EVIDENCE FOR THE BIOACTIVATION OF ZOMEPIRAC AND TOLMETIN BY AN OXIDATIVE PATHWAY: IDENTIFICATION OF GLUTATHIONE ADDUCTS IN VITRO IN HUMAN LIVER MICROSOMES AND IN VIVO IN RATS

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ABSTRACT:

Although zomepirac (ZP) and tolmetin (TM) induce anaphylactic reactions and form reactive acyl glucuronides, a direct link between the two events remains obscure. We report herein that, in addition to acyl glucuronidation, both drugs are subject to oxidative bioactivation. Following incubations of ZP with human liver microsomes fortified with NADPH and glutathione (GSH), a metabolite with an MH+ ion at m/z 597 was detected by LC/MS/MS. On the basis of collision-induced dissociation and NMR evidence, the structure of this metabolite was determined to be 5-4’-chlorobenzyol]-1,4-dimethyl-3-glutathionylpyrrole-2-acetic acid (ZP-SG), suggesting that the pyrrole moiety of ZP had undergone oxidation to an epoxide intermediate, followed by addition of GSH and loss of the elements of H2O to yield the observed conjugate. The oxidative bioactivation of ZP most likely is catalyzed by cytochrome P450 (P450) 3A4, since the formation of ZP-SG was reduced to ~10% of control values following pretreatment of human liver microsomes with ketoconazole or with an inhibitory anti-P450 3A4 IgG. A similar GSH adduct, namely 5-[4’-methylbenzoyl]-1-methyl-3-glutathionylpyrrole-2-acetic acid (TM-SG), was identified when TM was incubated with human liver microsomal preparations. The relevance of these in vitro findings to the in vivo situation was established through the detection of the same thiol adducts in rats treated with ZP and TM, respectively. Taken together, these data suggest that, in addition to the formation of acyl glucuronides, oxidative metabolism of ZP and TM affords reactive species that may haptenize proteins and thereby contribute to the drug-mediated anaphylactic reactions.

It is widely believed that certain carboxylic acid-containing drugs pose a toxicological concern because of their propensity to form reactive acyl glucuronides that covalently modify proteins (Faed, 1984; Spahn-Langguth and Benet, 1992). Two mechanisms have been invoked to account for such protein modifications, namely 1) transacylation, where the reactive center is the carbonyl carbon of the acyl glucuronide, from which the glucuronic acid moiety is displaced through nucleophilic attack by free cysteine, tyrosine, or lysine residues of target proteins; and 2) glycation, which involves migration of the aglycone to the 2-, 3- or 4-position of the sugar ring, followed by rearrangement to an α-hydroxyaldehyde that reacts with amine groups on proteins to afford stable 1-amino-2-keto adducts (Shipkova et al., 2003). Despite the fact that haptenization of proteins by such processes may be of toxicological relevance, the role of exposure to reactive acyl glucuronide metabolites of carboxylate drugs as mediators of the adverse effects of these agents remains to be established. For example, diclofenac forms a reactive acyl glucuronide and induces rare but severe hepatotoxicity in patients (Boelsterli, 2003). However, it was shown that the cytotoxicity of the drug in rat hepatocytes was inversely correlated with the amount of the glucuronide formed (Kretz-Rommel and Boelsterli, 1993). Recent in vitro and in vivo data indicate that diclofenac is subject to bioactivation by oxidation pathways to form electrophilic quinone imine and epoxide intermediates, identification of which was based on the detection of corresponding adducts with glutathione (GSH) and N-acetylcysteine (Tang et al., 1999; Poon et al., 2001; Yan et al., 2005; Yu et al., 2005). Hence, the hepatotoxic effects of diclofenac may not be related to the acyl glucuronidation pathway.

Zomepirac (ZP; 5-[4’-chlorobenzoxy]-1,4-dimethylpyrrole-2-acetic acid) and tolmetin (TM; 5-[4’-methylbenzoyl]-1-methylpyrrole-2-acetic acid) (Fig. 1) are nonsteroidal anti-inflammatory drugs (NSAIDs). Clinical use of ZP and TM has been associated with severe, sometimes fatal, anaphylactic reactions, as a result of which ZP was withdrawn from the market in 1983 (Restivo and Paulus, 1978; McCall and Cooper, 1980; Kiani and Kushner, 1983; Levy and Vasilomanolakis, 1984). It seems that glucuronidation represents a major pathway for the clearance of ZP and TM in patients (Muschek and Grindel, 1980; Hyneck et al., 1988). Both ZP and TM acyl glucuronides have been shown to be capable of covalently modifying

ABBREVIATIONS: GSH, reduced glutathione; ZP, zomepirac; TM, tolmetin; NSAID, nonsteroidal anti-inflammatory drug; P450, cytochrome P450; LC/MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; CID, collision-induced dissociation; SRM, selected reaction monitoring; ZP-SG, 5-[4’-chlorobenzoxy]-1,4-dimethyl-3-glutathionylpyrrole-2-acetic acid; TM-SG, 5-[4’-methylbenzoyl]-1-methyl-3-glutathionylpyrrole-2-acetic acid.
proteins via the glycation mechanism (Smith et al., 1986, 1990; Hyneck et al., 1988). Target proteins for modification include plasma albumin in humans, tubulin and dipeptidyl peptidase IV in rats, and CD26 in mice (Bailey et al., 1998; Wang et al., 2001, 2002). As a result, the reactive acyl glucuronides of ZP and TM have been hypothesized to serve as mediators of the serious adverse effects of these drugs (Zia-Amirhosseini et al., 1995). On the other hand, pyrrole derivatives in general, and 1,3-dimethylpyroles in particular also are known for their propensity to covalent modify proteins (Guengerich and Mitchell, 1980; Dalvie et al., 2002). In the present study, we report that the pyrrole moieties of ZP and TM are indeed subject to oxidative bioactivation, as evidenced by the detection of corresponding GSH adducts upon incubation of ZP and TM with human liver microsomal preparations fortified with NADPH and GSH, and following administration of these agents to rats.

Materials and Methods

Materials. ZP, TM, troleandomycin, GSH, and NADPH were purchased from the Sigma-Aldrich (St. Louis, MO). [4-Chlorophenyl-2-3H]ZP (radiochemical purity, 99.0%; and specific activity, 4.5 mCi/mg) and [4-methylphe-
nyl-2-$^3$H]TM (radiochemical purity, 99.9%; and specific activity, 21.3 mCi/mg) were synthesized by Labeled Compound Synthesis Group, Merck Research Laboratories (Rahway, NJ). Ketoconazole was obtained from Janssen Biotech NV (Olen, Belgium). BondElut C18 solid phase extraction cartridges were obtained from Varian, Inc. (Palo Alto, CA). Recombinant cytochromes P450 1A2, 2C8, 2D6, 2E1, and 3A4, coexpressed with NADPH-P450 oxidoreductase in baculovirus-insect cells, were purchased from BD Gentest (Woburn, MA). Monoclonal inhibitory antibodies against human hepatic P450 3A4 were prepared in mice by immunization with the corresponding P450 enzymes (Mei et al., 1999).

Instrumentation. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) was carried out on a PerkinElmerSciex API 3000 tandem mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) interfaced to an HPLC system consisting of a PerkinElmer Series 200 quaternary pump and a Series 200 autosampler (PerkinElmer Life and Analytical Sciences, Boston, MA). Turbo IonSpray was used for ionization with positive ion detection. The corresponding P450 enzymes is expressed as parts per million relative to tetramethylsilane.

FIG. 3. Partial $^1$H NMR spectrum of ZP (bottom trace) and its GSH adduct (top trace).

NMR spectra were recorded on a Varian Inova 600 spectrometer operated at 600 MHz. Isolated GSH adducts were dissolved in deuterated methanol. Chemical shifts are expressed as parts per million relative to tetramethylsilane.

Incubations with Human Liver Microsomes, Recombinant P450s, and Rat and Human Hepatocytes. Human liver samples from four male and five female donors were obtained from the Pennsylvania Regional Tissue Bank (Scranton, PA). The medical history of the donors has been reported elsewhere (Tang et al., 1999). Liver microsomes were isolated from individual livers by differential centrifugation (Raucy and Lasker, 1991). Aliquots from each preparation then were pooled on the basis of equivalent protein concentrations to yield a representative pool of human liver microsomes.

Human liver microsomes or recombinant P450 were suspended in phosphate buffer (pH 7.4) containing 5 mM GSH. The final concentration of P450 was 0.24 nmol/ml for human liver microsomes and 0.07 nmol/ml for the recombinant enzymes. ZP or TM in methanol was added to provide final drug concentrations ranging from 0 to 1 mM. The total incubation volume was 1 ml, containing 0.2% methanol (v/v). Incubations were performed in the presence of 1 mM NADPH at 37°C for 60 min, and the reaction was quenched by adding 60 μl of 10% aqueous trifluoroacetic acid.

In experiments involving a selective P450 3A4 inhibitor, ketoconazole in methanol was added to human liver microsomal suspensions containing 5 mM GSH. Controls contained the same amount of organic solvent but lacked the inhibitor. After preincubation at 37°C for 10 min, ZP or TM was added to reach a final concentration of 50 μM. Incubations (final volume, 1 ml) were performed for an additional 30 min at 37°C in the presence of 1 mM NADPH. The reaction was quenched by adding 60 μl of 10% aqueous trifluoroacetic acid.

Immunoinhibition experiments followed a protocol similar to that described above. Briefly, a monoclonal antibody against P450 3A4 (0.5–2 mg of IgG/nmol P450) was preincubated with human liver microsomes for 15 min at room temperature. Control incubations contained ascites from untreated animals. ZP or TM was added to provide a final concentration of 50 μM, and incubations were performed for an additional 30 min in the presence of 5 mM GSH and 1 mM NADPH. The reaction was quenched with 10% aqueous trifluoroacetic acid.

In time-dependent inhibition experiments, pooled human liver microsomes (2 mg/ml) were preincubated at 37°C with 10 μM ZP or TM in phosphate buffer (pH 7.4) containing an NADPH-generating system for a duration ranging from 5 to 30 min. The mixtures then were diluted 10-fold with phosphate buffer containing 250 μM testosterone and an NADPH-generating system and incubated for an additional 10 min to monitor testosterone 6β-hydroxylase activity. The reactions were terminated by adding 0.4 ml of
acetonitrile/water (75:25, v/v) containing 0.05% formic acid. All experiments were performed in duplicate.

Cryopreserved male rat hepatocyte and male human hepatocyte from three donors were obtained from In Vitro Technologies (Baltimore, MD). These hepatocytes exhibited viabilities of 70 to 80% based on testing with trypan blue. Incubations were performed with the hepatocytes suspended in Krebs-bicarbonate buffer followed by the addition of ZP or TM in methanol to reach a final concentration of 50 μM. Each incubation contained 5 million live cells in a final volume of 5 ml. Incubations proceeded for 2 h at 37°C, and reactions were quenched with 10% aqueous trifluoroacetic acid.

Animal Experiments. Experiments were performed according to procedures approved by the Merck Institutional Animal Care and Use Committee. Four male Sprague-Dawley rats, purchased from Harlan Laboratories (Indianapolis, IN) and weighing 270 to 360 g, were allowed free access to commercial rat chow and water. The animals were anesthetized with pentobarbital (Nembutal), and their bile ducts were cannulated with PE-10 tubing. Control bile was collected before treatment. An aqueous PEG400-ethanol solution (polyethylene glycol/ethanol/water, 20:10:70, v/v/v) of [3H]ZP or [3H]TM was administered at 10 mg/kg (40 μCi per rat) by oral gavage, and bile and urine were collected in 0.5 M formate (pH 3.0) for 24 h following dosing.

Preparation of Samples for LC/MS/MS and NMR Analyses. Samples from in vitro incubations or bile collected from treated rats were acidified to pH 2 and applied to a C18 extraction cartridge that had been prewashed with methanol and water. Controls contained the same amount of methanol (0.2%, v/v). Reactions were initiated by adding ZP or TM and NADPH and allowed to proceed for an additional 10 min. The formation of GSH adducts was analyzed by LC/MS/MS.

TABLE 1
Effect of anti-P450 3A4 IgG or ketoconazole on the formation of ZP-SG and TM-SG in incubations of X and TM with human liver microsomes

<table>
<thead>
<tr>
<th>IgG or Inhibitor</th>
<th>Concentration</th>
<th>Formation of ZP-SG</th>
<th>% Control</th>
<th>Formation of TM-SG</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-3A4 antibody</td>
<td>0.5 mg of IgG/nmol P450</td>
<td>15–17</td>
<td>27–29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-3A4 antibody</td>
<td>2 mg of IgG/nmol P450</td>
<td>13–14</td>
<td>18–19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1 μM</td>
<td>14–17</td>
<td>31–35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>10 μM</td>
<td>10–12</td>
<td>19–21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results

Following incubations of ZP with human liver microsomes in the presence of GSH, a metabolite with an MH⁺ ion at m/z 597 was detected by LC/MS. Subsequent CID of this species resulted in a product ion spectrum that exhibited neutral losses of 75 Da (glycine) and 129 Da (pyroglutamate), both of which are characteristic for a GSH adduct (Fig. 2) (Baillie and Davis, 1993). The fragment ion at m/z 553 is likely due to loss of the elements of carbon dioxide from the MH⁺, whereas the fragment ion at m/z 288 is possibly derived...
from cleavage of the pyrrole moiety following a neutral loss of 129 Da (pyroglutamate) with charge retention on the cysteine residue (Fig. 2). The metabolite was isolated, and its structure was confirmed to be that of a GSH adduct (ZP-SG) by NMR analysis. The site of attachment of the glutathionyl moiety was determined to be the 3-position of the pyrrole ring, since the C-3 proton was absent in the NMR spectrum of the adduct (Fig. 3).

A metabolite structurally similar to ZP-SG with an MH⁻/H⁻ ion at m/z 563 was detected in incubations of TM with human liver microsomes in the presence of GSH. The product ion spectrum of this metabolite, obtained by CID of m/z 563, again exhibited losses of the elements of glycine to give m/z 488 and pyroglutamate to give m/z 434 (Fig. 2). The fragment ion at m/z 519 likely derives from loss of the elements of carbon dioxide from the MH⁻, whereas the fragment ion at m/z 288 is ascribed to cleavage of pyrrole moiety following neutral loss of 129 Da (pyroglutamate) with charge retention on the cysteine residue (Fig. 2). The metabolite was isolated, and its structure was confirmed to be that of a GSH adduct (TM-SG) based on NMR analysis. The position of thiol substitution again was established to be the 3-position of the pyrrole ring, since the C-3 proton was absent and the adjacent proton at C-4 appeared as a singlet in the NMR spectrum (Fig. 4). No other GSH adducts were detected in incubations of ZP and TM with human liver microsomes.

In separate incubations of ZP and TM with a battery of recombinant P450s, the formation of ZP-SG and TM-SG was detected only with P450 3A4 (data not shown). In incubations with human liver microsomes, formation of ZP-SG and TM-SG was reduced to 20% of control values in the presence of ketoconazole, a selective P450 3A4 inhibitor, and by an inhibitory anti-P450 3A4 IgG (Table 1).

The possibility for irreversible inhibition of P450 3A4 was investigated following preincubations of ZP or TM with human liver microsomes. The rate constants for loss of 6β-testosterone hydroxylase activity in these cases were 0.004 min⁻¹, which were similar to the value of 0.007 min⁻¹ derived from the solvent controls. These data suggest that ZP and TM are not time-dependent inhibitors of

### TABLE 2

<table>
<thead>
<tr>
<th>Recovery of Radioactivity</th>
<th>Acylglucuronide</th>
<th>GSH Adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile %</td>
<td>Urine %</td>
<td>Bile %</td>
</tr>
<tr>
<td>ZP</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>TM</td>
<td>2</td>
<td>99</td>
</tr>
</tbody>
</table>

N.D., not detected.

Fig. 5. SRM LC/MS/MS detection of GSH adducts of ZP and TM in human hepatocytes incubated with ZP and TM, respectively. Three mass transitions, namely m/z 597 → 288, 597 → 424, and 597 → 278, were used for identification of ZP-SG, whereas three mass transitions, m/z 563 → 288, 563 → 434, and 563 → 315, were used for identification of TM-SG. The HPLC retention times of these adducts were the same as those observed in the microsomal incubations and rat bile samples.
P450 3A4. With troleandomycin, the rate constant for loss of testosterone 6-β-hydroxylase activity was 0.041 min⁻¹.

The GSH adducts of ZP and TM were detected in incubations with rat and human hepatocytes based on SRM LC/MS/MS analysis. Three mass transitions were used for the identification of ZP-SG, namely m/z 597 → 424, 597 → 288, and 597 → 278. In the case of TM-SG, the corresponding mass transitions were m/z 563 → 434, 563 → 315, and 563 → 288. Coincident responses were recorded in each ion current chromatogram at HPLC retention times corresponding to ZP-SG and TM-SG, respectively (Fig. 5).

Oxidative bioactivation of [³H]ZP and [³H]TM was further studied in vivo in bile duct-cannulated male rats dosed with either drug at 10 mg/kg. The recovery of radioactivity was high, ranging between 96 to 100%, most of which was in urine (Table 2). Acyl glucuronides of ZP and TM represented trace amounts of the recovered doses. Although this is different from observations in human and monkey, where glucuronidation represents the major elimination pathway, it is consistent with literature data derived from the rat (Sumner et al., 1975; Wu et al., 1980). The GSH adducts of ZP and TM were detected in bile by SRM LC/MS/MS analysis. Estimated from radiochromatograms of the bile samples, ZP-SG and TM-SG represented 0.3% or less of the recovered doses (Table 2).

Discussion

Identification of GSH adducts represents a practical, albeit indirect, experimental approach for elucidating the structures of reactive metabolites (Baillie and Davis, 1993; Tang and Miller, 2004). In the case of ZP and TM, the structures of their GSH conjugates suggested that bioactivation of these agents most likely involved epoxidation of the pyrrole moieties to form arene oxide intermediates, a process that has been well documented for several five-membered heterocycles (Dalvie et al., 2002). Subsequent nucleophilic attack on the epoxides by GSH, followed by loss of the elements of H₂O, would afford ZP-SG and TM-SG, respectively (Fig. 6). The identification of these two GSH adducts by LC/MS/MS in rat bile and human hepatocytes suggests that oxidative bioactivation of ZP and TM has the potential to take place in patients.

ZP-SG and TM-SG were detected in incubations of the drugs with recombinant P450 3A4 in the presence of GSH. The formation of the GSH adducts was inhibited nearly completely when human liver microsomes were pretreated with ketoconazole, a selective inhibitor of P450 3A4, or with a monoclonal anti-P450 3A4 IgG. These data indicate that oxidative bioactivation of ZP and TM in human liver microsomal incubations, based on GSH adduct formation, is catalyzed primarily by P450 3A4. In spite of this, ZP and TM did not exhibit mechanism-based inhibition of P450 3A4, since there was no apparent loss of testosterone 6β-hydroxylase activity following preincubation of the agents with human liver microsomes.

Both ZP and TM are carboxylate NSAIDs whose clinical use is associated with rare but potentially severe anaphylactic reactions (McCall and Cooper, 1980; Darwish et al., 1983; Bretza, 1985). It has been suggested that these adverse effects may be related to systemic exposure to ZP and TM acyl glucuronides, based on the observation that these metabolites covalently modify proteins (Smith et al., 1986, 1990; Hyneck et al., 1988; Munafò et al., 1993). In two separate studies, a correlation was observed between the amount of drug bound covalently to human serum albumin and the reactivity of the corresponding acyl glucuronides for a number of carboxylic drugs (Benet et al., 1993; Bolze et al., 2002). However, direct evidence is lacking to unambiguously link the formation of acyl glucuronides to clinical drug-related toxicity. The fact that the acyl glucuronides of TM and diclofenac are appreciably more reactive than those of ZP and suprofen, yet both TM and diclofenac are still used by patients with lower rates of side effects remains puzzling (Bolze et al., 2002). It seems reasonable, therefore, that other possible mechanisms, such as oxidative bioactivation, may contribute to the toxicities of ZP and suprofen. Evidence of this type is presented in the current report, i.e., ZP and TM undergo P450-catalyzed metabolism to what seems to be reactive pyrrole epoxides. These electrophilic intermediates are expected to be capable of haptenizing proteins and may thus contribute to drug-related anaphylactic reactions.

In summary, ZP and TM are subject to oxidative bioactivation in vitro with human liver microsomes and in vivo in rats, based on the identification of corresponding GSH adducts. The reactive intermediates are likely to be epoxides resulting from pyrrole oxidation, catalyzed in human liver preparations primarily by P450 3A4. Although speculative at present, our data suggest that P450-catalyzed oxidation of ZP and TM may be a contributing factor to the anaphylactic reactions observed clinically with these therapeutic agents.

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