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Cytotoxicity of 3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione (DCPT) and Analogues in Wild Type and CYP3A4 Stably Transfected HepG2 Cells

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Abstract

The thiazolidinedione (TZD) ring is a constituent of the glitazones that are used to treat type II diabetes. Liver injury has been reported following chronic glitazone use; however, they do not produce hepatic damage in common laboratory animal species. In contrast, 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT) causes hepatotoxicity in rats. DCPT toxicity is dependent upon the presence of an intact TZD ring and cytochrome P450 (CYP)-mediated biotransformation. To further investigate TZD ring-induced toxicity, DCPT and several structural analogues or potential metabolites were tested *in vitro* using wild type human hepatoma HepG2 and HepG2 cells stably transfected with the CYP3A4 isozyme. CYP3A4 activity was confirmed by measuring testosterone 6 β -hydroxylation. Both cell lines were treated with 0-250 μ M of the compounds in Hanks' balanced salt solution. Cell viability was measured after 24 hrs. DCPT and *S*-(3,5-dichlorophenyl)aminocarbonyl thioglycolic acid (DCTA) were the most toxic compounds of the series. Furthermore, DCPT was significantly more toxic in transfected cells ($LC_{50} = 160.2 \pm 5.9 \mu$ M) than in wild type cells ($LC_{50} = 233.0 \pm 19.7 \mu$ M). Treatment with a CYP3A4 inhibitor or inducer attenuated or potentiated DCPT cytotoxicity, respectively. These results suggest that DCPT-induced cytotoxicity in the transfected HepG2 cells is partially dependent on CYP3A4.

Keywords

3-(3,5-dichlorophenyl)-2,4-thiazolidinedione; HepG2 cells; cytochrome P450; thiazolidinedione; drug metabolism

1. Introduction

A thiazolidinedione (TZD) ring is a structural feature of the glitazones, a group of drugs used in the treatment of Type II diabetes. Troglitazone was the first drug in this class released in the United States, but was eventually removed from the market after causing severe liver damage in some patients (Gitlin et al., 1998; Kohlroser et al., 2000). TGZ-related hepatotoxicity was characterized by zone 3 necrosis, and in some cases infiltration of

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inflammatory cells (Gitlin et al., 1998; Kohlroser et al., 2000). There were also reports of cholestatic injury. Approximately 90 cases of liver toxicity were reported that required liver transplantation or resulted in death (Graham et al., 2003). While they remain on the market, there are also reports of mild hepatotoxicity associated with the routine administration of rosiglitazone (Forman et al., 2000; Gouda et al., 2001; Bonkovsky et al., 2002) and pioglitazone (Maeda 2001; May et al., 2002; Marcy et al., 2004). Patients currently taking this class of drugs must be free of pre-existing liver conditions and have their liver enzymes routinely monitored (Scheen, 2001).

Our lack of understanding of glitazone-induced hepatotoxicity is due, in part, to the absence of severe toxic effects in common laboratory animal models (Chojkier, 2005). Several studies, however, have provided information about the CYP-mediated metabolism of the glitazones as a possible mechanism of toxicity. While formation of a quinone species in the chromane ring of TGZ by CYP isozymes has been proposed (Yamazaki et al., 1999; Yamamoto et al., 2001; Yamamoto et al., 2002), this does not explain the hepatotoxicity observed with rosiglitazone and pioglitazone. Alternatively, glutathione (GSH) trapping studies have pointed to CYP3A-mediated oxidation of TGZ on the sulfur atom of the TZD ring (Kassahun et al., 2001; Tetley et al., 2001). A similar metabolic pathway was also reported for rosiglitazone and pioglitazone (Alvarez-Sánchez et al., 2006). *In vitro*, compared to the parent compound, the quinone metabolite of TGZ produced only slight toxicity on HepG2 cells (Yamamoto et al., 2001). Furthermore, a structural analog of TGZ containing a succinimide ring in place of the TZD ring was less toxic in human liver THLE-2 cells (Saha et al., 2010). Together, these data suggest that metabolism in the TZD ring may play a role in glitazone-induced toxicity.

During a study examining the structure-activity relationship (SAR) of cyclic imide containing compounds, we found that 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT; Fig. 1) was hepatotoxic in Fischer 344 rats (Kennedy et al., 2003; Patel et al., 2008). Because DCPT contains a TZD ring and produces hepatotoxicity in a common laboratory animal, it may be useful for investigating TZD ring-induced liver damage. Further investigations showed that the toxicity of DCPT in male rats is dependent on CYP3A-mediated biotransformation (Crincoli et al., 2008). Pre-treating rats with dexamethasone, a CYP3A inducer, exacerbated liver damage of an otherwise non-toxic dose of DCPT. In contrast, both 1-aminobenzotriazole (a non-specific CYP inhibitor) and troleandomycin (a CYP3A inhibitor) attenuated DCPT-induced hepatotoxicity. Additionally, an *in vivo* structure-activity relationship study with several analogues of DCPT found that hepatotoxicity is dependent on an intact TZD ring (Patel et al., 2011).

Although the TZD ring of DCPT has been shown to cause hepatotoxicity in rats, our ultimate goal is to explain the potential toxic effect of TZD ring-containing compounds in humans. To that end, human hepatoma derived HepG2 cells were used to investigate the metabolic activation and cytotoxicity of DCPT. While the wild type HepG2 cell line contains only low levels of CYP enzymatic activity (Rodríguez-Antona et al., 2002), previous studies have demonstrated that HepG2 cells transfected with CYP isozymes were a suitable model to investigate cytotoxicity (Yoshitomi et al., 2001; Vignati et al., 2005). Both our studies with DCPT in rats (Crincoli et al., 2008) and work with TGZ in rat and human systems (Kassahun et al., 2001; Tetley et al., 2001; He et al., 2004; Baughman et al., 2005; Alvarez-Sánchez, 2006) suggested the involvement of CYP3A in TZD ring biotransformation. In order to investigate the cytotoxicity of DCPT and make it relevant to human metabolism, we chose to utilize HepG2 cells stably transfected with CYP3A4. In addition, we also performed a SAR using compounds with modifications to the TZD ring. These include: *S*-(3,5-dichlorophenyl)aminocarbonyl thioglycolic acid (DCTA), a potential hydrolytic product of DCPT; 3,5-dichlorophenyl isocyanate (DPI), which could be formed

from degradation of DCTA; 3-(3,5-dichlorophenyl)-5-methyl-2,4-thiazolidinedione (DPMT), which contains a methyl group on the C-5 carbon of the TZD ring; and (3,5-dichlorophenyl)-2-thiazolidinone (2-DCTD) and 3-(3,5-dichlorophenyl)-4-thiazolidinone (4-DCTD), which contain isomeric thiazolidinone rings. The results of these studies suggest that *in vitro*, the cytotoxicity of DCPT is partially dependent on CYP3A4-mediated metabolism.

2. Materials and methods

2.1 Materials

DCPT, DCTA, DPMT, 2-DCTD, and 4-DCTD were previously synthesized in our laboratory (Kennedy et al., 2003, Patel et al., 2011). DPI was obtained from Alfa Aesar (Ward Hill, MA). The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Testosterone, 6 β -hydroxytestosterone, and cortisone were purchased from Steraloids, Inc. (Newport, RI). Dexamethasone (DEX), rifampicin (RIF), ketoconazole (KCZ), and CellTiterTM M were products of Sigma Chemical Co. (St. Louis, MO). The CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay kit (No. G5421) is a product of Promega Corp. (Madison, WI). Gibco[®] high glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen Corp. (Carlsbad, CA).

2.2 Cell culture

HepG2-CYP3A4 transfected and HepG2 wild type cells were cultured in Gibco[®] high glucose DMEM supplemented with 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% (v/v) fetal bovine serum. Prior to any treatment, cells were trypsinized, and plated with the above media at the densities specified below at 37 °C in a humidified atmosphere (5% CO₂/air). All of the compounds were dissolved in filter (0.2 μ m) sterilized DMSO, with a final concentration in HBSS of 0.1% (v/v).

2.3 Preparation of CYP3A4-transfected HepG2 cells

2.3.1 Cloning of CYP3A4—The coding sequence for CYP3A4 was amplified from a Quick-Clone cDNA library. The PCR fragment was cloned into pCRII (Invitrogen Corp., Carlsbad, CA), and the plasmid was transformed into *Escherichia coli*. Several clones were recovered, and the entire cDNA fragment was sequenced from both directions. The sequence was confirmed to be identical to that reported in GenBank accession no. NM_017460. A single clone was chosen and the cDNA recovered by digestion with EcoRI. The fragment was inserted into the expression vector pcDNA3 (Invitrogen Corp.) after digestion with the same restriction enzyme. After transformation into *E. coli*, several clones were recovered and sequenced from both ends to ensure that the cDNA was inserted in the proper orientation.

2.3.2 Stable Expression of CYP3A4 in HepG2 Cells—Stable cell lines were a kind gift of Merck Research laboratories, West Point, PA. Briefly; human HepG2 cells were maintained in MEM as previously described (Rushmore and Pickett, 1991). Stable cell lines were established for CYP3A4 after transfection and selection by G-418 (Rushmore et al., 1991). Briefly, cells were plated in 25 cm² flasks (approximately 1×10^4 cells), and after 24 h, cells in 24 plates were transfected with one of the expression constructs described above (10 μ g of DNA per flask). Twenty-four hours later, the medium was changed and supplemented with G-418 (50 μ g/ μ L) to a final concentration of 800 μ g/mL. The medium was changed every fourth day. The cells were allowed to grow in the selection medium for 4 weeks, at which time individual clonal expansions (clones) were visible. At confluence, each clone was tested for P450 activity by exposing the clone to a specific substrate for 16 h (250 μ M testosterone). After exposure for 16 h to the substrate, the medium was recovered and

monitored for both the parent and products using a generic high-pressure liquid chromatography method (Rushmore et al., 2000).

2.4 Transfectant characterization

2.4.1 Formation of 6 β -hydroxytestosterone—HepG2-CYP3A4 transfected cells were plated at a density of 5×10^5 cells/1 mL/well in 6-well plates and grown to confluence in media. Once confluent, the cells were incubated with media containing testosterone (0-250 μ M, 0.1% v/v DMSO). After 1 hr, a 250 μ L aliquot of media was collected and spiked with 100 μ L of methylene chloride containing 6 nmol of cortexolone, the internal standard, and extracted with acetonitrile (1:4, v/v). Samples were stored at 4 °C for 30 min and were then centrifuged at 6,000 rpm for 10 min. The organic fractions were collected in a clean microfuge tube and evaporated to dryness under vacuum in a SpeedVac evaporator (Savant Instruments Inc., Farmingdale, NY). The dried samples were stored at -80 °C and reconstituted in 100 μ L of the mobile phase (starting conditions, described below) before analysis. Cells were lysed using CellLytic™ reagent and the supernatant was quantitatively assayed for protein using the BioRad protein assay kit.

2.4.2 Inhibition of 6 β -hydroxytestosterone formation—HepG2-CYP3A4 transfected cells were plated as described above. Once confluent, the cells were incubated with media containing 250 μ M testosterone with or without ketoconazole (KCZ; 1 and 10 μ M, 0.1% v/v DMSO) for 1 hr. Samples were treated as described above.

2.4.3 Induction of 6 β -hydroxytestosterone formation—HepG2-CYP3A4 transfected cells were plated as described above. Once confluent, the cells were pretreated with either dexamethasone (DEX; 100 μ M, 0.1% v/v DMSO) or rifampicin (RIF; 100 μ M, 0.1% v/v DMSO) for 24 hr. Pretreatments were then removed; cells were washed with HBSS, and then incubated with media containing 250 μ M testosterone for 1 hr. Samples were treated as described above.

2.4.4 High performance liquid chromatography (HPLC)—HPLC analysis was performed on a Hewlett Packard 1050 HPLC system equipped with a pump, diode array detector (set at 247 nm), autoinjector and a column heater (set at 45 °C). A Beckman Ultrasphere C-18 (5 μ m, 4.2 mm $\times$ 250 mm) column was used with a mobile phase consisting of 10% methanol in H₂O (A) and 100% methanol (B) at a constant flow rate of 1.0 mL/min. The linear gradient was as follows: 0-8 min, 47% A; 8-14 min., 42% A. The column was then reequilibrated to 47% A before starting a new injection.

2.5 In vitro toxicity of TZD ring-containing compounds

2.5.1 Treatments—HepG2 and HepG2-CYP3A4 transfected cells were plated at a density of 2×10^4 cells/200 μ L/well in 96-well stripwell plates and incubated for 24 hr. On the following day, the media was removed and the cells were washed with HBSS. The HBSS was then removed, replaced with 200 μ L of compound (0-250 μ M) in HBSS and incubations were continued for 24 hr. Cell viability was assayed as described below.

2.5.2 Inhibition of CYP3A4 enzymatic activity—HepG2 and HepG2-CYP3A4 transfected cells were plated as described above. On the following day, the media was removed, the cells were washed with HBSS, and then replaced with 200 μ L of DCPT (250 μ M) in HBSS alone or in the presence of KCZ (2 μ M) and incubated for 24 hr. Cell viability was assayed as described below.

2.5.3 Induction of CYP3A4 enzymatic activity—HepG2 and HepG2-CYP3A4 transfected cells were plated as described above. On the following day, the media was

removed, the cells were washed with HBSS, and then replaced with 200 μ L of media in the presence or absence of DEX (100 μ M) and incubated for 24 hr. The pretreatment was removed, the cells were washed with HBSS, and 200 μ L of DCPT (100 μ M) in HBSS was added and incubated for 24 hr. Cell viability was assayed as described below.

2.5.4 Cell viability (MTS assay)—After the respective treatments, media containing the compounds was removed, the cells were washed with HBSS, and then 100 μ L of HBSS was added to the wells. Viability was assessed using the CellTiter 96[®] AQueous non-radioactive cell proliferation assay according to the manufacturer's instructions (Promega, Madison, WI). Absorbance at 492 nm was measured on an HTS 7000 Plus microplate reader (Perkin Elmer, Inc., Waltham, MA).

2.6 DCPT hydrolysis

2.6.1 Incubations with HBSS—A 1 mL volume of DCPT (250 μ M, 0.1% v/v DMSO) in HBSS was added to each well of a 24-well plate and incubated at 37 °C in a humidified atmosphere (5% CO₂/air). Aliquots were collected at 0.5, 1, 1.5, 2 and 24 hr, spiked with 300 μ L of 4 nmol N-(3,5-dichlorophenyl)succinamic acid (NDPSA) in acetonitrile as the internal standard, and extracted with acetonitrile (1:4, v/v). The mixture was vortexed and centrifuged at 6,000 rpm for 5 min. Organic fractions were collected in microfuge tubes and evaporated to dryness under vacuum in a SpeedVac evaporator. The dried samples were stored at -80°C and reconstituted in 100 μ L of the mobile phase (starting conditions described below) before analysis.

2.6.2 High Performance Liquid Chromatography (HPLC)—HPLC analysis was performed on a Hewlett Packard 1050 HPLC system equipped with a pump, diode array detector (set at 230 and 254 nm) and an autoinjector. A Tosohaas C-18 (5 μ m, 4.6 mm $\times$ 250 mm) reverse phase column was used with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a constant flow rate of 1.0 mL/min. Samples were analyzed for 10 min with an isocratic elution of 41% A. The column was then flushed for 7 min before starting a new injection.

2.7 Calculations and statistics

For the testosterone assay, the K_m and V_{max} values were calculated using scatter plots and polynomial trend lines generated from Microsoft[®] Office Excel 2003. This program was also used to calculate LC₅₀ values for the cell viability assays, using bar graphs and linear or polynomial trend lines. Results generated using SigmaPlot[®] 11 graphing and statistics software (Systat Software, Inc., Chicago, IL) are expressed as means $\pm$ SE (N = 4). The data were analyzed by a one way ANOVA followed by a Student-Newman-Keuls post hoc test and/or Student's *t*-test. If the equal variance test failed, the Mann-Whitney Rank Sum test was used. Differences in the means or medians were considered significant when $p < 0.05$.

3. Results

3.1 Transfectant characterization

To determine the metabolic activity of the HepG2-CYP3A4 transfected cells, the rate of conversion of testosterone to 6 β -hydroxytestosterone was measured monthly. The stable transfectants and wild type cells were incubated with 0-250 μ M testosterone for 1 hr as a marker for CYP3A4 activity. The calculated V_{max} (mean $\pm$ SE) was 92.7 $\pm$ 13.3 pmol/min/mg protein and the K_m (mean $\pm$ SE) was 81.1 $\pm$ 5.7 μ M. There was no detectable 6 β -hydroxylation of testosterone by the HepG2 wild type cells.

3.2 Effect of CYP3A4 inhibition/induction on testosterone 6 β -hydroxylation

To further investigate the enzymatic activity of the HepG2-CYP3A4 transfectants, cells were exposed to 250 μ M testosterone along with CYP inhibitors and inducers. Ketoconazole (KCZ, 1 μ M), a reversible CYP3A inhibitor, significantly decreased the conversion of testosterone to 6 β -hydroxytestosterone by approximately 30% (Table 1). With 10 μ M KCZ no 6 β -hydroxytestosterone was detected. Pretreatment with 0.1% DMSO in HBSS produced a modest, but significant increase in 6 β -hydroxytestosterone formation (Table 2). The CYP3A inducer DEX increased the rate of testosterone metabolism approximately 3-fold. In contrast, RIF, another CYP3A inducer, had no effect.

3.3 In vitro toxicity of DCPT and analogues

DCPT and several structurally similar compounds were incubated for 24 hr with both HepG2 wild type and HepG2-CYP3A4 transfectants as previously described in the Methods section. Concentrations of DCPT up to 75 μ M had no significant effect on cell viability compared to the control group (Fig. 2). With 100-150 μ M DCPT, cell viability was significantly decreased in the HepG2-CYP3A4 transfected cells, but not in the HepG2 wild type cells. A significant decrease in cell viability was observed in both cell lines starting at 175 μ M and up to 250 μ M (the limit of the solubility of DCPT in HBSS as observed by turbidity of solutions at higher concentrations). At 200 μ M and above, there was a significantly greater decrease in cell viability in the CYP3A4 transfected cells compared to the wild type cells.

The cytotoxicity of DCTA and DPI was also evaluated. Although it was not different from the control, the viability of HepG2-CYP3A4 transfected cells treated with 25 μ M DCTA was lower than the wild type cells (Fig. 3). This was not observed at higher concentrations and is probably not toxicologically relevant. In contrast, DCTA caused a significant loss of cell viability in both cell lines beginning at 50 μ M. Cytotoxicity with DPI was observed in the wild type cells beginning at 25 μ M (Fig. 4). A significant decrease in the viability of the HepG2-CYP3A4 transfected cells was observed with 100 μ M DPI. However, even with the 250 μ M treatment, viability following DPI treatment did not go below 50% in either cell line.

Treatment with DPMT resulted in no observed decrease in viability in either of the cell lines (results not shown). It should be noted that there were issues regarding the solubility of DPMT in HBSS, evident at 250 μ M. Since the lower concentrations were prepared by serial dilution, this problem may have contributed to the lack of cytotoxicity. We found that 2-DCTD was not cytotoxic in the wild type cell line (results not shown). However, there was a steep decline in viability from $99.6 \pm 4.6\%$ at 100 μ M to $37.3 \pm 7.1\%$ at 250 μ M in the CYP3A4 transfectants. 4-DCTD was not cytotoxic in either cell line over the concentration range examined (results not shown).

The LC₅₀ values in the HepG2-CYP3A4 transfected and HepG2 wild type cells were calculated for all six compounds (Table 3). These data suggest that DCPT is significantly more cytotoxic in the HepG2-CYP3A4 transfectants compared to the HepG2 wild type cells, while DCTA is highly cytotoxic in both cell lines. With DPI, DPMT, 2-DCTD, and 4-DCTD, the LC₅₀ values in both cell lines were greater than 250 μ M, the highest concentration of each compound that was tested.

3.4 Effect of CYP3A4 inhibition/induction on DCPT cytotoxicity

To further investigate the potential bioactivation of DCPT by CYP3A isozymes, transfected cells were treated with CYP inhibitors and inducers. Treatment with 2 μ M KCZ (Table 4) alone was not cytotoxic. In the presence of DCPT, there was a significant decrease in the

viability of the HepG2-CYP3A4 transfectants compared to controls. However, viability in the presence of KCZ was significantly increased when compared to cells treated with 250 μ M DCPT alone.

Transfected cells were pretreated with DEX, then treated with a lower (100 μ M vs. 250 μ M) concentration of DCPT to determine if enzymatic induction would enhance cytotoxicity. HepG2-CYP3A4 transfectants pretreated with DEX, followed by exposure to 100 μ M DCPT exhibited a slight (approximately 10%), but statistically significant decrease in cell viability when compared to controls (Table 5).

3.5 DCPT hydrolysis

The hydrolysis of 250 μ M DCPT in HBSS over time was measured using HPLC (Fig. 5). Compared to the 0 hr time point, the percentage of DCPT was significantly decreased starting at 0.5 hr and remained so through 2 hr, with less than 50% remaining after 24 hr. Alternatively, the percentage of DCPT converted to DCTA was significantly increased from the 0 hr time point beginning at 0.5 hr and continuing to 2 hr. Although there was still a significant increase in DCTA at 24 hr relative to the 0 hr time point, there was an obvious decrease in the percentage of DCTA from that observed at the 2 hr time point. DPI was not detected in these incubations; however, it is a low melting compound (m.p. 28-29 $^{\circ}$ C, b.p. 242-244 $^{\circ}$ C) and may have been lost on the vacuum concentrator.

4. Discussion

CYP3A-mediated biotransformation of the TZD ring may be involved in hepatotoxicity of the glitazones in humans (Kassahun et al., 2001; Tettey et al., 2001; He et al., 2004; Baughman et al., 2005; Alvarez-Sánchez, 2006) and DCPT in rats (Crincoli et al., 2008). This information prompted us to use HepG2 cells stably transfected with CYP3A4 to study the potential toxicity associated with DCPT and similar compounds in a human cell line.

We initially needed to confirm that the CYP3A4 enzyme stably transfected in HepG2 cells was present and functional. Although the turnover was lower than that observed with human hepatocytes, the characteristics of the HepG2-CYP3A4 transfected cells used in these experiments were similar to other transfectant cells reported in the literature (Yoshitomi et al., 2001; Vignati et al., 2005). In contrast, wild type HepG2 cells had no detectable CYP3A4 activity. This was not unexpected since the wild type HepG2 cell line contains only low levels of CYP activity (Rodríguez-Antona et al., 2002).

We also demonstrated the ability of the transfected CYP3A4 enzyme to undergo inhibition and induction. A reversible CYP3A inhibitor, KCZ, blocked the 6 β -hydroxylation of testosterone by the transfectants. While DEX, a CYP3A inducer, was able to increase the formation of 6 β -hydroxytestosterone, no induction was observed with a second CYP3A inducer, RIF. These findings are probably due to differential expression of the receptors for DEX and RIF in HepG2 cells. DEX interacts with the nuclear glucocorticoid receptor (GR), while RIF selectively binds to pregnane X receptor (PXR) (Moore et al., 2006). DEX induces CYP3A expression in rats (Schuetz et al., 1984) and CYP3A4 mRNA expression in HepG2 cells (Swales et al., 2003). The lack of induction by RIF may be due to the fact that the basal level of PXR expression in HepG2 cells is low (Naspinski et al., 2008).

With proof of the functionality of our model, we then examined the cytotoxicity of DCPT and structurally similar analogues in the transfected HepG2 cells to determine whether CYP3A4-mediated biotransformation plays a role. DCPT-induced cytotoxicity exhibited some dependence on CYP3A4-mediated metabolism. A concentration-response was observed in the HepG2-CYP3A4 transfected cells beginning at 100 μ M DCPT. With 250

μM DCPT, cell viability in transfectants was reduced by 80%. The calculated LC₅₀ for DCPT in the HepG2-CYP3A4 transfectant cells was $160.2 \pm 5.9 \mu\text{M}$, while in the HepG2 wild type cells the LC₅₀ value was $233.0 \pm 19.7 \mu\text{M}$. These results suggest that CYP3A4 can convert DCPT to a toxic metabolite and are consistent with what we previously observed in rats (Crincoli et al., 2008). DCPT reduced cell viability in the wild type HepG2 cells by approximately 60% at the highest concentration, which suggests either that the parent compound itself is toxic or that a non CYP3A-dependent pathway is contributing to the formation of a cytotoxic metabolite.

As noted above, we found that KCZ can inhibit the formation of 6β -hydroxytestosterone in HepG2-CYP3A4 transfectant cells, while DEX induces its formation, suggesting that CYP3A4 transcriptional machinery is functional. If CYP-mediated oxidation plays a role in the cytotoxicity of DCPT, these compounds should modulate cell viability. Treatment with KCZ attenuated cytotoxicity in the transfectants. However, the viability of these cells remained significantly lower when compared to controls. Pretreating HepG2-CYP3A4 transfectant cells with DEX potentiated the effect of $100 \mu\text{M}$ DCPT on cell viability. We believe that DEX increased production of more CYP3A4 enzyme which, in turn, could increase formation of a toxic metabolite from DCPT. Taken together, the inhibitor and inducer studies provide further evidence for a partial role of CYP3A4-mediated metabolism in the cytotoxicity of DCPT.

We also conducted a structure activity relationship (SAR) study using several analogues and potential degradation products of DCPT (Fig 1). Both DCTA and DPI produced a concentration-dependent loss of cell viability, although there was no difference between the two cell lines with either compound. This suggests that the cytotoxicity of these compounds is not CYP3A4-dependent. DCTA could result directly from hydrolysis of the TZD ring in DCPT and we previously found that it was toxic in rats (Patel et al., 2011). DPI is an isocyanate and is therefore a potentially reactive compound that could cause protein carbamylation and toxicity (Brown et al., 1987). DPMT, which possesses a methyl group on the C-5 position of the TZD ring, was non-toxic in both cell lines. In contrast, this compound was hepatotoxic when evaluated in rats (Patel et al., 2011). However, solubility problems were encountered with DPMT in cell culture, and it is possible that the cells were not exposed to a sufficient concentration of compound to cause toxicity. The isomeric compounds 2-DCTD and 4-DCTD contain thiazolidinone rings instead of the TZD ring found in DCPT. 4-DCTD was not toxic in either cell line. 2-DCTD produced a decrease in HepG2-CYP3A4 transfectant viability, but only at the highest concentration tested ($250 \mu\text{M}$). One conceivable explanation for this finding could be CYP3A4-dependent conversion of 2-DCTD to DCPT. The lack of in vitro toxicity of 4-DCTD and low cytotoxicity of 2-DCTD are generally consistent with what we previously found in rats, where neither compound produced liver damage (Patel et al., 2011). Overall, the in vivo and in vitro SAR studies support a role for the TZD ring in toxicity.

In conclusion, our HepG2-CYP3A4 stably transfectant cells appear to be a suitable human model system to study the cytotoxicity of DCPT. We found that DCPT-induced toxicity was partially due to CYP3A4-dependent metabolism in the transfectants. In analogy to the glitazones (Kassahun et al., 2001; Tetey et al., 2001; Alvarez-Sánchez et al., 2006), CYP3A4-mediated metabolic activation of DCPT could conceivably occur via a sulfoxidation step (Fig. 6, pathway A). However, DCPT was cytotoxic in the wild type HepG2 cells and the CYP3A4 inhibitor KCZ was only partially protective in the transfectants. These results suggest that either the parent compound is directly cytotoxic or that non CYP3A4-dependent pathways can contribute to the formation of toxic metabolites. One possibility would involve hydrolysis of DCPT to DCTA, followed by further degradation of DCTA to DPI (Fig. 6, pathway B). This reaction sequence has been shown to

occur for 3-methyl-1,3-thiazolidine-2,4-dione, a structural analogue of DCPT (Macháček et al., 1981). The first step involves base-catalyzed hydrolysis of 3-methyl-1,3-thiazolidine-2,4-dione to S-(methylaminocarbonyl)thioglycolic acid (analogous to DCTA). Subsequent degradation of the thioglycolic acid resulted in formation of methyl isocyanate (analogous to DPI). In fact, we found that DCPT undergoes time-dependent, spontaneous hydrolysis to DCTA in HBSS. Furthermore, DCTA and DPI were cytotoxic in both HepG2 cell lines. Thus, it is possible that formation of DCTA, and possibly DPI, in the incubations could contribute to the observed non CYP-dependent cytotoxicity of DCPT. These results suggest that toxic metabolites from the putative hydrolytic pathway may also play a role in the cytotoxicity of DCPT, or in the case of the HepG2 wild type cells, may predominate. We believe that formation of the putative DCPT sulfoxide (Fig. 6, pathway A) is CYP-dependent and therefore it would not be produced when DCPT was incubated with HBSS only. Additional experiments are needed to further explore the contribution of the oxidative and hydrolytic pathways (Fig. 6, pathways A and B, respectively) to DCPT cytotoxicity in HepG2 cells.

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Highlights

- 3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione (DCPT) was cytotoxic in HepG2 cells.
- Cytotoxicity was enhanced in HepG2 cells transfected with CYP3A4.
- CYP3A4 inhibition and induction modulated toxicity in the transfectants.
- A potential DPCT hydrolysis product also reduced cell viability in both cell lines.
- CYP3A4-dependent and independent metabolites may contribute to DCPT cytotoxicity.

DCPT

DCTA

DPI

DPMT

2-DCTD

4-DCTD

Figure 1.
Structures of DCPT and analogues.

Figure 2.

Effect of DCPT on the viability of HepG2-CYP3A4 transfected and HepG2 wild type cells. Values are means $\pm$ SE (N = 4). Asterisks (*) indicate values that are significantly different ($p < 0.05$) from the 0 μ M DCPT control value. Daggers (†) indicate values that are significantly different ($p < 0.05$) between the two cell lines.

Figure 3. Effect of DCTA on the viability of HepG2-CYP3A4 transfected and HepG2 wild type cells. Values are means $\pm$ SE (N = 4). Asterisks (*) indicate values that are significantly different ($p < 0.05$) from the 0 μ M DCTA control value. Daggers (†) indicate values that are significantly different ($p < 0.05$) between the two cell lines.

Figure 4. Effect of DPI on the viability of HepG2-CYP3A4 transfected and HepG2 wild type cells. Values are means $\pm$ SE (N = 4). Asterisks (*) indicate values that are significantly different ($p < 0.05$) from the 0 μM DPI control value.

Figure 5. Hydrolysis of DCPT (250 μM) in HBSS over time. Values are means ± SE (N = 4). Asterisks (*) indicate values that are significantly different (p < 0.05) from the 0 hour time point.

Figure 6.
Proposed DCPT biotransformation pathways.

Table 1

Effect of CYP inhibition on 6 β -hydroxytestosterone in HepG2-CYP3A4 transfected cells. Cells were incubated with 250 μ M testosterone for 1 hr in the absence or presence of ketoconazole (KCZ). Values are means $\pm$ SE (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the testosterone treatment alone. N.D. = Not Detected.

Treatment	6 β -Hydroxytestosterone Formation (pmol/min/mg protein)
Testosterone	51.2 $\pm$ 8.4
1 μ M KCZ Co-incubation w/ Testosterone	14.0 $\pm$ 1.3*
10 μ M KCZ Co-incubation w/ Testosterone	N.D.

Table 2

Effect of CYP inducers on 6 β -hydroxytestosterone in HepG2-CYP3A4 transfected cells. Cells were incubated with 250 μ M testosterone for 1 hr following pretreatment with 0.1% DMSO, dexamethasone (DEX) or rifampicin (RIF). Values are means $\pm$ SE (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the testosterone treatment alone.

Treatment	6 β -Hydroxytestosterone Formation (pmol/min/mg protein)
Testosterone	79.5 $\pm$ 5.7
0.1% DMSO Pretreatment	110.5 $\pm$ 2.9*
100 μ M DEX Pretreatment	233.2 $\pm$ 11.3*
100 μ M RIF Pretreatment	83.2 $\pm$ 5.1

Table 3

LC₅₀ values for DCPT and analogues in HepG2-CYP3A4 transfected and HepG2 wild type cells. The maximum concentration tested for each compound was 250 μ M. Values were determined via linear or polynomial regression and are means $\pm$ SE (N = 4). An asterisk (*) indicates that the value is significantly different ($p < 0.05$) from the LC₅₀ value in the HepG2-CYP3A4 transfectant cells.

Compound	HepG2-CYP3A4 Transfectant LC ₅₀ (μ M)	HepG2 Wild Type LC ₅₀ (μ M)
DCPT	160.2 $\pm$ 5.9	233.0 $\pm$ 19.7*
DCTA	67.3 $\pm$ 8.1	65.3 $\pm$ 3.9
DPI	> 250	> 250
DPMT	> 250	> 250
2-DCTD	> 250	259.6 $\pm$ 12.2
4-DCTD	> 250	> 250

Table 4

Effect of DCPT (250 μ M) on the viability of HepG2-CYP3A4 transfected cells in the presence of 2 μ M ketoconazole (KCZ). Values are means $\pm$ SE (N = 4). Asterisks (*) indicate values that are significantly different ($p < 0.05$) from incubations conducted in the absence of inhibitor and DCPT. Pound signs (#) indicate values that are significantly different ($p < 0.05$) from DCPT treatment alone.

Inhibitor	DCPT	% Cell Viability
-	-	100.0 $\pm$ 5.8
2 μ M KCZ	-	105.7 $\pm$ 4.5
-	250 μ M DCPT	21.3 $\pm$ 3.2*
2 μ M KCZ	250 μ M DCPT	46.6 $\pm$ 1.5*#

Table 5

Effect of DCPT (100 μ M) on the viability of HepG2-CYP3A4 transfected cells following pretreatment with 100 μ M dexamethasone (DEX). Values are means $\pm$ SE (N = 4). Asterisks (*) indicate values that are significantly different ($p < 0.05$) from incubations conducted in the absence of DEX pretreatment and DCPT. Pound signs (#) indicate values that are significantly different ($p < 0.05$) from DCPT treatment alone.

Pre-treatment	DCPT	% Cell Viability
-	-	100.0 $\pm$ 0.8
100 μ M DEX	-	114.9 $\pm$ 5.1
-	100 μ M DCPT	82.6 $\pm$ 2.8*
100 μ M DEX	100 μ M DCPT	73.0 $\pm$ 1.0*#