-mediated ZP-SG degradation product is drawn as the amide-linked cysteinylglycine adduct (ZP-NCG). This is because the degradation of S-acyl-glutathione thioesters by γ-GT results in the cleavage of the glutamyl-residue of the conjugate forming an unstable S-acyl-cysteinylglycine product that rapidly undergoes an intramolecular transacylation reaction leading to a stable N-acyl-cysteinylglycine derivative (Tate, 1975; Grillo and Benet, 2001). Together, these results provide strong evidence for the formation of ZP-SG in rat hepatocytes incubated with ZP.

**Time Course of ZP-SG Formation in Freshly Isolated Rat Hepatocytes.** When freshly isolated rat hepatocytes were incubated with ZP (100 µM), the formation of ZP-SG was shown to reach a maximum concentration in the incubation (0.24 ± 0.03 nM) by the 4-min time point (Fig. 8), followed by a gradual decrease in concentration to 0.07 ± 0.03 nM ZP-SG at the 60-min time point (Fig. 8). No ZP-SG formation was detected in similar incubations with heat-
denatured hepatocytes (data not shown), which indicates the need for viable hepatocytes and functional enzymes to metabolically activate ZP. In addition, the time course for the degradation of authentic ZP-SG (1 μM) during incubations with rat hepatocytes revealed the derivative to be metabolically unstable, resulting in its rapid hydrolysis (Fig. 9, apparent half-life ~0.8 min). This indicates that ZP-SG formed in hepatocytes is efficiently hydrolyzed to ZP free acid and never builds up in the incubation. In these experiments, it was shown that no γ-GT-mediated degradation product, ZP-NCG, was detected in extracts from incubations of ZP-SG with rat hepatocytes, which is consistent with results showing the lack of sufficient γ-GT activity in rat liver tissue (Hinchman and Ballatori, 1990).

Discussion

Carboxylic acid-containing drugs withdrawn from the market occurred due to severe and unexplained immunotoxic side effects. For many discontinued acidic drugs, frequent types of adverse reactions such as idiosyncratic liver damage, skin reactions, and renal toxicity occurred (Zimmerman, 1994; Bakke et al., 1995). The biochemical mechanisms responsible for the production of these side effects are not well understood. A possible mechanism to explain this idiosyncratic toxicity proposes that covalent modification of cellular proteins by chemically reactive species formed during the metabolism of acidic drugs leads to immunotoxic reactions (Faed, 1984; Spahn-Langguth and Benet, 1992).

In the present studies, we found that ZP is bioactivated to reactive acylating species that transacylate GSH, in vitro in rat hepatocytes and in vivo in rat, forming ZP-SG. Two alternative routes of metabolism that lead to reactive acylating metabolites of acidic drugs are acyl glucuronidation (Benet et al., 1993; Fig. 1) and acyl-coenzyme A (acyl-CoA) formation (Faed, 1984; Boelsterli, 2002; Grillo and Benet, 2002). Irreversible binding of acidic drugs to plasma proteins via acyl

![Fig. 4. Cumulative excretion of ZP-SG into bile after a single dose of ZP (100 mg/kg i.p.) to a male Sprague-Dawley rat (230 g, n = 1).](image)

![Fig. 5. Representative reverse-phase gradient LC/MS-MS MRM chromatograms of extract from rat hepatocytes incubated with 100 μM ZP (4-min time point) (A), and extract from control rat hepatocytes spiked with ZP-SG authentic standard (B).](image)

The transitions used for LC/MS-MS MRM analyses were m/z 581 to m/z 274 and m/z 237 to m/z 194 for ZP-SG and carbamazepine internal standard, respectively.
glucuronide metabolites has been well documented (Spahn-Langguth et al., 1997) and has shown that for varied acyl glucuronides having increasing chemical instability, there is a correlation with increased covalent binding to protein in vitro and in vivo (Benet et al., 1993). By contrast, evaluation of the importance of reactive acyl-CoA intermediates in the metabolic activation of acidic drugs is a relatively new focus toward understanding the mechanism(s) of formation of the associated drug-protein adducts (Boelsterli, 2002; Grillo and Benet, 2002).

Clofibryl-S-acyl-glutathione thioester (CA-SG) was the first example of a GSH thioester formed in vivo (Shore et al., 1995). The authors from that study detected CA-SG in the bile of clofibric acid-dosed rats (75 mg/kg i.v.), which showed it to be a very minor metabolite [0.1% of the amount of clofibryl-1-acyl-glucuronide (CA-1-O-G) excreted in bile]. Studies on the chemical reactivity of CA-1-O-G with GSH in vitro in buffer demonstrated the formation of CA-SG, but the major reaction occurring was acyl migration to isomers that were not able to react with GSH (Shore et al., 1995). ZP-1-O-G is an unstable acyl glucuronide that degrades with an apparent first order half-life of ~0.45 h as compared with the more stable CA-1-O-G derivative (apparent first order half-life ~7.3 h) when measured during incubations in buffer at pH 7.4 and 37°C (Boelsterli, 2002). Therefore, acyl migration of ZP-1-O-G would be predicted to lead to less reactive acyl glucuronide isomers and possibly to decreased formation of ZP-SG.

Comparative in vitro studies on the reactivity of clofibryl-S-acyl-CoA, a known intermediary metabolite of the drug, with GSH showed it to be 40-fold more reactive with glutathione, forming CA-SG (Grillo and Benet, 2002), relative to similar studies with CA-1-O-G (Shore et al., 1995). Along the same line as the clofibryl acid studies, experiments with 2-phenylpropionic acid acyl-CoA thioester showed it to be approximately 70-fold more reactive with GSH, forming 2-phenylpropionyl-S-acyl-glutathione, than the respective 1-O-acyl-glucuronide in vitro in buffer (Li et al., 2002a). Therefore, we propose that the formation of ZP-SG could arise from reactions of either ZP-1-O-G or ZP-S-acyl-CoA thioester (ZP-CoA) with GSH. In fact, a recent report showed that ZP (1 nM), in incubations with freshly isolated rat hepatocytes, formed both ZP-1-O-G and ZP-CoA thioester derivatives (Olsen, 2002). At the present time, it is not known which of these two reactive conjugates is more important in the transacylation of GSH forming ZP-SG. However, a recent study by Li et al. (2002b) showed that the covalent binding of 2-phenylpropionic acid to protein in incubations with rat hepatocytes correlated more with bioactivation by acyl-CoA formation than by acyl glucuronidation. In addition, in a report by Bailey and Dickinson (1996), it was indicated that ZP-protein adduct concentrations in the livers of ZP-dosed rats could not be related to acyl glucuronide reactivity, and that it may have been the reactive ZP-CoA intermediate which mediated the acylation of hepatic proteins.

The formation of ZP-SG in rat hepatocyte incubations, as shown in Fig. 8, occurred in a time-dependent fashion with a maximum concentration reached very early (at the 4-min time point) and then decreased in concentration, in a fairly linear fashion, for the remainder of the incubation. Unfortunately, we did not quantify the formation of the reactive metabolites, ZP-CoA and ZP-1-O-G, in hepatocyte extracts in the present work, but we might assume that the metabolite responsible for the formation of ZP-SG would have kinetics that demonstrate a rapid, but short-lived, formation. Since the method used for the isolation and incubation of rat hepatocytes allowed for sustained cell viability and GSH concentration (up to 3 h of incubation; Nakagawa and Moldeus, 1998), we would assume that the decrease in ZP-SG formation may be due to a decrease in the amount of reactive metabolite formed after the 4-min time point, rather than being due to a decrease in GSH concentration.

The reactivity of both acyl glucuronides and acyl-CoA thioesters with GSH has been shown to occur by second order reaction kinetics that is first order with respect to both the concentration of the acyl glucuronide and the concentration of the acyl-CoA derivative (Li et al., 2002a). Assuming that ZP-1-O-G and ZP-CoA also react with GSH in a second order fashion, then a decrease in the concentration of the reactive metabolite may, we propose, lead to a corresponding decrease in ZP-SG formation.

Incubations of ZP-SG with hepatocytes showed the derivative to be
metabolically unstable toward hydrolysis (apparent half-life ~0.8 min, Fig. 9). We assume that this hydrolysis is being catalyzed by thioesterases located extracellularly in the hepatocyte incubation, or intracellularly if ZP-SG is able to cross the cell membrane. Glutathione thioesters have recently been shown to be cleaved by glutathione S-transferase (GST)-mediated hydrolysis (Dietze et al., 1998), where the ability of GST A1–1 to hydrolyze ethacrynyl-S-acyl-glutathione was shown in vitro with purified enzyme. Therefore, the ZP-SG conjugate, after being formed intracellularly in rat hepatocyte incubations, might be hydrolyzed by intracellular thioesterases or GSTs, or extracellularly by thioesterases after transport of the conjugate out of the cell (Boelsterli, 2002). Glutathione conjugation with reactive metabolites of drugs usually leads to chemically nonreactive products. However, the ZP-SG thioester derivative has been shown to be chemically reactive, in a transacylation-type reaction, with N-acetylcysteine (NAC) forming ZP-S-acyl-NAC thioester (Grillo and Benet, 2002).

Therefore, we propose that ZP-SG, although a very minor metabolite, might also contribute to the covalent binding to protein in ZP-exposed liver.

The focus of the present studies was to determine the ability of reactive acylating metabolites of ZP to transacylate GSH, in vitro in rat hepatocytes and in vivo in rat. We do not discount the proposed importance of the ability of the acyl migration isomers of ZP-1-O-G to contribute to the covalent binding to protein in vivo via a glycation mechanism (Smith et al., 1986, 1990), where it was shown that the irreversible binding to protein in vivo correlated with overall exposure to ZP glucuronide.

Studies with ZP that are ongoing in our laboratory include 1) kinetic in vitro experiments with rat hepatocytes, to measure the time- and ZP-concentration-dependent formation of ZP-1-O-G and ZP-CoA and to determine whether a correlation exists with ZP-SG formation; and 2) mechanistic studies with inhibitors of ZP-1-O-G and ZP-CoA formation to examine the subsequent effect on ZP-SG production in incubations with rat hepatocytes. Through the use of sensitive LC/MS-MS SRM modes of detection and the utilization of freshly isolated rat hepatocyte in vitro techniques as tools, results from these proposed experiments should provide insight into the reactive metabolite(s) involved in the formation of covalent drug-protein adducts related to the use of ZP and other carboxylic acid-containing drugs.

Acknowledgments. We thank Terri L. VandeGiessen, Lori R. Norris, and Brian W. Jones from the Preclinical Research Laboratory, Pharmacokinetics, Dynamics, and Metabolism (PDM), Pfizer, Inc. (Kalamazoo, MI), for assistance in the preparation of rat hepatocytes. We acknowledge Dr. Chunze Li, Department of Bio-
Fig. 8. Time course for the formation of ZP-SG in freshly isolated rat hepatocytes (2 million viable cells/ml in suspension) incubated with 100 μM ZP.

Values are expressed as the mean ± S.E. of triplicate experiments.

Fig. 9. Time-dependent degradation of ZP-SG (1 μM) in incubations with freshly isolated rat hepatocytes (2 million viable cells/ml) leading to the rapid formation of ZP free acid.

Values are expressed as the mean ± S.E. of triplicate incubations.

pharmaceutical Sciences, University of California at San Francisco, for performing rat bile collection studies. We also acknowledge Dr. Chungang Gu (PDM, Pfizer, Inc., Kalamazoo, MI) for support in the mass spectrometric analysis of ZP-SG and ZP-NCG derivatives.

References


