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Cytotoxicity of Thiazolidinedione-, Oxazolidinedione- and Pyrrolidinedione-Ring Containing Compounds in HepG2 Cells

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Abstract

Liver damage occurred in some patients who took troglitazone (TGZ) for type II diabetes. The 2,4-thiazolidinedione (TZD) ring in TGZ's structure has been implicated in its hepatotoxicity. To further examine the potential role of a TZD ring in toxicity we used HepG2 cells to evaluate two series of compounds containing different cyclic imides. N-phenyl analogues comprised 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT); 3-(3,5-dichlorophenyl)-2,4-oxazolidinedione (DCPO) and N-(3,5-dichlorophenyl)succinimide (NDPS). Benzylic compounds, which closely resemble TGZ, included 5-(3,5-dichlorophenylmethyl)-2,4-thiazolidinedione (DCPMT); 5-(4-methoxyphenylmethyl)-2,4-thiazolidinedione (MPMT); 5-(4-methoxyphenylmethylene)-2,4-thiazolidinedione (MPMT-I); 5-(4-methoxyphenylmethyl)-2,4-oxazolidinedione (MPMO); 3-(4-methoxyphenylmethyl)succinimide (MPMS) and 3-(4-methoxyphenylmethylene)succinimide (MPMS-I). Cytotoxicity was assessed using the MTS assay after incubating the compounds (0-250 μ M) with HepG2 cells for 24 h. Only certain TZD derivatives (TGZ, DCPT, DCPMT and MPMT-I) markedly decreased cell viability, whereas MPMT had low toxicity. In contrast, analogues without a TZD ring (DCPO, NDPS, MPMO, MPMS and MPMS-I) were not cytotoxic. These findings suggest that a TZD ring may be an important determinant of toxicity, although different structural features, chemical stability, cellular uptake or metabolism, etc., may also be involved. A simple clustering approach, using chemical fingerprints, assigns each compound to one of three classes (each containing one active compound and close homologues), and provides a framework for rationalizing the activity in terms of structure.

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Keywords

Clustering; Cytotoxicity; HepG2 cells; Molecular fingerprints; Oxazolidinedione; Pyrrolidinedione; Succinimide; Thiazolidinedione; Troglitazone

1. Introduction

Troglitazone (TGZ, Fig. 1) was the first drug of its class to be marketed in the United States for the treatment of type 2 diabetes (Owens, 2002). It exerted therapeutic activity via a unique mechanism of action as a ligand for the peroxisome proliferator-activated receptor γ (PPAR- γ , Owens, 2002). However, TGZ was removed from the US market within three years after it was associated with severe liver damage in some patients (Scheen, 2001; Chojkier, 2005). The hepatotoxicity of this drug is believed to be independent of its binding to PPAR- γ (Lebovitz, 2002). Rosiglitazone (RGZ) and pioglitazone (PGZ) were marketed for the same therapeutic indication (Fig. 1). There have been several reports of mild hepatic injury in some patients who used these drugs (Scheen, 2001). However, these two glitazones are given at lower doses than TGZ which could be a factor in their reduced toxicity (Goldstein, 2002). Another member of this family, ciglitazone (Fig. 1), was discontinued during clinical development due to hepatic injury (Day, 1999). Other adverse effects have also been associated with this drug class, and pioglitazone is the only one still commonly used (Kung and Henry, 2012; Consoli and Formoso, 2013). TZD rings are also found in drugs being evaluated as potential aldose reductase inhibitors (Bruno et al., 2002; Rakowitz et al., 2006), COX2 inhibitors (Ali et al., 2007), antimicrobial drugs (Avupati et al., 2012) and neuroprotective agents (Oppermann et al., 2014), and thus there is the potential for continued exposure of patients to this structural moiety.

The mechanism by which TGZ exerts liver damage is not well understood (Smith, 2003). Furthermore, there is conflicting evidence in the literature as to whether TGZ itself or a metabolite causes liver damage (Masubuchi, 2006). Glutathione (GSH) trapping experiments and quantum chemical modeling studies have provided evidence that reactive intermediates can be generated from an initial cytochrome P450 (CYP)-mediated sulfoxidation in the thiazolidinedione (TZD) ring of TGZ (Alvarez-Sanchez et al., 2006; He et al., 2004; Kassahun et al., 2001; Taxak et al., 2012; Tetey et al., 2001).

3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione (DCPT, Fig. 2) is another TZD ring-containing compound. Unlike TGZ, DCPT is hepatotoxic in rats (Kennedy et al., 2003; Patel et al., 2008). Liver damage was time- and dose-dependent, and male rats were more susceptible than females (Patel et al., 2008). Furthermore, we also found that the CYP3A isozymes have a role in converting DCPT to a hepatotoxic metabolite in rats (Crincoli et al., 2008). In vivo structure-activity relationship (SAR) studies with DCPT analogues (Kennedy et al., 2003; Patel et al., 2012) suggested that the TZD ring is an important structural feature in hepatotoxicity.

We have also evaluated the effect of DCPT on cell viability in wild-type and CYP3A4 stably transfected human hepatoma HepG2 cells (Frederick et al., 2011). DCPT was more cytotoxic in the CYP3A4 transfectants than wild-type cells (LC_{50} values, $160.2 \pm 5.9 \mu M$ vs.

233.0 $\pm$ 19.7 μ M, respectively). The wild-type HepG2 cell line contains only low levels of CYP activity (Rodríguez-Antona et al., 2002; Donato et al., 2008). Therefore, these results suggest that DCPT itself and CYP3A4-derived metabolite(s) can contribute to a loss of cell viability in the transfected cells, whereas only the parent compound is exerting toxicity in the wild-type cell line. Similar results have been reported for TGZ (Vignati et al., 2005).

Although DCPT is a useful model compound for exploring toxicity of TZD ring-containing compounds, it has several distinct structural differences from TGZ (cf. Figs. 1 and 2). On the TZD ring of DCPT, a 3,5-dichlorophenyl group is attached to the imide nitrogen, and the 5-methylene position (adjacent to the sulfur atom) is unsubstituted. In contrast, on the TZD ring of TGZ, the imide nitrogen is unsubstituted and a bulky benzylic group is attached to the 5-position. These structural differences could alter the stability, susceptibility to metabolism, etc., of the TZD rings in DCPT and TGZ.

The experiments described here were designed to further explore the SAR in DCPT and TGZ analogues. We postulated that a TZD ring is an important factor in cytotoxicity. To address this hypothesis we evaluated two series of compounds: (1) N-phenyl analogues of DCPT, in which the TZD ring was replaced with other 5-membered cyclic imides and (2) benzylic compounds that more closely mimic the structure of TGZ. In addition to DCPT, the N-phenyl series (Fig. 2) consisted of 3-(3,5-dichlorophenyl)-2,4-oxazolidinedione (DCPO) and N-(3,5-dichlorophenyl)succinimide (NDPS, a pyrrolidinedione derivative). The benzylic compounds (Fig. 2) included 5-(4-methoxyphenylmethyl)-2,4-thiazolidinedione (MPMT); 5-(4-methoxyphenylmethyl)-2,4-oxazolidinedione (MPMO); 3-(4-methoxyphenylmethyl)succinimide (MPMS) and 5-(3,5-dichlorophenylmethyl)-2,4-thiazolidinedione (DCPMT). These compounds were chosen to evaluate the effect of sulfur (DCPT, MPMT, DCPMT), oxygen (DCPO, MPMO) and carbon (NDPS, MPMS) on cytotoxicity in both series. MPMT, MPMO and MPMS are “truncated” derivatives of TGZ (cf. Figs. 1 and 2). DCPMT contains features of both series, i.e., a benzylic backbone with the 3,5-dichlorophenyl substitution pattern that is common to the N-phenyl compounds (Fig. 2). Finally, we also evaluated the cytotoxicity of two unsaturated compounds, 5-(4-methoxyphenylmethylene)-2,4-thiazolidinedione (MPMT-I) and 3-(4-methoxyphenylmethylene)succinimide (MPMS-I), which are synthetic intermediates in the preparation of MPMT and MPMS, respectively. All compounds were incubated (0-250 μ M) with wild-type HepG2 cells and cytotoxicity was evaluated after 24 h exposure. TGZ was included as a positive control. With the exception of MPMT, the TZD ring-containing compounds were highly cytotoxic. In contrast, none of the oxazolidinedione or pyrrolidinedione derivatives reduced HepG2 cell viability. Our results suggest that the presence of a TZD ring may be important for cytotoxicity; other factors that may contribute will be discussed.

2. Materials and Methods

2.1. Materials

All chemicals were of the highest purity commercially available. Lithium borohydride (LiBH_4), 3,5-dichlorophenyl isocyanate and 2,4-thiazolidinedione were obtained from Alfa Aesar (Ward Hill, MA, USA). Ethyl 2-mercaptoacetate, 4-methoxybenzaldehyde, 3,5-

dichlorobenzaldehyde, piperidine, pyridine, maleimide, toluene, triethylamine, ethyl acetate, chloroform, hexane, tetrahydrofuran, triphenylphosphine, DMSO, palladium on charcoal and acetone were all obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Hydrogen Zero Grade was purchased from Airgas (Essington, PA, USA).

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit ("MTS assay", kit no. G5421) is a product of Promega Corp. (Madison, WI, USA). Human hepatoma HepG2 cells (cat. no. HB-8065) were obtained from American Type Culture Collection (Manassas, VA, USA). Trypsin-EDTA (cat. no. 25-053-CI), penicillin-streptomycin (cat. no. 30-001-CI) and HBSS 1× (cat. no. 21-023-CM, with calcium and magnesium, without phenol red) were products of Mediatech, Inc. (Manassas, VA, USA). DMEM 1× (cat. no. 11995-040) was purchased from Life Technologies, Inc. (Carlsbad, CA, USA).

2.2. Test Compounds and Syntheses

DCPT was prepared as described by Kennedy et al. (2003). The other N-phenyl analogues, DCPO and NDPS, were available from previous work in our laboratory (Kennedy et al., 2003). TZD ring-containing benzylic compounds were synthesized by Knoevenagel condensation of 2,4-thiazolidinedione and the appropriate, substituted benzaldehyde to yield the unsaturated 5-phenylmethylene derivatives (e.g., MPMT-I). These intermediates were then reduced with lithium borohydride to the saturated 5-phenylmethyl compounds (e.g., MPMT). MPMS-I was synthesized by condensing the triphenylphosphine derivative of succinimide with 4-methoxybenzaldehyde, followed by catalytic hydrogenation to yield MPMS. MPMO was obtained from Chemgenx, LLC (New Brunswick, NJ, USA); m.p. 108-110 °C (m.p. 115 °C, provided by vendor). TGZ was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

Melting points were determined on a Mel-Temp capillary melting point apparatus and are uncorrected. Reactions were monitored by TLC on Silica Gel GF plates (Analtech, Inc., Newark, DE, USA) using ethyl acetate or 9:1 ethyl acetate/chloroform as mobile phase with visualization under short wave UV light. ¹H-NMR spectra were recorded at 400 MHz on Bruker AVANCE II or AVANCE III spectrometers. Chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane. The abbreviations used in reporting the ¹H-NMR data are: s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), t (triplet), m (multiplet). Elemental analysis for the novel compound DCPMT was performed by Galbraith Laboratories, Inc. (Knoxville, TN, USA).

2.2.1. 5-(3,5-Dichlorophenylmethylene)-2,4-thiazolidinedione (DCPMT)—

DCPMT was synthesized in two steps by the method of Rakowitz et al. (2006). Thus, 2,4-thiazolidinedione (1.7 g, 14.5 mmol) was added to a mixture of ethanol (80 mL) and piperidine (0.98 g, 11.5 mmol). Once dissolved, this was added dropwise to a solution of 3,5-dichlorobenzaldehyde (2.5 g, 14.5 mmol) and ethanol (22 mL). The reaction mixture was refluxed for 24 h, then added to an equal volume of distilled water and acidified with acetic acid (pH ≈ 4). The precipitate was collected by vacuum filtration and dried in a vacuum oven at 55 °C. Once dry, the crude product was recrystallized from methanol; m.p. 221-223 °C. ¹H NMR (DMSO-d₆) δ: 7.63 (d, 2H, ArH), 7.75 (t, 1H, ArH), 7.78 (s, 1H, CH), and 12.8 (s, 1H, NH).

The intermediate 5-(3,5-dichlorophenylmethylene)-2,4-thiazolidinedione (1.63 g, 6.0 mmol) was dissolved in a mixture of pyridine (12 mL) and anhydrous THF (5 mL). After the addition of LiBH_4 (2.5 equiv.), the mixture was refluxed for 3 h, allowed to cool, and then slowly added to an ice-cold solution of hydrochloric acid (12 N, 3.3 mL) and distilled water (20 mL). The aqueous solution was extracted with ethyl acetate. Organic fractions were collected and washed with distilled water, then dried with anhydrous magnesium sulfate. The solution was concentrated under vacuum in a rotary evaporator and dried in a vacuum oven at 55°C. Purification of the crude product was performed via silica gel column chromatography with ethyl acetate as the eluent then subsequently recrystallized using a mixed solvent system of absolute ethanol-water; m.p. 125-126 °C. ^1H NMR (DMSO-d_6) δ : 3.19 (dd, 1H, CH_2), 3.40 (dd, 1H, CH_2), 4.99 (dd, 1H, CH), 7.35 (d, 2H, ArH), 7.50 (t, 1H, ArH), and 12.80 (s, 1H, NH). Anal. calc'd. for $\text{C}_9\text{H}_7\text{Cl}_2\text{NO}_2\text{S}$ (276.15): C, 43.50; H, 2.56; N, 5.07; O, 11.58; S, 11.61; Cl, 25.68. Found: C, 43.77; H, 2.62; N, 5.07; S, 11.83; Cl, 25.71.

2.2.2. 5-(4-methoxyphenylmethylene)-2,4-thiazolidinedione (MPMT-I)—MPMT-I was synthesized using a method similar to that described in Bruno et al. (2002). Ethanol (22 mL) and 4-methoxybenzaldehyde (1.75 mL, 14.5 mmol) were combined in a round bottom flask. A solution of 2,4-thiazolidinedione (1.7 g, 14.5 mmol) and piperidine (1.14 mL) in ethanol (80 mL) was added dropwise and the reaction mixture was refluxed for 24 h. After cooling to room temperature the reaction mixture was poured into distilled water (100 mL) and was acidified to pH 4 with glacial acetic acid, resulting in a fluffy yellow precipitate. The precipitate was vacuum-filtered and dried in a vacuum oven at 55 °C for 24 h. Recrystallization was performed using ethyl acetate and hexane; m.p. 218-219 °C (lit. m.p. 216-218 °C, Bruno et al. (2002)). ^1H -NMR (DMSO-d_6): δ 3.8 (s, 3H, CH_3), 7.1 (d, 2H, Ar-H), 7.5 (d, 2H, Ar-H), 7.75 (s, 1H, CH), 12.5 (s, 1H, NH).

2.2.3. 5-(4-methoxyphenylmethyl)-2,4-thiazolidinedione (MPMT)—MPMT was synthesized using a method similar to those described by Giles et al. (2000) and Rakowitz et al. (2006). MPMT-I (2 g, 8.5 mmol) was dissolved in pyridine (16 mL) and THF (5.88 mL). LiBH_4 (0.8 g) was added slowly until bubbling ceased. The solution was flushed with nitrogen gas and was refluxed for 3 h, resulting in a dark brown color. The flask was allowed to cool to room temperature and 10% HCl (20 mL) was added dropwise to destroy any remaining LiBH_4 . The reaction mixture was vacuum-filtered, and the filtrate was extracted with 3×10 mL portions of ethyl acetate. The organic fraction was successively washed with 10 mL water, 10 mL 9% saline, 10 mL water, then dried with anhydrous magnesium sulfate. The ethyl acetate was evaporated under vacuum, and the crude product was placed in a freezer for 48 h. The resulting precipitate was recrystallized using ethyl acetate and hexane; m.p. 108-110 °C (lit. m.p. 115 °C, Giles et al. (2000); 110 °C, Rakowitz et al. (2006)). ^1H -NMR (DMSO-d_6): δ 3.1 (m, 1H, CH), 3.3 (dt, 1H, CH), 3.75 (s, 3H, CH_3), 4.8 (m, 1H, CH), 6.8 (d, 2H, Ar- H_2), 7.2 (d, 2H, Ar- H_3), 12.0 (s, 1H, NH).

2.2.4. 3-(4-Methoxyphenylmethylene)succinimide (MPMS-I)—MPMS-I was synthesized in two steps using methods similar to those described in Saha et al. (2010). First, triphenylphosphine (2.70 g, 10.3 mmol), maleimide (1.0 g, 10.3 mmol), and acetone (30

mL) were heated to reflux for 1 h. After cooling, the precipitate was filtered and washed with cold acetone (100 mL). The precipitate was dried under vacuum overnight to give 3-(triphenylphosphoranylidene)pyrrolidine-2,5-dione as a colorless solid; m.p. 221-223 °C (lit. m.p. 227 °C, Saha et al. (2010)). In the second step, 3-(triphenylphosphoranylidene)pyrrolidine-2,5-dione (5.15 g, 14.5 mmol), 4-methoxybenzaldehyde (1.75 mL, 14.5 mmol) and methanol (280 mL) were refluxed for 1.5 h. The reaction mixture was cooled to room temperature, gravity filtered and dried under vacuum for 4 h; m.p. 238-239 °C. ¹H-NMR (DMSO-d₆): δ 3.81 (s, 3H, CH₃), 7.03 (d, 2H, Ar-H), 7.34 (t, 1H, CH), 7.58 (d, 2H, Ar-H), 11.35 (s, 1H, NH).

2.2.5. 3-(4-methoxyphenylmethyl)succinimide (MPMS)—MPMS was synthesized using a method similar to those described in Christoffers et al. (2004). MPMS-I (0.5 g, 2.3 mmol) was dissolved in EtOH (10 mL) in a Schlenk tube, degassed and placed under an argon atmosphere. Pd/C (5% Pd, 0.020 g) was added to the tube, and it was placed under 1 atm of H₂, sealed, and heated to 60 °C for 4 h. The solution was cooled and filtered through celite. The ethanol was evaporated under vacuum to isolate the product which required no further purification; m.p. 108-110 °C (lit. m.p. 109-111 °C, Goehring et al. (1991)). ¹H-NMR (CDCl₃): δ 2.53 (dt, 1H, TZD CH), 2.99-2.86 (m, 1H, TZD CH), 3.17 (tdd, 2H, benzylic CH₂), 3.81 (s, 3H, CH₃), 6.90-6.79 (m, 2H, Ar-H), 7.16-7.05 (m, 2H, Ar-H), 7.95 (s, 1H, NH).

2.3. Cell Culture and Cytotoxicity Determination

2.3.1. Cells—HepG2 cells were cultured in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS) in T-75 flasks (Corning 430641, Corning, Inc., Corning, NY, USA). The cells were incubated at 37 °C under a humidified atmosphere of 5% CO₂-95% air. Prior to treatment with any compounds the cells were trypsinized and plated as described below.

2.3.2. Incubations—Replicative, independent experiments consisted of at least four individual cell culture plates with freshly prepared reagents. HepG2 cells were seeded at 20,000 cells/well (minimum 4 wells per treatment per plate) in 96-well stripwell plates (Costar 9102, Corning, Inc., Corning, NY, USA) in DMEM and were allowed to grow for 24 h at 37°C under 5% CO₂-95% air. The media was removed, and the cells were then treated with 0-250 μM of each compound for 24 h in HBSS (containing up to 0.1% DMSO). Controls were incubated with HBSS (0.1% DMSO) only. The concentration range was chosen based on our prior studies with DCPT (Frederick et al., 2011). Due to very limited solubility of some of the compounds in HBSS, concentrations higher than 250 μM were generally not used.

2.3.3. MTS Assay—After the respective treatments, media containing the compounds was removed, the cells were washed with HBSS, and then 100 μL of MTS reagent was added to the wells. Viability was assessed using the CellTiter 96® AQueous non-radioactive cell proliferation assay according to the manufacturer's instructions. Absorbance at 492 nm was measured on an HTS 7000 Plus (PerkinElmer, Inc., Waltham, MA, USA) or a Victor 3 Model 1420 Multilabel Counter (PerkinElmer, Inc., Wellesley, MA, USA) plate reader.

2.3.4 Determination of Median Lethal Concentrations—LC₅₀ values for the toxic compounds were calculated in Excel 2010 (Microsoft, Redmond, WA, USA) from the best fit curves determined using regression analyses. The LC₅₀ values for the non-toxic compounds were not calculated since they would be greater than our maximum working concentration of 250 μ M, above which solubility is a limiting factor.

2.4. Stability of DCPMT

The stability of DCPMT in HBSS at 37 °C under a humidified atmosphere of 5% CO₂-95% air was measured as previously described for DCPT (Frederick et al., 2011). Samples were analyzed by reversed phase HPLC at 24, 48 and 72 h. Using the published assay method, DCPMT had a retention time of 8.3 min.

2.5. Partition Coefficients

To estimate relative lipophilicities for the compounds, partition coefficients (expressed here as log P values) were calculated using ChemBioDraw Ultra 14.0 (PerkinElmer, Inc., Waltham, MA, USA).

2.6. Statistics

Results are expressed as means $\pm$ standard errors (SE). SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA, USA) was used for graphing and statistical analyses. The data were analyzed by a one-way analysis of variance (ANOVA) followed by a Student Newman-Keuls post hoc test. Differences in the means were considered significant when $p < 0.05$.

2.7. Molecular Fingerprints and Clustering

Molecular fingerprints (Xue and Bajorath, 2000; Duan et al., 2010) were computed for all the compounds discussed here using the Canvas application (Canvas, version 2.0, Schrödinger, LLC, New York, NY, 2014) of the Schrodinger Suite (www.schrodinger.com). Specifically, radial extended connectivity fingerprints (ECFPs) were generated. In the ECFP approach, each heavy atom is used in turn as an initiation site for fragment (substructure) growth, with fragments iteratively extended from the initial atom by following consecutive bonds, thus creating chemical ‘neighborhoods’ of increasing size. The unique chemical substructures so constructed are retained, and the atom types and bond orders for each fragment are mapped to a unique integer using a hashing function; in turn, this integer is used as an index into a binary bit string (with all bit positions initially ‘0’), and the bit at the selected position is set to ‘1’. The final bit string is thus the logical OR of all the fragment contributions, and in this way each compound is mapped to a binary string (32 bits long using default Canvas settings), which serves as a compact one-dimensional descriptor of the chemical structure.

All pair-wise distances between molecules were computed from their fingerprints, using a metric derived from the Tanimoto coefficient for the corresponding binary strings. The resulting distance matrix was used to generate a hierarchical clustering of the compounds, and by application of an appropriate distance threshold the hierarchical grouping gave rise to a set of defined clusters with specific compound membership.

3. Results

3.1. Cytotoxicity of the N-phenyl compounds

Results of the cytotoxicity assays for DCPT, DCPO and NDPS are shown in Fig. 3. Consistent with our previous findings (Frederick et al., 2011), 24 h treatment with DCPT resulted in HepG2 cell death (Fig. 3A). Compared to control incubations (0 μ M DCPT) the loss of viability was approximately 55% at the highest DCPT concentration used in these experiments (250 μ M). At lower concentrations there were no statistically significant changes in cell viability. By contrast, no cytotoxicity was observed when the oxazolidinedione (DCPO, Fig. 3B) or pyrrolidinedione (succinimide) ring (NDPS, Fig. 3C) containing analogues were incubated with HepG2 cells for 24 h. Even at the highest concentrations used (250 μ M), the loss of cell viability did not exceed 10% for either of these compounds (Figs. 3B and 3C).

3.2. Cytotoxicity of the benzylic compounds

Troglitazone (TGZ) was included in these experiments as a positive control for cytotoxicity. When HepG2 cells were treated with TGZ for 24 h, there was a statistically significant decline in viability (approximately 40% loss) starting at a concentration of 17.5 μ M that reached ca. 80% at 250 μ M TGZ (Fig. 4).

DCPMT combines structural features of both DCPT (i.e., the 3,5-dichlorophenyl ring) and TGZ (i.e., a benzylic substituent on the 5-position of the TZD ring, cf. Figs. 1 and 2). Following 24 h incubation with DCPMT, cell viability in the MTS assay exhibited a sharp decline starting at 12.5 μ M (ca. 55% loss) and plateaued at approximately 80% loss (similar to TGZ) with increasing concentrations (Fig. 5).

Results for the 4-methoxy substituted benzylic compounds (which are truncated analogues of TGZ, cf. Figs. 1 and 2) are summarized in Fig. 6. Following 24 h treatment, the TZD derivative MPMT exerted a small (ca. 17%), but statistically significant, decrease on HepG2 cell viability at 250 μ M (Fig. 6A). The limited solubility of MPMT in HBSS prevented us from determining if cytotoxicity would increase at higher concentrations. As shown in Fig. 6A, MPMT-I, the unsaturated, synthetic precursor to MPMT, was highly cytotoxic to HepG2 cells following 24 h exposure. The decrease in cell viability became statistically significant at 50 μ M (ca. 65% loss) and continued to decline up to 250 μ M (approximately 95% loss). With the oxazolidinedione analogue, MPMO, there was a ca. 17% loss of cell viability at 250 μ M (similar to MPMT); although this was not significantly different from the control (Fig. 6B). Due to its greater solubility in HBSS than MPMT, further cytotoxicity experiments were conducted with MPMO. However, even at higher concentrations (300-500 μ M), no significant loss of cell viability was observed with this compound (results not shown). In contrast to the TZD ring analogues, incubation of HepG2 cells with the pyrrolidinedione ring derivatives (MPMS and MPMS-I) resulted in no more than a 7% loss in viability in 24 h over the entire concentration range examined (Fig. 6C).

3.3. Median lethal concentrations

All TZD analogues, except for MPMT, had LC_{50} values less than 250 μM (Table 1). Based on its LC_{50} , DCPT ranked as the least toxic TZD derivative. In contrast, DCPMT and TGZ were the most highly cytotoxic analogues and had comparable LC_{50} values. MPMT-I had an LC_{50} approximately 3-fold higher than DCPMT and TGZ; and therefore ranked between these two compounds and DCPT in terms of toxicity.

3.4. Estimated lipophilicities

Log P values were calculated (Table 1) to determine if lipophilicity had any correlation with cytotoxicity of the compounds. TGZ was the most lipophilic of all of the compounds, followed by DCPMT. The N-phenyl series had intermediate log P values (1.98-2.73), and the TZD derivative DCPT was the most lipophilic compound of this group. Values for the 4-methoxy substituted benzylic derivatives (0.77-1.76) suggested that these compounds were less lipophilic than the N-phenyl analogues. The TZD ring-containing compounds (MPMT and MPMT-I) again had the highest log Ps among this series. Unsaturated compounds (MPMT-I and MPMS-I) were less lipophilic than their saturated analogues (MPMT and MPMS, respectively).

3.5. Clustering/SAR

The hierarchical clustering generated using the ECFP descriptors is shown as a dendrogram in Fig. 8, along with defined cluster assignments found by ‘cutting’ the hierarchical grouping at a distance threshold of 0.92 (the threshold was generated automatically by the Canvas application to realize the selected number of three clusters). The most toxic compounds (TGZ, DCPMT) are assigned to the same cluster (#1) on the basis of structural similarity, along with some close homologues which are inactive. Likewise, one active compound is assigned to each of clusters #2 and #3 (MPMT-I and DCPT respectively), again in the company of inactive homologues. In each case, the active compound is characterized by the specific TZD ring feature (which is found in compounds in all three clusters), although the toxicity for actives within each group is modulated by their structural class, with cluster #1 > #2 > #3. Thus, the fingerprint-based clustering provides a straightforward but useful approach to constructing a structure-activity relationship (SAR), and can be readily extended to include additional compounds, by expanding the membership of existing clusters, and/or increasing the number of clusters to accommodate more structural classes.

3.6. Stability of DCPMT

Our previous work (Frederick et al., 2011) demonstrated that DCPT is a labile compound and that the TZD ring is prone to hydrolysis even in HBSS. Degradation of DCPMT (initial concentration 250 μM) in HBSS over time was therefore measured using reversed phase HPLC. There was no significant change from the initial percentage (at time zero) of DCPMT at 24 and 48 h (Fig. 7). Although there was a small decrease (approximately 16% loss) in DCPMT at 72 h, it was not statistically significant. These results suggest that DCPMT is very stable under the incubation conditions used in these studies.

4. Discussion

The TZD ring may be an important structural feature in hepatotoxicity of the glitazones (Alvarez-Sanchez et al., 2006; He et al., 2004; Kassahun et al., 2001; Taxak et al., 2012; Tetley et al., 2001) and DCPT (Kennedy et al., 2003; Patel et al., 2012). In addition, structural modifications to DCPT, where the TZD ring had a 5-methyl substituent or was replaced with isomeric 2- or 4-thiazolidinone rings, reduced or eliminated cytotoxicity to HepG2 cells (Frederick et al., 2011). Herein we investigated compounds with bioisosteric replacements for the TZD ring sulfur atom (Meanwell, 2011)- either an oxygen atom (oxazolidinedione ring) or a methylene group (pyrrolidinedione ring). We evaluated two different series (Fig. 2): (1) N-phenyl compounds, which included analogues of DCPT and (2) benzylic compounds, which are truncated derivatives of TGZ. HepG2 cells were used for comparison to our previous work.

The LC₅₀ for DCPT was ca. 240 μ M (Table 1), similar to our previous studies (233 ± 19.7 μ M, Frederick et al. 2011). Since this cell line expresses low levels of CYP activity (Rodríguez-Antona et al., 2002; Donato et al., 2008), metabolic activation via TZD ring sulfoxidation should be minimal. This suggests that an oxidative DCPT metabolite does not contribute substantially to the loss of viability in wild-type HepG2 cells. The lack of effect seen with DCPO and NDPS supports our hypothesis that the TZD ring has a role in toxicity. Since all three compounds have the 3,5-dichlorophenyl moiety attached to the imide nitrogen, cytotoxicity is less likely to be dependent on this structural feature. The relative lipophilicities of compounds can be compared by calculating their octanol-water partition coefficients and are commonly expressed as log P values (Giagnis and Tsantili-Kakoulidou, 2008). Based on the calculated log P values (DCPT > DCPO > NDPS), DCPT is the most lipophilic compound in this series. Thus, it is conceivable that the greater cytotoxicity of DCPT could be due to higher cellular permeability and intracellular accumulation compared to the other N-phenyl compounds. However, as described below, the chemical lability of the TZD ring in DCPT may also be a factor in its cytotoxicity.

We observed a similar trend in the benzylic series, where only TZD derivatives produced a significant loss of cell viability. TGZ was used as a positive control based on its previously reported cytotoxicity in HepG2 cells (Tirmenstein et al., 2002; Vignati et al., 2005). Our calculated LC₅₀ of ca. 20 μ M for TGZ compares favorably with the literature values of 34 μ M (Yamamoto et al., 2001) and 55 μ M (Guo et al., 2006). A TZD ring is common to all of the glitazones (Fig. 1). However, compared to TGZ, RGZ and PGZ either exhibited reduced cytotoxicity (Guo, et al., 2006) or had no effect on HepG2 cell viability (Yamamoto et al., 2001). The difference between compounds was attributed to the chromane ring in TGZ, which is absent in both RGZ and PGZ (Yamamoto et al., 2001). However, ciglitazone, which lacks a chromane ring, had a similar LC₅₀ to TGZ in HepG2 cells (Guo et al., 2006). Thus, TZD rings may contribute to the toxicity of some, but not all, glitazones. Based on their LC₅₀ values, TGZ, DCPMT and MPMT-I were the most cytotoxic among the compounds we evaluated, although MPMT-I had a markedly lower log P value than the other two compounds.

Since we used HepG2 cells, CYP-mediated biotransformation should be minimal (Rodríguez-Antona et al., 2002; Donato et al., 2008). Therefore, these results suggest that the parent compounds or non CYP-derived metabolites are responsible for the cytotoxicity. As in the N-phenyl series, the benzylic oxazolidinedione (MPMO) and pyrrolidinedione (MPMS and MPMS-I) derivatives did not reduce cell viability. A similar bioisosteric replacement approach was used by Saha et al. (2010, 2012) in an effort to improve the safety profile of the glitazones. This group synthesized and evaluated pyrrolidinedione (succinimide) ring analogues of TGZ, PGZ and RGZ. Trosuccinimide and piosuccinimide (but not rosisuccinimide) were less toxic than the corresponding glitazones in THLE-2 human hepatocytes, supporting a contribution to cytotoxicity from the TZD ring. Among our benzylic series, TZD derivatives were more lipophilic than their bioisosteric oxazolidinedione- and pyrrolidinedione-ring containing analogues, which could affect intracellular accumulation. However, in contrast to the potent cytotoxicity of its synthetic, unsaturated precursor MPMT-I, MPMT was only marginally toxic (95% vs. 17% loss of viability, respectively, over 24 h). Since MPMT has a slightly higher log P value than MPMT-I, lipophilicity does not correlate with toxicity for these two compounds.

A potentially important structural difference between MPMT and MPMT-I is the presence of an α,β -unsaturated ketone in the latter compound. This functional group may be electrophilic and can undergo Michael addition reactions between the β -alkene carbon atom and cellular nucleophiles, such as thiol groups (LoPachin et al., 2008; LoPachin and Gavin, 2014). Compounds containing α,β -unsaturated aldehydes or ketones have been shown to be cytotoxic or mutagenic (Chan et al., 2008; Janzowski et al., 2003). However, since MPMS-I (the pyrrolidinedione analogue of MPMT-I) is not cytotoxic, the presence of an α,β -unsaturated ketone by itself is not sufficient to confer cytotoxicity on these compounds. It is conceivable that the greater toxicity of MPMT-I is due to an additive or synergistic effect between the TZD ring and the α,β -unsaturated ketone.

The cytotoxicity of other truncated TGZ analogues, similar to the benzylic compounds described here, has also been evaluated. Haskins et al. (2001) reported that 5-(4-hydroxyphenylmethyl)-2,4-thiazolidinedione was not toxic in rat hepatocytes. In comparison to MPMT, this compound has a free phenolic hydroxy group in the aromatic ring. Thiazolidinedione phenoxyacetic acid, which contains an O-acetic acid moiety in place of the O-methyl group of MPMT, did not affect cell viability in rat N1S1 hepatoma cells (Narayanan et al., 2003). However, both of these compounds have polar aromatic substituents, which could impact cellular uptake.

One difference between the two series of compounds we investigated here that could impact cytotoxicity is the stability of the cyclic imide groups, particularly the TZD rings. DCPT is a chemically labile compound in HBSS (Frederick et al., 2011) and undergoes spontaneous hydrolysis to [[[3,5-dichlorophenyl)amino]carbonyl]thio]glycolic acid (DCTA). In fact, less than 50% of the original DCPT remained in the HBSS incubations after 24 h. DCTA can then further degrade into 3,5-dichlorophenyl isocyanate (DPI, unpublished results). A similar hydrolytic sequence in buffer was reported for the DCPT analogue 5-methyl-2,4-thiazolidinedione (Machálek et al., 1981). These findings are relevant since both DCTA and DPI are cytotoxic to HepG2 cells (Frederick et al., 2011). Thus, the loss of HepG2 cell

viability seen with DCPT may be due to the combined effects of DCPT, DCTA and DPI. In contrast, we found that DCPMT is a very stable compound and does not degrade in HBSS under our typical incubation conditions. Therefore, the cytotoxicity we observed with DCPMT is more likely due to the parent compound itself. The different substitution patterns in the DCPT and DCPMT TZD rings can account for the stabilities of these two compounds. In fact, cyclic imides (pyrrolidinedione derivatives) with a phenyl group attached to the imide nitrogen (comparable to DCPT) were much more prone to hydrolysis than their hydrogen atom substituted analogues (comparable to DCPMT) (Hargreaves et al., 1970). This was attributed to the conjugation of the imide nitrogen lone pair with the phenyl group, an effect that was enhanced by the presence of electron-withdrawing groups in the aromatic ring. Thus, the difference in stability of the TZD rings in DCPT and DCPMT can be explained by the effects of the 3,5-dichlorophenyl group that is directly attached to the imide nitrogen of DCPT, rendering this molecule more prone to hydrolysis.

Overall, the results support our contention that the presence of a TZD ring in these compounds can be an important determinant in their cytotoxicity to wild-type HepG2 cells. This effect was common to both series of compounds, suggesting that it may be due to some inherent property of the TZD ring, when compared to the oxazolidinedione and pyrrolidinedione rings. However, even among TZD derivatives, we observed differential effects on cell viability, ranging from minimally cytotoxic (e.g., MPMT) to highly cytotoxic (e.g., MPMT-I) in the same series of compounds. Furthermore, cell death may be due to the parent compound (e.g., DCPMT) or a combination of parent compound and hydrolysis products (e.g., DCPT), but is unlikely to involve CYP-derived metabolites. Although the TZD ring-containing analogues were the most lipophilic compounds in both series, the relative lipophilicities (expressed as log P values) were of limited utility for predicting cytotoxicity. In conclusion, in addition to a TZD ring, other factors, including other structural features, chemical stability, extent of cellular uptake, non CYP-mediated metabolism, different intracellular targets, etc., may also be important determinants of cytotoxicity.

While many factors may play a role in determining the toxicities of the compounds discussed here, the simple structure-based clustering approach developed provides an effective SAR that assigns each active compound to a rank-ordered category. We recognize that there is a level of subjectivity in assigning a similarity threshold to determine the number of clusters, but this can be guided by chemical intuition in recognizing the concomitant definition of a set of distinct and coherent structural classes. The approach is easily extended to include additional compounds and additional chemical classes.

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Highlights

- Cyclic imide cytotoxicity was evaluated in HepG2 cells
- Oxazolidinedione and pyrrolidinedione ring-containing analogues were non-toxic
- A thiazolidinedione (TZD) ring conferred cytotoxicity on several compounds
- Molecular fingerprints were used to assign the compounds into 3 clusters
- The TZD ring may be an important determinant of cytotoxicity in HepG2 cells

Figure 1. Structures of the glitazones. The dashed rectangle represents the “truncated” benzylic, saturated backbone of TGZ that is present in MPMT, MPMO and MPMS

N-Phenyl Compounds

DCPT

DCPO

NDPS

Benzylic Compounds

DCPMT

MPMT

MPMT-I

MPMO

MPMS

MPMS-I

Figure 2. Structures of the N-phenyl and benzylic compounds

Figure 3.

Effect of (A) DCPT, (B) DCPO and (C) NDPS on the viability of HepG2 cells as measured by the MTS assay (means $\pm$ SE; $n = 4$). The results are from replicative, independent experiments consisting of at least four individual cell culture plates with freshly prepared reagents. Asterisks indicate values that are significantly different ($p < 0.05$) from the 0 μ M control value for each compound.

Figure 4.

Effect of TGZ on the viability of HepG2 cells at 24 h as measured by the MTS assay (means $\pm$ SE; $n = 4$). The results are from replicative, independent experiments consisting of at least four individual cell culture plates with freshly prepared reagents. Asterisks indicate values that are significantly different ($p < 0.05$) from the 0 μ M TGZ control value.

Figure 5.

Effect of DCPMT on the viability of HepG2 cells at 24 h as measured by the MTS assay (means $\pm$ SE; n = 4). The results are from replicative, independent experiments consisting of at least four individual cell culture plates with freshly prepared reagents. Asterisks indicate values that are significantly different ($p < 0.05$) from the 0 μ M DCPMT control values.

Figure 6.

Effect of (A) MPMT and MPMT-I, (B) MPMO and (C) MPMS and MPMS-I on the viability of HepG2 cells at 24 h as measured by the MTS assay (means $\pm$ SE; $n = 4$). The results are from replicative, independent experiments consisting of at least four individual cell culture plates with freshly prepared reagents. Asterisks indicate values that are significantly different ($p < 0.05$) from the 0 μ M control value for each compound.

Figure 7.

Effect of time on DCPMT concentration in HBSS. The initial concentration was 250 μM . Loss of DCPMT was measured by reversed phase HPLC. Values are means $\pm$ SE ($n = 4$) from replicative, independent experiments. There were no significant differences at 24, 48 or 72 h when compared back to the 0 h time point.

Figure 8.

Dendrogram of hierarchical clustering generated using ECFP descriptors. The dashed line shows the approximate position of the cut threshold applied to generate three distinct clusters. The color-coded blocks at the bottom represent the ten compounds, labeled by cluster number, with left-to-right order corresponding to the top-to-bottom sequence of the structures as depicted for each cluster. Active compound(s) in each cluster are marked with an asterisk.

Table 1
Calculated LC₅₀ and log P values for the N-phenyl and benzylic cyclic imide ring-containing compounds

Compound	Abbreviation	LC ₅₀ (μM) ^a	log P ^b
3-(3,5-dichlorophenyl)-2,4-thiazolidinedione	DCPT	241.2 ± 8.3*	2.73
3-(3,5-dichlorophenyl)-2,4-oxazolidinedione	DCPO	> 250	2.16
N-(3,5-dichlorophenyl)succinimide	NDPS	> 250	1.98
5-(4-methoxyphenylmethylene)-2,4-thiazolidinedione	MPMT-I	50.5 ± 13.8 [†]	1.36
5-(4-methoxyphenylmethyl)-2,4-thiazolidinedione	MPMT	> 250	1.76
5-(4-methoxyphenylmethyl)-2,4-oxazolidinedione	MPMO	>250	1.19
3-(4-methoxyphenylmethylene)succinimide	MPMS-I	> 250	0.77
3-(4-methoxyphenylmethyl)succinimide	MPMS	> 250	1.01
5-(3,5-dichlorophenylmethyl)-2,4-thiazolidinedione	DCPMT	14.1 ± 1.4 [#]	3.00
troglitazone	TGZ	20.6 ± 0.6 [#]	5.08

^aLC₅₀ values (means ± SE, n =4) were determined in Microsoft Excel 2010 using regression analyses. Different symbols indicate that the corresponding LC₅₀ values are significantly different from each other (p < 0.05). The results are from replicative, independent experiments consisting of at least four individual cell culture plates with freshly prepared reagents. The LC₅₀ values for DCPT and MPMT-I were calculated from quadratic ($r^2 = 0.9942 \pm 0.0022$) or exponential ($r^2 = 0.9147 \pm 0.0384$) equations, respectively, over the complete 0-250 μM concentration range. Due to the steep decline in cell viability, LC₅₀ values for DCPMT and TGZ were calculated from 0-25 μM, using linear regression analyses with $r^2 = 0.8730 \pm 0.0294$ and 0.7176 ± 0.1421 , respectively.

^blog P values were calculated using ChemBioDraw Ultra version 14.0.