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Role of Biotransformation in 3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione-induced Hepatotoxicity in Fischer 344 Rats

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Abstract

Cytochrome P450 (CYP)-mediated metabolism in the thiazolidinedione (TZD) ring may contribute to the hepatotoxicity of the insulin-sensitizing agents such as troglitazone. We were interested in determining if biotransformation could also be a factor in the liver damage associated with another TZD ring containing compound, 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT). Therefore, hepatotoxic doses of DCPT (0.6 or 1.0 mmol/kg, i.p.) were administered to male Fischer 344 rats after pretreatment with vehicle, 1-aminobenzotriazole (ABT, non-selective CYP inhibitor) and troleandomycin (TAO, CYP3A inhibitor). Alternatively, rats were pretreated with vehicle or the CYP3A inducer dexamethasone (DEX) prior to a non-toxic DCPT dose (0.2 mmol/kg, i.p.). Vehicle-, ABT-, TAO- and DEX-only control groups were also run. Toxicity was assessed 24 hours after DCPT administration. Both hepatotoxic doses of DCPT induced elevations in serum alanine aminotransferase (ALT) levels that were attenuated by ABT or TAO pretreatment. Liver sections from rats that received vehicle + DCPT revealed areas of gross necrosis and neutrophil invasion, whereas sections from ABT + DCPT and TAO + DCPT rats showed minor changes compared to controls. DEX pretreatment potentiated ALT levels associated with the non-toxic DCPT dose. Furthermore, DEX + DCPT rat liver sections exhibited hepatic injury when compared against rats that received vehicle + DCPT. Blood urea nitrogen levels, urinalysis and kidney morphology were not markedly altered by any combination of pretreatments or treatments. Enzyme activity and Western blotting experiments with rat liver microsomes confirmed the effects of the various pretreatments. Our results suggest that hepatic CYP3A isozymes may be involved in DCPT-induced liver damage in male rats. We believe this is the first report demonstrating that modulation of the biotransformation of a TZD ring-containing compound can alter hepatotoxicity in a common animal model.

Keywords

hepatotoxicity; thiazolidinediones; 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione; rats

1. Introduction

The cyclic imide 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT, Fig. 1) is one of a series of chemicals that were originally synthesized as agricultural fungicides (Fujinami et al.,

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1971). As part of a structure-activity relationship study into the mechanism of toxicity for these types of compounds, we discovered that DCPT produced hepatotoxicity in male Fischer 344 rats (Kennedy et al., 2003). The liver damage associated with DCPT was subsequently found to be dose-, time- and gender-dependent (Patel et al., 2008). DCPT-induced hepatotoxicity was characterized by centrilobular necrosis, hepatocyte swelling, nuclear condensation and elevated serum alanine aminotransferase (ALT) levels. In contrast, DCPT had only mild toxic effects on rat kidney function and morphology (Kennedy et al., 2003; Patel et al., 2008).

Structurally, DCPT consists of 2,4-thiazolidinedione (TZD) and 1,3-dichlorobenzene (1,3-DCB) rings (Fig. 1). Although liver damage in rats has been reported with the DCBs (Stine et al., 1991; Valentovic et al., 1993), we believe that the TZD ring is the crucial structural feature for DCPT-induced hepatotoxicity. This premise is based partly on the fact that close structural analogues of DCPT, which contain either a 2,5-pyrrolidinedione (succinimide) or 2,4-oxazolidinedione ring instead of a TZD ring, did not produce any liver damage in rats (Kennedy et al., 2003). Even among the DCBs, toxicity is dependent on the chlorine substitution pattern, with 1,2-DCB producing the largest elevations in serum ALT levels at comparable doses of the three isomers (Stine et al., 1991; Valentovic et al., 1993). Furthermore, ALTs were elevated by 0.4 mmol/kg DCPT (Patel et al., 2008), whereas 4 mmol/kg 1,3-DCB produced no such effect (Valentovic et al., 1993). Collectively, these findings suggest that hepatotoxicity is primarily due to the TZD ring, not the 1,3-DCB moiety, of DCPT.

A TZD ring is also found in the insulin-sensitizing agents or “glitazones” that are used to treat non-insulin dependent type-II diabetes. Troglitazone (TGZ, Fig. 1) was the first drug of this class to be approved for clinical use in the United States, starting in 1997. However, TGZ was withdrawn from the U.S. market in 2000 due to reports of drug-induced hepatotoxicity and liver failure (Smith, 2003). The liver pathology in some patients taking TGZ showed centrilobular necrosis (Gitlin et al., 1998; Kohloser et al., 2000). Other members of this family of drugs include rosiglitazone (RGZ) and pioglitazone (PGZ). Although RGZ and PGZ are both still prescribed, there have also been reports of mild liver toxicity associated with their use (Gouda et al., 2001; Maeda, 2001) and routine monitoring of liver enzymes is recommended. These drugs are not indicated in patients with pre-existing liver disease (Scheen, 2001).

While the mechanism of TGZ-induced hepatotoxicity is unknown, metabolism in its TZD and chromane rings has been implicated. These studies generally involved characterization of glutathione (GSH) conjugates formed in vitro and in vivo. Several groups have shown that TZD ring oxidation and subsequent scission could potentially lead to the formation of reactive intermediates from TGZ (Kassahun et al., 2001; Tetey et al., 2001; He et al., 2004; Reddy et al., 2005). These reactions appear to be catalyzed primarily by the cytochrome P450 3A (CYP3A) isozyme subfamily. NADPH-dependent covalent binding was also observed in microsomal incubations with radiolabeled TGZ (He et al., 2004). Baughman et al. (2005) have reported that PGZ metabolism resulted in the formation of reactive TZD ring-opened products which were identified in rat and human liver microsomes. Finally, Álvarez-Sánchez et al. (2006) have suggested that there may be a common mechanism for metabolic activation of the TZD ring in all three glitazones that involves an initial sulfoxidation step. In spite of these studies, the role of metabolism in TGZ hepatotoxicity remains controversial (Masubuchi, 2006). Further investigation of DCPT may help address the question of whether biotransformation in a TZD ring can be a factor in chemically-induced hepatotoxicity.

In addition to the glitazones, a TZD ring is also present in prototype aldose reductase inhibitors that may be of value in treating diabetes-related complications (Bruno et al., 2002; Rakowitz et al., 2006). As there is a potential for continued human exposure to compounds that contain a TZD ring, it is important to determine whether this structural feature can contribute to

hepatotoxicity. Unfortunately, there have been no reported studies demonstrating whether metabolism of a TZD ring-containing compound can contribute to liver damage in vivo. This is due, at least in part, to the fact that the glitazones are not hepatotoxic in any common laboratory animal species (Smith, 2003; Chojkier, 2005; Ong et al., 2007). Since it produces liver damage in rats (Kennedy et al., 2003; Patel et al., 2008) DCPT may be a useful model compound to explore the potential role of a TZD ring in chemically-induced hepatotoxicity. However, it should be noted that the substitution pattern in the TZD ring of DCPT differs from that in the glitazones (Fig. 1), which could impact on biotransformation and toxicity.

As described above, metabolism may contribute to glitazone-induced toxicity. We were therefore interested in determining if biotransformation could have a similar role in DCPT-induced liver damage. Previously, we found that DCPT was less hepatotoxic in female versus male rats, which could be due to a gender-based difference in metabolism (Patel et al., 2008). The centrilobular nature of DCPT-induced liver damage (Kennedy et al., 2003; Patel et al., 2008) suggests that hepatic CYPs may be involved in DCPT metabolic activation (Lindros, 1997). To achieve our goals we used two approaches: (1) rats were pretreated with CYP inhibitors prior to hepatotoxic doses of DCPT; and (2) rats were pretreated with a CYP inducer prior to a non-hepatotoxic dose of DCPT. Our results suggest that DCPT must undergo CYP-mediated biotransformation to produce liver damage in male rats. To the best of our knowledge, this is the first report documenting a correlation between modulation of metabolism and in vivo toxicity of a TZD ring-containing compound in rats.

2. Materials and Methods

2.1. Chemicals/reagents

Troleandomycin (TAO), 2,4-dinitrophenylhydrazine, sodium pyruvate, DL-alanine, α -keto-glutaric acid, 7-ethoxyresorufin, 7-pentoxyresorufin and resorufin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dexamethasone (DEX) and 1-aminobenzotriazole (ABT) were purchased from TCI (Portland, OR, USA). Steroid standards for HPLC analysis were obtained through Steraloids, Inc. (Newport, RI, USA). All other chemical reagents were of the highest purity available. DCPT was prepared by the method of Kennedy et al. (2003) and was recrystallized from ethanol. Purity was established by a sharp melting point (m.p. 163–165°C; lit. 164.5–165.5 °C; Fujinami et al., 1971).

2.2. Clinical chemistry/histology

Serum samples were analyzed for ALT using a procedure similar to that found in Sigma kit #505-P from Sigma Chemical (St. Louis, MO, USA). Blood urea nitrogen (BUN, kit # B7551-120) and urine glucose (kit # G7518-150) levels were measured using the indicated assay kits from Pointe Scientific (Detroit, MI, USA). Urine protein was measured using the Bio-Rad protein assay (Hercules, CA, USA) with bovine serum albumin as standard (Bradford, 1976). Kidney and liver samples were fixed in 10% formalin, stained with hematoxylin and eosin and/or periodic acid-Schiff (PAS) stain, and mounted by American Histolabs, Inc. (Gaithersburg, MD, USA). Slides were coded and read by a person blind to the code.

2.3. Animals

Male Fischer 344 rats (ca. 200 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and were held in the USP Vivarium for one week prior to use. The animals were housed under a 12 h light/dark cycle at ca. 22°C and 45–50% relative humidity. Food (laboratory rodent diet #5001, PMI Foods, Inc., St. Louis, MO) and water were freely available during this period. All experiments involving rats were approved by the USP Institutional Animal Care and Use Committee.

2.4. In vivo toxicity

Experimental parameters (hepatotoxic and non-hepatotoxic DCPT doses, time point, etc.) were based on our prior work (Patel et al., 2008). Appropriate controls were included to monitor the effects of dosing vehicles and other experimental manipulations (i.e. food and water deprivation period) on toxicity and hepatic enzyme activities.

Rats were randomly divided into treatment groups (N=4 rats/group) and transferred to stainless steel metabolism cages (Allentown Caging Equipment Co., Allentown, NJ, USA). The animals were kept in the metabolism cages for a minimum of two days before use. Vehicle control rats received either saline (4 ml/kg, i.p.) followed by corn oil (4 ml/kg, i.p.), or corn oil (4 ml/kg, i.p.) followed by corn oil (4 ml/kg, i.p.). For the enzyme inhibition studies, animals were pretreated with TAO (500 mg/kg, i.p. in acidified saline), ABT (100 mg/kg, i.p. in saline) or saline (4 ml/kg, i.p.), followed 1–2 hours later by a hepatotoxic dose of DCPT (0.6 or 1.0 mmol/kg, i.p. in corn oil). In the enzyme induction studies, rats were pretreated with DEX (50 mg/kg/day for 3 days, i.p. in corn oil) or corn oil (4 ml/kg/day for 3 days, i.p.) prior to administration of a non-hepatotoxic dose of DCPT (0.2 mmol/kg, i.p. in corn oil). To ensure that the pretreatments themselves had no adverse effects on the rats, separate groups of animals were administered ABT, TAO or DEX prior to corn oil (4 ml/kg, i.p.).

On the control day of the experiment urine was collected for 6 h to determine baseline protein content. During this time food and water were temporarily removed from all rats to avoid possible urine dilution or contamination. On the dosing day, rats were treated with either DCPT or corn oil. Immediately after dosing, a contaminant free urine sample was again collected for 6 h. Body weight and 24 h urine volume were noted on both days of the experiment. Twenty four hours after dosing with DCPT or corn oil, a blood sample was obtained via cardiac puncture under general anesthesia (isoflurane) for assessment of ALT and BUN levels. While still anesthetized, the rats were euthanized by cervical dislocation. The kidneys and livers were removed, weighed and the right kidney and a section of liver were fixed in 10% buffered formalin for histological analysis.

2.5. Preparation of microsomes

Rat liver microsomes were prepared and isolated by differential centrifugation (Lake, 1987) from separate groups of animals (N = 4 rats/group) that received ABT, TAO, DEX or corn oil pretreatments and were stored at –80°C until use. The total protein concentrations in microsomes were determined by a Bio-Rad protein assay kit using BSA as the standard.

2.6. Testosterone 6 β - and 16 α -hydroxylation

The microsomes (2.0 mg/ml) were incubated for 10 minutes at 37° C in 1 ml incubation mixtures containing potassium phosphate buffer (100 mM, pH 7.4), MgCl₂ (3 mM), NADP⁺ (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 U/ml), the steroid 5 α -reductase inhibitor 17 β -N,N-diethylcarbamoyl-4-methyl-4-aza-5 α -androstane-3-one (1 μ M, 4-MA) and testosterone (250 μ M), at the final concentrations indicated. Reactions were started by the addition of the NADPH-generating system and were stopped by the addition of 6 ml ice-cold methylene chloride. Each sample was spiked with 100 μ l methylene chloride containing 6 nmol corticosterone (internal standard) and vortex-mixed for 10 minutes. Samples were centrifuged (2000 $\times$ g for 10 min), the phases were separated, and the aqueous layers were discarded. Aliquots (4 ml) of the organic phases were transferred to 12 $\times$ 75 mm culture tubes and evaporated under nitrogen. The residues were reconstituted in 200 μ l 1:1 methanol:water mixture and 50 μ L aliquots were analyzed by HPLC.

6 β - or 16 α -Hydroxytestosterone were quantitated by the generation of standard curves using concentrations ranging from 5 μ M to 500 μ M under mock incubation conditions and run under

the same HPLC conditions. The analyses were conducted on a Hewlett Packard 1050 system equipped with a pump, diode array detector (set at 247 nm), autoinjector, and column heater (set at 45°C). Baseline separation between 6 β -hydroxytestosterone (retention time 5.0 min), 16 α -hydroxytestosterone (6.0 min), the internal standard cortexolone (8.1 min) and testosterone (11.5 min) was achieved on a Beckman Ultrasphere C18 column (5 μ m, 4.2 $\times$ 250 mm) using conditions described in the Gentest catalogue (modified from Buters et al., 1994). The mobile phase consisted of (A) 10% methanol in water and (B) 100% methanol, and was pumped at a flow rate of 1.0 ml/min. Initial conditions were 53% B, which was increased via a linear gradient to 58% B over 8 min, followed by isocratic elution at 58% B for 6 minutes. A final gradient (1 min) returned the system to starting conditions.

2.7. Ethoxyresorufin O-deethylase (EROD)/pentoxyresorufin O-depentylase (PROD) activities

Incubations were conducted in 48 well cell culture plates (Costar 3548, Corning, NY, USA). In short, microsomes (0.1 mg) from each pretreatment group were incubated for 30 minutes at 37°C with MgCl₂ (120 mM), glucose-6-phosphate (200 mM), NADP⁺ (40 mM) and glucose-6-phosphate dehydrogenase (2 units) in a potassium phosphate buffer (100 mM, pH 7.4) containing EDTA (1.0 mM). After the preincubation period either 7-ethoxyresorufin (5 μ M final concentration) or 7-pentoxyresorufin (5 μ M final concentration) were added. The plates were incubated at 37°C with shaking for 30 minutes with a fluorescence reading taken every two minutes. Resorufin concentration was measured against a standard curve generated using concentrations ranging from 0–1000 pmol/ml. Specific activity was calculated from the amount of resorufin formed over the incubation period divided by the final protein concentration. Fluorescence readings were taken on a PerkinElmer (Wellesley, MA, USA) plate reader (Model # HTS 7000 Plus) set at excitation wavelength of 530 nm and emission wavelength of 590 nm using HTSoft version 2.0 software.

2.8. Western blotting

Microsomal samples from corn oil- and DEX-pretreated animals were boiled in Laemmli buffer. Five to ten micrograms of protein from each sample were loaded and resolved on 7.5% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) gels. Proteins were transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in Tris-buffered saline-Tween 20 (TBST, 0.01% v/v Tween 20) and probed for 1 h with dilutions of primary antibodies raised in rabbit against rat CYP3A1 (1:1000) and CYP3A2 (1:1000), followed by an overnight incubation with the secondary antibody (1:5000) raised in goat against rabbit. For normalization all blots were re-probed for β -actin. Anti-CYP3A1 and anti-CYP3A2 antibodies were purchased from Chemicon, Inc. (Temecula, CA, USA). The normalization antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The protein/antibody complex was visualized by enhanced chemiluminescence according to manufacturer's recommendations (Pierce Biotechnology Inc., Rockford, IL, USA). Blots were photographed and densitometric analysis was performed using LabWorks Image Acquisition and Analysis software (UVP Inc., Upland, CA, USA).

2.9. Statistical analyses

Results are expressed as means $\pm$ SE (N=4 or 5). The data were analyzed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc. Differences between the means were considered significant when $p < 0.05$.

3. Results

3.1. Effect of CYP inhibition on DCPT hepatotoxicity

Serum alanine aminotransferase (ALT) levels were determined as an index of liver damage (Fig. 2). Compared to the saline (pretreatment) + corn oil (treatment) controls, rats that received saline followed by 0.6 or 1.0 mmol/kg DCPT (known hepatotoxic doses, Patel et al., 2008) exhibited ca. 10- and 34-fold elevations in ALT values, respectively. In contrast, after pretreatment with either ABT (non-specific CYP inhibitor) or TAO (CYP3A inhibitor), animals which received hepatotoxic doses of DCPT (0.6 mmol/kg) appeared to be protected from liver damage, as evidenced by markedly reduced serum ALT levels. Similar results following pretreatment with either of the CYP inhibitors were also obtained at the higher DCPT dose (1.0 mmol/kg). ABT or TAO pretreatment followed by administration of corn oil did not result in significant elevations in serum ALT concentrations (Fig. 2). ALT values for the CYP inhibitors plus DCPT (0.6 and 1.0 mmol/kg) were not significantly different from the inhibitors plus corn oil (e.g. TAO + DCPT vs. TAO + saline). BUN and urine protein content were not significantly different among any of the treatment groups (Table 1). However, diuresis was observed in all rats that received a hepatotoxic dose of DCPT, regardless of pretreatment (Table 1). Compared to the saline + corn oil controls, body, liver and kidney weights were not altered in any of the animals (Table 2).

The histology of vehicle control (e.g. saline + corn oil) livers showed slightly swollen sinusoids, but was otherwise normal in appearance (Fig. 3A). Histological evidence of injury was seen in the liver sections of animals that received saline followed by the hepatotoxic doses (0.6 or 1.0 mmol/kg) of DCPT (Figs. 3D and 3G, respectively). The histological changes consisted of swollen hepatocytes with occasional condensed nuclei, and occluded sinuses (Fig. 3D). At the higher dose (Fig. 3G) regions of centrilobular necrosis were evident. Pretreatment with either of the CYP inhibitors protected rat livers from DCPT-induced necrosis at either hepatotoxic dose (ABT + DCPT, Figs. 3E and 3H; TAO + DCPT, Figs. 3F and 3I). The histology of liver sections from rats that received a CYP inhibitor followed by corn oil (ABT + corn oil, Fig. 3B; TAO + corn oil, Fig. 3C) was generally normal. Compared to the controls, minor swelling in the glomerular and proximal tubular spaces was observed in kidney sections from rats that received either hepatotoxic dose of DCPT, regardless of pretreatment (data not shown).

3.2. Effect of CYP induction on DCPT hepatotoxicity

The effects of the CYP3A inducer DEX on DCPT-induced changes in serum ALT levels are summarized in Fig. 4. Corn oil-pretreated animals that were administered a non-hepatotoxic dose (0.2 mmol/kg) of DCPT (Patel et al., 2008) did not exhibit any significant elevations in ALT levels when compared to the corn oil + corn oil controls. Although serum ALT values were elevated approximately two-fold in the DEX + corn oil group, this increase was not significantly different from the control group ($p = 0.055$). In contrast, the combination of DEX pretreatment plus the normally non-hepatotoxic dose of DCPT resulted in a statistically significant (ca. 5-fold) increase in ALT levels compared to corn oil + DCPT. The ALT values were also significantly elevated in the DEX + DCPT group compared to the corn oil + corn oil and the DEX + corn oil groups (Fig. 4). BUN levels, urine volume and urine protein content were not significantly altered in any of the treatment groups (Table 1). Final body weights were decreased in rats that were pretreated with DEX versus corn oil (Table 2). In contrast, liver and kidney weights were significantly elevated by DEX pretreatment, regardless of whether the rats subsequently received corn oil or DCPT (Table 2).

Liver sections from animals that received corn oil followed by a non-hepatotoxic dose (0.2 mmol/kg) of DCPT were not altered compared to corn oil + corn oil control rats, and exhibited open sinusoids and clear veins (Figs. 5A and 5C, respectively). In contrast, animals which were

pretreated with DEX prior to the non-hepatotoxic dose of DCPT showed evidence of hepatic damage, such as protein condensation and deposits; in addition, cells were vacuolated with a lacy appearance (Fig. 5D). Liver sections from DEX + corn oil treated rats exhibited the same lacy structure, but with less protein condensation (Fig. 5B). Periodic acid-Schiff (PAS) staining of liver sections from both groups of rats that were pretreated with DEX (Figs. 6B and 6D) showed vacuolization of the cell structure with depletion of glycogen staining. Patchy glycogen staining was not observed in the livers of control animals or rats that received a non-toxic dose of DCPT in the absence of DEX pretreatment (Figs. 6A and 6C, respectively). Renal morphology in the corn oil + DCPT (0.2 mmol/kg) treated animals was not altered when compared to the corn oil + corn oil controls (data not shown). In rats that received DEX followed DCPT; kidney histology showed slight accumulation of proteinaceous deposits and occasional dead cells (data not shown). These histological changes were not different from the DEX + corn oil controls.

3.3. Effect of pretreatments on hepatic CYP activities

There were statistically significant decreases in 6 β -hydroxytestosterone formation using microsomes prepared from animals that were pretreated with either ABT or TAO versus rats that were pretreated with corn oil (Table 3). Microsomes prepared from livers of animals pretreated with DEX showed a significant increase in the 6 β -hydroxylation of testosterone when compared to corn oil pretreated rats (Table 3). A significant decrease in 16 α -hydroxylation of testosterone was seen in animals pretreated with ABT when compared to animals pretreated with corn oil (Table 3). By contrast, TAO and DEX pretreatment had no effect on formation of 16-hydroxytestosterone (Table 3). The conversion of ethoxyresorufin and pentoxyresorufin to resorufin (EROD and PROD activities, respectively) was significantly decreased in microsomes prepared from animals pretreated with the CYP inhibitor ABT (Table 4). In contrast, there were no significant changes in EROD or PROD activities in microsomes from the TAO- or DEX-pretreated rats (Table 4).

3.4. Effect of DEX pretreatment on hepatic CYP3A levels

Western blot analysis showed the effect of DEX pretreatment on CYP3A1 and CYP3A2 protein expression levels (Fig. 7A). Animals pretreated with DEX exhibited significantly higher amounts of CYP3A1 and CYP3A2 (2.5- and 1.2-fold, respectively) compared to corn oil only pretreated animals (Fig. 7B). CYP3A2 levels were approximately twice as high in corn oil treated rats compared to CYP3A1 expression levels (Fig. 7B).

4. Discussion

We previously reported that there was a gender-dependent difference in susceptibility to DCPT-induced hepatotoxicity in Fischer 344 rats and speculated that males may convert it to a toxic metabolite more extensively than females (Patel et al., 2008). The centrilobular location of DCPT-induced hepatic injury (Kennedy et al., 2003; Patel et al., 2008) is similar to troglitazone-induced liver damage in human patients (Gitlin et al., 1998; Kohlroser et al., 2000) and could also be consistent with a role for CYPs in generating a toxic metabolite from DCPT (Lindros, 1997). Furthermore, CYP-mediated metabolic activation is a common factor in chemically-induced liver damage (Venkatakrisnan et al., 2001; Cullen, 2005). The present study was designed to further explore the possible relationship between DCPT biotransformation and its hepatotoxicity in male rats.

Based on serum ALT levels and hepatic morphology, we found that the ABT- and TAO-pretreatments effectively protected rats against DCPT-induced liver damage. ABT is a non-specific, irreversible CYP inhibitor (Mugford et al., 1992), whereas TAO exhibits selectivity for inhibition of the CYP3A isoforms (Kostrubsky et al., 1997). ABT effectively reduces

hepatic CYP activity by about 70% within one hour of administration (Mugford et al., 1992), which is the timeframe we employed for the pretreatment. The decision to use a CYP3A inhibitor was based on the evidence implicating this CYP isozyme subfamily in glitazone TZD ring metabolic activation (Kassahun et al., 2001; Tettey, 2001). Our results with these two inhibitors suggest that CYPs, more specifically the CYP3A1/3A2 isozymes in male rats, have an important role in the generation of a toxic metabolite from DCPT. In rats, CYP3A isozymes have been shown to participate in the generation of hepatotoxic metabolites from other compounds, including diclofenac (Tang et al., 1999) and clivorine, a hepatotoxic pyrrolizidine alkaloid (Lin et al., 2003). Control experiments, in which rats received ABT or TAO, but no DCPT, confirmed that neither CYP inhibitor produced any hepatic effects when administered alone. Serum BUN levels and urine protein content were not altered in any of the treatment groups. Although diuresis was observed with both hepatotoxic doses of DCPT, this outcome would appear to be independent of DCPT biotransformation, since it was not altered by the CYP inhibitors. The lack of significant effects on renal function and morphology are consistent with our previous findings for DCPT in rats (Kennedy et al., 2003; Patel et al., 2008).

To confirm that ABT and TAO were having the desired effects on hepatic CYPs, we measured enzyme activities using liver microsomes prepared from rats that received the same pretreatments. In male rat liver, testosterone is metabolized to 6 β - and 16 α -hydroxytestosterone by the CYP3A1/3A2 and CYP2C11 isozymes, respectively (Arlotto et al., 1991). CYP2C11 activity was assessed since it is the predominant isozyme in uninduced male rat liver (Arlotto et al., 1991; Kato and Yamazoe, 1992) and therefore could have a significant role in DCPT metabolic activation. CYP-mediated EROD and PROD activities were also measured in the microsomes. Although these are often associated with the CYP1A and CYP2B isozyme families, respectively, Burke et al. (1994) have shown that they are relatively non-selective. Nevertheless, we felt that they were still valuable endpoints for assessing the impact of the ABT and TAO pretreatments on overall CYP enzyme activity. In fact, microsomes prepared from ABT-pretreated animals showed a significant decrease in all measured CYP activities, as would be expected by the non-specific inhibitory activity of this compound (Mugford et al., 1992). In contrast, only the CYP3A1/3A2-mediated activity was significantly inhibited in TAO microsomes. TAO forms a metabolic intermediate complex that can persist from pretreatment of the rats through the preparation of the microsomes (Kostrubsky et al., 1997).

For the enzyme induction studies, we again chose to focus the potential role of CYPs in DCPT-induced hepatotoxicity in rats by using the CYP3A inducer DEX (Tettey et al., 2001). Serum ALT levels were elevated in the DEX + DCPT animals compared to the other groups, suggesting that DEX was potentiating DCPT toxicity. Liver sections from these rats confirmed the start of injury. These results provide further evidence supporting a role for the CYP3A isozymes in the metabolic activation of DCPT. DEX pretreatment alone also produced an elevation in serum ALT levels, although it was not significantly different from what was seen in the vehicle control rats. Upon histological analysis we found that DEX pretreatment resulted in hepatocytes which were lacy in appearance, regardless of whether or not DCPT was subsequently administered. Times of stress, hypoxia or shock can cause the liver to rely on glycogenolysis for energy (Thogmartin et al., 2001). This loss of glycogen (also known as "glycogen fallout") was seen in all animals receiving DEX pretreatment and was confirmed by periodic acid-Schiff (PAS) staining, which is specific for glycogen. DEX pretreatment resulted in decreased body weight and increased liver and kidney weights in the rats. This effect was observed regardless of whether the rats were subsequently administered corn oil or DCPT, and is likely due to the DEX only. Similar to what we found in the inhibition studies, there was no evidence of significant nephrotoxicity in any of the DEX pretreatment groups, again confirming the selective effects of DCPT on rat liver.

Although DEX has been used as a CYP3A inducer (Tettey et al., 2001), it has also been reported to alter the activity of other hepatic CYPs. For example, high doses (125 mg/kg/day for 6 days) decreased hepatic CYP2C11 activity in rat liver, whereas low doses (0.2 mg/kg/day for 6 days) had no impact (Murray, 2000). Using quantitative RT-PCR, Caron et al. (2005) reported that DEX treatment (100 mg/kg/day for 3 days) had variable effects on rat liver CYP isozyme mRNA levels. Our DEX pretreatment regimen (50 mg/kg/day for 3 days) could therefore alter multiple CYP activities. However, using microsomes from DEX-pretreated rats, we found that only CYP3A activity was elevated. In particular, there were no statistically significant alterations in CYP2C11, EROD or PROD activities, validating the lack of upregulation or downregulation of multiple CYP isozymes by DEX. Further confirmation of CYP3A1/3A2 induction by DEX pretreatment was obtained by Western blotting experiments that demonstrated increased levels of both isozymes, particularly CYP3A1, in the microsomes.

Collectively, the results of these studies suggest that hepatic CYP3As have a critical role in conversion of DCPT to a hepatotoxic species in male rats, although the participation of other CYP isozymes cannot be definitively excluded. In analogy to what has been suggested for the glitazones, it is possible that DCPT metabolic activation could involve an initial sulfoxidation reaction in its TZD ring. However, we cannot reject the possibility that reactive metabolites may be formed from the 1,3-DCB ring of DCPT. In vitro glutathione trapping experiments may help determine whether and where DCPT metabolic activation occurs. The lack of kidney damage suggests that the potential for metabolism of DCPT into a toxicant is probably confined to the liver.

In conclusion, we have shown for the first time that the in vivo hepatotoxicity of a TZD ring-containing compound, DCPT, can be altered by modulation of rat hepatic CYP activity. Although our findings suggest the possible formation of a toxic species from DCPT, additional experiments are necessary to determine the nature of this metabolite and a mechanism for its toxicity. This knowledge may ultimately be beneficial in studying the mechanism behind the hepatotoxicity associated with the use of the glitazones.

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DCPT

Troglitazone (Rezulin®)

Fig. 1.
Structures of 3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione (DCPT) and Troglitazone (Rezulin®).

Fig. 2.

Effect of 1-Aminobenzotriazole (ABT) and Troleandomycin (TAO) Pretreatments on DCPT-induced Alterations in Rat Serum Alanine Aminotransferase (ALT) Levels. Pretreatment groups are indicated along the X-axis. Treatments are indicated by white (clear) bars (corn oil), grey bars (DCPT, 0.6 mmol/kg) and black bars (DCPT, 1.0 mmol/kg). Rats were pretreated with saline (vehicle, 4 ml/kg, i.p.), ABT (100 mg/kg, i.p. in saline) or TAO (500 mg/kg, i.p. in saline), and were then administered corn oil (vehicle, 4 ml/kg, i.p.) or DCPT (0.6 or 1.0 mmol/kg, i.p. in corn oil). Values indicate means $\pm$ standard errors (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the Saline + Corn oil control group. A dagger (†) indicates that the value is significantly different ($p < 0.05$) from the Saline + DCPT (0.6 mmol/kg) group. A double dagger (‡) indicates that the value is significantly different ($p < 0.05$) from the Saline + DCPT (1.0 mmol/kg) group.

Fig. 3.

Effect of 1-Aminobenzotriazole (ABT) and Troleandomycin (TAO) Pretreatments on DCPT-induced Alterations in Rat Liver Histology. Groups are indicated by pretreatment + treatment as follows: (A) Saline + Corn oil; (B) ABT + Corn oil; (C) TAO + Corn oil; (D) Saline + DCPT (0.6 mmol/kg); (E) ABT + DCPT (0.6 mmol/kg); (F) TAO + DCPT (0.6 mmol/kg); (G) Saline + DCPT (1.0 mmol/kg); (H) ABT + DCPT (1.0 mmol/kg); (I) TAO + DCPT (1.0 mmol/kg). Rats were pretreated with saline (vehicle, 4 ml/kg, i.p.), ABT (100 mg/kg, i.p. in saline) or TAO (500 mg/kg, i.p. in saline), and were then administered corn oil (vehicle, 4 ml/kg, i.p.) or DCPT (0.6 or 1.0 mmol/kg, i.p. in corn oil). The black arrow heads point to swollen hepatocytes (panel D) or regions of necrosis (panel G); and the white arrow heads point to condensed nuclei. Sections were stained with hematoxylin-eosin. Magnification is 400 $\times$.

Fig. 4.

Effect of Dexamethasone (DEX) Pretreatment on DCPT-induced Alterations in Rat Serum Alanine Aminotransferase (ALT) Levels. Pretreatment groups are indicated along the X-axis. Treatments are indicated by white (clear) bars (corn oil) or black bars (DCPT, 0.2 mmol/kg). Rats were pretreated with corn oil (vehicle, 4 ml/kg, i.p.) or DEX (50 mg/kg/day for 3 days, i.p. in corn oil), and were then administered corn oil (vehicle, 4 ml/kg, i.p.) or DCPT (0.2 mmol/kg, i.p. in corn oil). Values indicate means $\pm$ standard errors (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the Corn oil + Corn oil control group. A dagger (†) indicates that the value is significantly different ($p < 0.05$) from the Corn oil + DCPT (0.2 mmol/kg) group. A double dagger (§) indicates that the value is significantly different ($p < 0.05$) from the DEX + corn oil group.

Fig. 5.

Effect of Dexamethasone (DEX) Pretreatment on DCPT-induced Alterations in Rat Liver Histology. Groups are indicated by pretreatment + treatment as follows: (A) Corn oil + Corn oil; (B) DEX + Corn oil; (C) Corn oil + DCPT (0.2 mmol/kg); (D) DEX + DCPT (0.2 mmol/kg). Rats were pretreated with corn oil (vehicle, 4 ml/kg, i.p.) or DEX (50 mg/kg/day for 3 days, i.p. in corn oil), and were then administered corn oil (vehicle, 4 ml/kg, i.p.) or DCPT (0.2 mmol/kg, i.p. in corn oil). The black arrow heads point to condensed proteins in cells, and the black arrows point to vacuolization. Sections were stained with hematoxylin-eosin. Magnification is 400×.

Fig. 6.

Effect of Dexamethasone (DEX) Pretreatment on DCPT-induced Alterations in Rat Liver Glycogen Content. Groups are indicated by pretreatment + treatment as follows: (A) Corn oil + Corn oil; (B) DEX + Corn oil; (C) Corn oil + DCPT (0.2 mmol/kg); (D) DEX + DCPT (0.2 mmol/kg). Rats were pretreated with corn oil (vehicle, 4 ml/kg, i.p.) or DEX (50 mg/kg/day for 3 days, i.p. in corn oil), and were then administered corn oil (vehicle, 4 ml/kg, i.p.) or DCPT (0.2 mmol/kg, i.p. in corn oil). The black arrows point to vacuolization due to loss of glycogen. Sections were stained with Periodic Acid-Schiff. Magnification is 400×.

Fig. 7. Effect of Dexamethasone (DEX) Pretreatment on CYP3A1/CYP3A2 Rat Liver Microsomal Protein Level Expression. Rats were pretreated with Corn oil (vehicle, 4 ml/kg, i.p.) or DEX (50 mg/kg/day for 3 days, i.p. in corn oil) before preparation of the microsomes. (A) Representative Western blots, (B) Quantitative results (means $\pm$ standard errors) following densitometric analysis. Protein levels were normalized to β -actin as a loading control. Asterisks (*) indicate values significantly different from Corn oil control. A dagger (†) indicates values different from corn oil pretreated CYP3A1. Lane 1 is a representative blot obtained using pooled corn oil pretreated microsomes, lanes 2–5 contain DEX pretreated microsomes from individual rats. The graphical representation summarizes the densitometry results obtained

with each antibody from four separate blots using the DEX pretreated microsomes or pooled corn oil microsomes.

Table 1

The effect of pretreatments and treatments on rat blood urea nitrogen concentration, urine volume and urine protein content. Rats were pretreated with saline or corn oil (vehicle, 4 ml/kg, i.p.), 1-aminobenzotriazole (ABT, 100 mg/kg, i.p.), troleandomycin (TAO, 500 mg/kg, i.p.) or dexamethaxone (DEX, 50 mg/kg/day for 3 days, i.p.) prior to treatment. Values indicate means $\pm$ standard errors (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the saline + corn oil control group in the inhibitor study. There were no significant differences from the corn oil + corn oil control group in the inducer study.

Pretreatment Group	Treatment Group	Blood Urea Nitrogen(mg/dl)	24 hour Urine Volume (ml)	6 hour Urine Protein Content (mg)
Saline	Corn oil	24.5 $\pm$ 0.8	5.4 $\pm$ 1.3	2.7 $\pm$ 0.4
ABT	Corn oil	24.0 $\pm$ 0.6	3.1 $\pm$ 1.2	3.6 $\pm$ 0.1
TAO	Corn oil	22.7 $\pm$ 1.4	3.9 $\pm$ 0.7	3.5 $\pm$ 0.7
Saline	DCPT (0.6 mmol/kg)	22.5 $\pm$ 1.2	12.9 $\pm$ 1.3*	3.1 $\pm$ 0.2
ABT	DCPT (0.6 mmol/kg)	23.8 $\pm$ 1.0	17.6 $\pm$ 1.4*	3.7 $\pm$ 0.7
TAO	DCPT (0.6 mmol/kg)	23.0 $\pm$ 1.9	11.4 $\pm$ 2.7*	3.1 $\pm$ 1.4
Saline	DCPT (1.0 mmol/kg)	25.4 $\pm$ 1.9	17.0 $\pm$ 1.5*	2.0 $\pm$ 0.3
ABT	DCPT (1.0 mmol/kg)	25.6 $\pm$ 0.5	12.6 $\pm$ 3.9*	2.9 $\pm$ 0.7
TAO	DCPT (1.0 mmol/kg)	26.7 $\pm$ 0.7	20.5 $\pm$ 0.5*	3.0 $\pm$ 0.6
Corn oil	Corn oil	24.0 $\pm$ 1.8	7.1 $\pm$ 1.4	2.5 $\pm$ 0.2
DEX	Corn oil	21.3 $\pm$ 1.0	11.0 $\pm$ 1.9	6.2 $\pm$ 2.6
Corn oil	DCPT (0.2 mmol/kg)	22.3 $\pm$ 0.9	7.9 $\pm$ 0.8	6.6 $\pm$ 0.7
DEX	DCPT (0.2 mmol/kg)	20.6 $\pm$ 1.2	10.0 $\pm$ 1.5	2.5 $\pm$ 0.6

Table 2

The effect of pretreatments and treatments on rat body, liver and kidney weights. Rats were pretreated with saline or corn oil (vehicle, 4 ml/kg, i.p.), 1-aminobenzotriazole (ABT, 100 mg/kg, i.p.), troleandomycin (TAO, 500 mg/kg, i.p.) or dexamethaxone (DEX, 50 mg/kg/day for 3 days, i.p.) prior to treatment. Values indicate means $\pm$ standard errors (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the corn oil + corn oil control group in the inducer study. There were no significant differences from the saline + corn oil control group in the inhibitor study.

Pretreatment Group	Treatment Group	Body Weight(g)	Liver Weight(g/100 g BW)	Kidney Weight(g/100 g BW)
Saline	Corn oil	204.3 $\pm$ 8.5	3.38 $\pm$ 0.25	0.41 $\pm$ 0.02
ABT	Corn oil	209.2 $\pm$ 6.8	3.62 $\pm$ 0.14	0.41 $\pm$ 0.01
TAO	Corn oil	202.6 $\pm$ 3.1	3.53 $\pm$ 0.08	0.39 $\pm$ 0.02
Saline	DCPT (0.6 mmol/kg)	191.5 $\pm$ 2.9	3.57 $\pm$ 0.06	0.41 $\pm$ 0.01
ABT	DCPT (0.6 mmol/kg)	199.9 $\pm$ 3.7	3.76 $\pm$ 0.07	0.400 $\pm$ 0.004
TAO	DCPT (0.6 mmol/kg)	203.2 $\pm$ 1.5	3.70 $\pm$ 0.08	0.41 $\pm$ 0.01
Saline	DCPT (1.0 mmol/kg)	195.5 $\pm$ 2.6	4.00 $\pm$ 0.05	0.46 $\pm$ 0.01
ABT	DCPT (1.0 mmol/kg)	183.4 $\pm$ 10.1	3.81 $\pm$ 0.21	0.44 $\pm$ 0.02
TAO	DCPT (1.0 mmol/kg)	201.8 $\pm$ 1.6	3.36 $\pm$ 0.23	0.42 $\pm$ 0.02
Corn oil	Corn oil	222.5 $\pm$ 7.0	3.57 $\pm$ 0.29	0.370 $\pm$ 0.004
DEX	Corn oil	185.5 $\pm$ 4.5*	5.73 $\pm$ 0.18*	0.48 $\pm$ 0.01*
Corn oil	DCPT (0.2 mmol/kg)	216.3 $\pm$ 3.0	4.10 $\pm$ 0.05	0.39 $\pm$ 0.01
DEX	DCPT (0.2 mmol/kg)	168.3 $\pm$ 4.7*	5.90 $\pm$ 0.44*	0.53 $\pm$ 0.02*

Table 3

6 β - and 16 α -Hydroxytestosterone Formation in Rat Liver Microsomes. Rats were pretreated with corn oil (vehicle, 4 ml/kg, i.p.), 1-aminobenzotriazole (ABT, 100 mg/kg, i.p.), troleandomycin (TAO, 500 mg/kg, i.p.) or dexamethaxone (DEX, 50 mg/kg/day for 3 days, i.p.) prior to preparation of the microsomes. Values indicate means $\pm$ standard errors (N = 4). An asterisk indicates that the value is significantly different ($p < 0.05$) than the value obtained using microsomes obtained from corn oil-treated rats.

Pretreatment Group	6 β -Hydroxytestosterone Formation(pmol/mg protein/min)	16 α -Hydroxytestosterone Formation(pmol/mg protein/min)
Corn oil	2.10 $\pm$ 0.05	419 $\pm$ 70
ABT	0.43 $\pm$ 0.05*	212 $\pm$ 14.7*
TAO	0.98 $\pm$ 0.03*	485 $\pm$ 52.4
DEX	13.2 $\pm$ 0.4*	396 $\pm$ 30.8

Table 4

Ethoxyresorufin (EROD) and Pentoxyresorufin (PROD) Activities in Rat Liver Microsomes. Rats were pretreated with corn oil (vehicle, 4 ml/kg, i.p.), 1-aminobenzotriazole (ABT, 100 mg/kg, i.p.), troleandomycin (TAO, 500 mg/kg, i.p.) or dexamethaxone (DEX, 50 mg/kg/day for 3 days, i.p.) prior to preparation of the microsomes. Values indicate means $\pm$ standard errors (N = 5). An asterisk indicates that the value is significantly different ($p < 0.05$) than the value obtained using the microsomes obtained from corn oil-treated rats.

Pretreatment Group	EROD Activity (pmol/mg protein/min)	PROD Activity (pmol/mg protein/min)
Corn oil	173.94 $\pm$ 35.82	19.71 $\pm$ 2.70
ABT	48.50 $\pm$ 12.05*	9.61 $\pm$ 1.49*
TAO	114.24 $\pm$ 20.83	15.31 $\pm$ 1.39
DEX	104.04 $\pm$ 15.13	25.07 $\pm$ 3.02