Cinnamic acid based thiazolidinediones inhibit human P450c17 and 3 β -hydroxysteroid dehydrogenase and improve insulin sensitivity independent of PPAR γ agonist activity

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Abstract

Thiazolidinediones improve insulin sensitivity in type 2 diabetes mellitus by acting as peroxisome proliferator-associated receptor gamma (PPAR γ) agonists, and decrease circulating androgen concentrations in polycystic ovary syndrome by unknown mechanisms. Some thiazolidinediones directly inhibit the steroidogenic enzymes P450c17 and 3 β -hydroxysteroid dehydrogenase type II (3 β HSDII) by distinct mechanisms. We synthesized five novel thiazolidinediones, CLX-M1 to -M5 by linking a 2,4-thiazolidinedione moiety to a substituted α -phenyl cinnamic acid previously shown to have glucose-lowering effects. Using yeast microsomes expressing human P450c17 and 3 β HSDII we found that cinnamic acid methyl esters with a double bond in the thiazolidinedione core structure (M3, M5) were stronger inhibitors of P450c17 than methyl esters with the conventional core (M1, M4). These four compounds inhibited 3 β HSDII equally well, while the free cinnamic acid analog (M2) did not inhibit either enzyme. Thus, the inhibition of P450c17 and 3 β HSDII by these novel thiazolidinediones reveals structure-activity relationships independent of PPAR γ transactivation. PPAR γ transactivation was moderate (M1), weak (M2, M3) or even absent (M4, M5). While the PPAR γ agonist activity of M1 was only 3% of that of rosiglitazone, both increased glucose uptake by 3T3-L1 adipocytes and reduced serum glucose levels in *ob/ob* and *db/db* mice to a similar extent. The similar glucose-lowering effects of M1 and rosiglitazone, despite their vast differences in PPAR γ agonist activity, suggests these two actions may occur by separate mechanisms.

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Introduction

Thiazolidinediones (TZDs), a recently introduced class of drugs (Schoonjans & Auwerx 2000), decrease hepatic gluconeogenesis and improve insulin sensitivity, particularly in adipose tissue (Lehmann *et al.* 1995, Spiegelman 1998, Olefsky 2000) and hence are useful in treating type 2 diabetes. The TZD drug troglitazone improves both insulin sensitivity and hyperandrogenemia in women with the polycystic ovary syndrome (PCOS) (Dunaif *et al.* 1996, Azziz *et al.* 2001). PCOS is a heterogeneous disorder, variably consisting of

menstrual irregularities and hyperandrogenemia, and is the leading cause of female infertility (Ehrmann *et al.* 1995, Dunaif & Thomas 2001). PCOS is frequently associated with obesity, insulin resistance and increased risk of type 2 diabetes (Ehrmann *et al.* 1995, Franks 1995, Dunaif & Thomas 2001). While the insulin-sensitizing effects of TZD drugs are clearly associated with their action as agonists of the nuclear peroxisome proliferator-associated receptor gamma (PPAR γ) (Lehmann *et al.* 1995), the mechanism by which they lower circulating androgen concentrations is less clear. The androgen-lowering effect of

troglitazone has been thought to be secondarily due to its insulin-sensitizing effect (Dunaif *et al.* 1996, Ehrmann *et al.* 1997). However, we have recently demonstrated that troglitazone also directly inhibits the catalytic activities of two key enzymes in human androgen biosynthesis (Arlt *et al.* 2001). Troglitazone at concentrations achieved in clinical use competitively inhibits both the 17 α -hydroxylase and 17,20-lyase activities of P450c17 and non-competitively inhibits 3 β -hydroxysteroid dehydrogenase type 2 (3 β HSDII) (Arlt *et al.* 2001). Two other TZDs, rosiglitazone and pioglitazone, also inhibited both P450c17 and 3 β HSD but only at supra-pharmacologic concentrations (Arlt *et al.* 2001). Thus, inhibition of P450c17 and 3 β HSD may be a general characteristic of TZD drugs, possibly independent of PPAR γ agonist activity.

We previously reported that a substituted α -phenyl cinnamic acid derivative exerts weak antihyperglycemic effects in several animal models, possibly by an interaction with the insulin receptor (Dey *et al.* 1999, 2001). We hypothesized that linking this molecule with a 2,4-TZD moiety might generate bifunctional molecules that exert hypoglycemic actions by interacting with both the insulin receptor and PPAR γ . Thus we synthesized five novel cinnamic or phenyl propionic acid based 2,4-TZD derivatives and investigated their PPAR γ agonist activity, their glucose-lowering effects *in vitro* and *in vivo*, and their ability to inhibit P450c17 or 3 β HSD.

Materials and methods

Drug preparations

Reagent-grade 2,4-TZD derivatives of cinnamic acid were synthesized and purified. Rosiglitazone was purified from commercially available tablets (Avandia, Smith Kline Beecham). The chemical equivalence and purity of the novel TZDs and of rosiglitazone prepared from tablets were confirmed by their HPLC and ^1H -NMR analysis.

Transfection of HEK293 cells and reporter gene assay for PPAR γ agonist activity

A PPAR γ 2 expression vector was constructed by inserting the PPAR γ 2 coding region into pcDNA3.1+ vector (Invitrogen, Carlsbad, CA, USA). The luciferase reporter vector was con-

structed by ligating a PPRE response element (a gift from K. Feingold, San Francisco VA Medical Center, CA, USA) upstream from the firefly luciferase coding region. Control vector pRL-SV40 expressing Renilla luciferase was purchased from Promega (Madison, WI, USA).

About 2.7×10^4 HEK293 cells (ATCC, Manassas, VA, USA) were plated onto a 35 mm culture well and maintained in Eagle's Minimal Essential Medium (EMEM, ATCC, Manassas, VA, USA) supplemented with 10% heat inactivated horse serum (ATCC) for 24 h. The expression, reporter and control vectors (2.5 ng control and 100 ng others per culture well) were transfected by lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Transfection reagent and DNA were prepared according to the manufacturer's recommendations and incubated with cells for 3 h followed by adding an equal volume of EMEM supplemented with 20% horse serum. Twenty-four hours after transfection, cells were treated with vehicle or TZD compounds at the indicated final concentrations for 24 h. The final concentration of DMSO in the medium was 0.01%. Treatments with vehicle or compound were all conducted in triplicate. Each culture well was then assayed for firefly luciferase activity and Renilla luciferase using the Dual-luciferase Reporter Assay System (Promega). Final reporter activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

Enzyme preparations

Saccharomyces cerevisiae strain W303B (Pompon *et al.* 1996) was transformed by the lithium acetate procedure (Gietz *et al.* 1992) with the yeast expression vector V10 (Pompon *et al.* 1996) containing the cDNA sequences for human P450c17 or 3 β HSDII, as described previously (Auchus *et al.* 1998, Lee *et al.* 1999). For transformation with P450c17, yeast were co-transformed with the vector pYcDE2 (Hadfield *et al.* 1986) expressing human P450 oxidoreductase cDNA (Auchus *et al.* 1998).

Enzyme assays

Yeast microsomes were incubated in 0–100 μM of each TZD drug. Compounds were dissolved in DMSO, and 4 μl DMSO containing the respective

amount of CLX-M1–M5 were added to 196 µl of a 50 mM potassium phosphate buffer (pH 7.5). Enzymatic assays were performed with 1 µM progesterone or 17 α -hydroxypregnenolone for P450c17 activities and 5 µM pregnenolone for 3 β HSDII activity. These steroid substrate concentrations are above the apparent K_m values of the respective enzymes in this system (Auchus *et al.* 1998, Lee *et al.* 1999). Each reaction contained 20 000 c.p.m. of [14 C]-pregnenolone (55.4 mCi/mmol) (Amersham, Arlington Heights, IL, USA) for analysis of 3 β HSDII activity, 20 000 c.p.m. of [14 C]-progesterone (55.4 mCi/mmol) (NEN Life Science Products, Boston, MA, USA) for 17 α -hydroxylase activity or 100 000 c.p.m. of [3 H]-Preg (21.1 Ci/mmol) (NEN Life Science Products) for 17,20-lyase activity. Catalysis was initiated by adding 1 mM NADPH for P450c17 or 1 mM NAD $^+$ for 3 β HSDII activity, and assays were carried out in the linear time range of the enzymatic reaction. Assays of 17,20-lyase activity were performed with addition of purified recombinant human cytochrome b $_5$ (PanVera, Madison, WI, USA).

Steroids were extracted from the reaction mixtures with 400 µl ethyl acetate/isooctane (1:1), concentrated by evaporation under continuous nitrogen flow, and assayed by thin layer chromatography (TLC) on PE SIL G/u.v. silica gel plates (Whatman, Maidstone, UK) using 3:1 chloroform:ethyl acetate as the solvent system (Lin *et al.* 1991). Substrates and conversion products were identified by comparison with reference steroids and quantified by phosphorimager analysis on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The percentage of transformation, the residual percentage of enzymatic activity after inhibitor administration (= %baseline activity), and the percentage of inhibition were calculated from the equation (% baseline activity = % transformation with inhibitor / % transformation without inhibitor $\times$ 100).

Basal glucose uptake by 3T3-L1 adipocytes

Basal glucose uptake was measured in 3T3-L1 adipocytes (Tafari 1996). 3T3-L1 fibroblasts were procured from ATCC (Manassas, VA, USA) and differentiated to adipocytes by treating cells with porcine insulin (1 µg/ml for 4 days), dexametha-

sone (0.25 µM for the initial 2 days) and isobutyl methyl xanthine (0.5 mM for the initial 2 days) (all from Sigma Chemicals, St Louis, MO, USA) (Frost & Lane 1985). The differentiated adipocytes were incubated in DMEM containing 10% fetal bovine serum (Gibco) with either rosiglitazone or compounds M1–M5 (0.1 and 1.0 µM) or vehicle (0.1% DMSO) for 48 h in 24-well plates. The cells were then washed with phosphate-buffered saline and incubated in glucose-free DMEM containing 1.0% BSA for 1 h at 37 °C, then 14 C-deoxyglucose (300 mCi/mmol, American Radiolabeled Chemicals Inc., St Louis, MO, USA) was added and the cells were incubated for another 10 min at room temperature. The cells were then washed three times with ice cold Dulbecco's PBS containing 10 mM cold deoxyglucose, lysed with 0.5% SDS, and counted in a scintillation counter. All assays were performed in triplicate.

In vivo studies

All procedures performed were in compliance with the Animal Welfare Act and US Department of Agriculture regulations and were approved by the Calyx Therapeutics Institutional Animal Care and Use Committee. Animals were housed at 22 °C and 50% relative humidity, with a 12 h light : 12 h darkness cycle, and received a regular rodent diet (Harlan Teklad, Madison, WI, USA) with free access to water; food was available *ad libitum*. Male C57BL/KsJ-*db/db* and C57BL/6J-*ob/ob* mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at age 5 weeks. Seven- to 8-week-old animals were given compound M1, rosiglitazone or vehicle (0.5% carboxymethyl cellulose (CMC), Sigma) in water or 0.5% CMC in water containing 10% PEG-300 orally once daily by gavage. Blood glucose levels were monitored at 0, 1, 4, 6, 24, 48 and 72 h with a One Touch Glucose Meter (Life Scan, Inc., Milpitas, CA, USA) and/or a glucose oxidase assay (Glucose Trinder, Sigma) prior to administration of the next dose or 24 h after the last dose and in the fed state. Body weights were monitored throughout the study.

Hepatic cytochrome P450 inhibition assay

The activities of hepatic cytochrome P450 enzymes, including those that metabolize rosiglitazone, were measured using established assays

Table 1 Differential effects of compounds M1 to M5 and rosiglitazone on PPAR γ transactivation as assessed by luciferase reporter gene assay, glucose uptake by 3T3-L1 adipocytes, and inhibition of enzymatic activities of the steroidogenic enzymes P450c17 and of β HSD expressed in yeast microsomes

Compound	PPAR γ transactivation EC ₅₀ (μ M) $\pm$ SD	% glucose uptake $\pm$ SD		Inhibition of steroidogenic enzymes IC ₅₀ (μ M)		
		0.1 μ M	1.0 μ M	17 α -hydroxylase	17:20-lyase	β HSD
Rosiglitazone	0.01 $\pm$ 0.007	204 $\pm$ 8.5	214 $\pm$ 36.5	86	90	54
M1	0.28 $\pm$ 0.036	161 $\pm$ 11.4	218 $\pm$ 10.0	56	>100	16
M2	0.69 $\pm$ 0.038	109 $\pm$ 19.9	200 $\pm$ 11.6	–	–	–
M3	1.14	143 $\pm$ 7.4	209 $\pm$ 22.6	32	85	9
M4	57.7	104 $\pm$ 5.7	137 $\pm$ 10.1	69	>100	20
M5	23.9	102 $\pm$ 6.8	130 $\pm$ 27.0	28	72	16

(Baldwin *et al.* 1999, Crespi & Miller 1999, Nakajima *et al.* 1999). Six pre-warmed reaction vials with 100 μ l of a solution containing 50 mM potassium phosphate, pH 7.4, 2.6 mM NADP⁺, 6.6 mM glucose 6-phosphate, 0.8 U of glucose 6-phosphate dehydrogenase/ml and a 1:6 serial dilution of the test compound were prepared along with six tubes of a 1:6 serial dilution of a suitable positive control inhibitor. The reactions were initiated by adding 100 μ l of a prewarmed solution of recombinant enzyme (purchased from Gentest, Woburn, MA, USA) and substrate (purchased either from Gentest or Sigma) in 0.05–0.35 M potassium phosphate, pH 7.4. A zero time-point control reaction was prepared by adding 40 μ l of acetonitrile to 100 μ l of cofactor solution to inactivate the enzymes, then adding 100 μ l of enzyme/substrate solution. A control reaction with no inhibitor was also prepared. After a 10-min incubation at 37 °C, the reactions were terminated by the addition of 40 μ l of acetonitrile. The reactions were centrifuged for 3 min and the supernatants were analyzed for the metabolite and/or parent forms of the probe substrate using an RPAmide C16 column maintained at 40 °C. The probe substrates and metabolites were detected by HPLC–u.v. Each assay was performed in duplicate. The enzyme–substrate and control inhibitor combinations are as follows: CYP1A2 (20 nM)–phenacetin (10 μ M)–furafylline (0.0026–3.33 μ M); CYP2A6 (13 nM)–coumarin (10 μ M)–tranylcypromine (0.0064–50 μ M); CYP2C8

(100 nM)–paclitaxel (10 μ M)–quercetin (0.013–16.67 μ M); CYP2C9 (20 nM)–diclofenac (20 μ M)–sulaphenazole (0.0064–50 μ M); CYP2C19 (100 nM)–(S)-mephenytoin (100 μ M)–tranylcypromine (0.064–500 μ M); CYP2D6 (20 nM)–dextromethorphan (14 μ M)–quinidine (0.0013–1.67 μ M); CYP2E1 (200 nM)–chlorzoxazone (50 μ M)–4-methylpyrazole (0.013–16.67 μ M); CYP3A4 (8, 13 or 13 nM)–midazolam (6 μ M), testosterone (100 μ M) or nifedipine (20 μ M)–ketoconazole (0.0013–1.67 μ M). The final concentrations of M1 and M2 were 0.0064, 0.039, 0.23, 1.39, 8.33 and 50 μ M.

Results

Structural features of the cinnamic acid based TZDs

The five compounds are 2,4-TZD derivatives of cinnamic or phenyl propionic acid (Fig. 1). Analogs M1, M2 and M3 are cinnamic acid derivatives and M4 and M5 are phenyl propionic acid derivatives. Compounds M1, M3, M4 and M5 are methyl esters and M2 is the corresponding free acid of M1. In M3 and M5, the TZD ring is connected to rest of the molecule with a double bond instead of a single bond as in M1, M2 and M4.

PPAR γ transactivation assay

Using a luciferase reporter assay, we determined PPAR γ agonist activity of the five novel TZDs

Figure 1 Chemical structures of the established TZD compounds pioglitazone, rosiglitazone and troglitazone, of an α -phenyl substituted cinnamic acid derivative, and of the five novel cinnamic acid based 2,4-TZD analogs, CLX-M1 to -M5. The closed and open arrows indicate the double bonds and ester groups discussed in the text.

(Table 1). The most potent compound in this series was M1 (EC_{50} of $0.28 \mu\text{M}$), which had one-thirtieth the activity of rosiglitazone (EC_{50} of $0.009 \mu\text{M}$) in the same assay. Compounds M2 and M3 also showed reasonable potency, though less than M1. Compounds M4 and M5, in which the cinnamic acid double bond was reduced, were essentially inactive. Thus the presence of the cinnamic acid double bond is essential for the maintenance of PPAR γ agonist activity.

Assays of P450c17 and 3 β HSD activities

P450c17 catalyzes both 17 α -hydroxylase and 17,20-lyase activities, and is required for biosynthesis of human androgens (Nakajin *et al.* 1981, Zuber *et al.* 1986, Lin *et al.* 1991). The 17 α -hydroxylase activity of human P450c17 converts pregnenolone to 17 α -OH-pregnenolone (17-Preg) and progesterone to 17 α -OH-progesterone (17 OHP) with equivalent efficiencies (Auchus *et al.* 1998). However, the 17,20-lyase activity is 30–50-fold more effective in converting 17-Preg to DHEA than in converting 17 OHP to androstenedione (Lin *et al.* 1993, Auchus *et al.* 1998). Each catalytic cycle of P450c17 requires electron donation from P450 oxidoreductase, with cytochrome b_5 , allosterically facilitating the 17,20-lyase reaction but not the 17 α -Hydroxylase reaction (Auchus *et al.* 1998). Therefore, all P450c17 assays were carried out with microsomes from yeast co-transformed with vectors expressing human P450c17 and human oxidoreductase (Auchus *et al.* 1998, Arlt *et al.* 2001). 17 α -hydroxylase activity was measured as the conversion of progesterone to 17 OHP, which is not metabolized further, and 17,20-lyase activity was measured as the conversion of 17-Preg to DHEA in the presence of cytochrome b_5 .

Two catalytically equivalent forms of 3 β -hydroxysteroid dehydrogenase (3 β HSD) catalyze the conversions of pregnenolone to progesterone, of 17-Preg to 17 OHP and of DHEA to androstenedione (Thomas *et al.* 1989, Rhéaume *et al.* 1992). Androstenedione, in turn, is converted to testosterone and dihydrotestosterone, which bind to and activate the androgen receptor. We measured 3 β HSD activity in microsomes of transformed yeast as the conversion of pregnenolone to progesterone, which also reflects the rate of conversion of DHEA to androstenedione because all three substrates for 3 β HSD have equivalent apparent K_m and similar

$V_{\max}$ values in our yeast expression system (Lee *et al.* 1999).

Compounds M3 and M5 readily inhibited the 17 α -hydroxylase activity in a dose-dependent manner, whereas M1 and M4 were less effective and M2 had virtually no inhibitory effect (Fig. 2A). The IC_{50} values for M3 and M5 were 28–32 μM , and those for M1 and M4 were 56 and 69 μM respectively, whereas and IC_{50} value for M2 could not be calculated (Table 1). A similar pattern was seen with the 17,20-lyase activity of P450c17. M3 and M5 were better inhibitors than M1 and M4, while M2 exhibited virtually no inhibitory effect (Fig. 2B and Table 1). By contrast, the IC_{50} values for the two P450c17 activities were 86–90 μM for rosiglitazone and >100 μM for pioglitazone, but only 11–12 μM for troglitazone (Arlt *et al.* 2001).

The effects on 3 β HSD activity were somewhat different (Fig. 3). All the cinnamic or phenyl propionic acid methyl esters (M1, M3, M4 and M5) were effective 3 β HSD inhibitors, with IC_{50} values ranging from 9 to 20 μM (Table 1), while M2, the free cinnamic acid derivative, again was devoid of inhibitory activity. Thus the 3 β HSD inhibitory activity of the cinnamic acid methyl ester TZDs was equal to or greater than that of troglitazone (IC_{50} = 24 μM) (Arlt *et al.* 2001), but the inhibitory activity of the free acid derivative was less than that of rosiglitazone and similar to that of pioglitazone (Arlt *et al.* 2001). Thus the methyl group, which is present in all compounds but M2, seems to be of crucial importance for the inhibition of both P450c17 and 3 β HSD. Furthermore, the introduction of a double bond into the conventional TZD core structure in M3 and M5 results in significantly enhanced inhibition of 17 α -hydroxylase activity.

Glucose uptake by 3T3-L1 adipocytes

Glucose uptake by adipocytes was measured by incorporation of ^{14}C -deoxyglucose into 3T3-L1 cells, which had first been induced to differentiate from fibroblasts to adipocytes by treatment with insulin, dexamethasone and methylxanthine (Frost & Lane 1985). Adding 1.0 μM of compounds M1, M2, M3 or rosiglitazone doubled glucose uptake by 3T3-L1 cells while compounds M4 and M5 had minimal activity (Table 1). At 0.1 μM , compounds M1 and M3 retained some activity, but less than that of rosiglitazone, while the other compounds were inactive. Compounds M1 and M3 differ in the

A **B**

Figure 2 Effect of CLX-M1 to -M5 on the activities of human P450c17. (A) 17 α -Hydroxylase activity. Microsomes prepared from yeast co-expressing human P450c17 and human P450 oxidoreductase were incubated with 1 μ M [14 C]progesterone (Prog) and 0–100 μ M of CLX-M1 to -M5. 17 α -Hydroxylase activity was assessed as the conversion rate of Prog to 17 OHP. (B) 17,20-Lyase activity. Microsomes co-expressing P450c17 and oxidoreductase were incubated with 1 μ M [3 H]17 α -hydroxypregnenolone (17-Preg) and 0–100 μ M CLX-M1 to -M5. 17,20-Lyase activity was assessed as the conversion rate of 17-Preg to DHEA. Each data point represents the mean $\pm$ s.d. of triplicate determinations.

Figure 3 Effect of CLX-M1 to -M5 on 3β HSDII activity. Microsomes prepared from yeast expressing human 3β HSDII were incubated with $5\ \mu\text{M}$ [^{14}C]pregnenolone (Preg) and 0–100 μM of CLX-M1 to -M5. 3β HSDII activity was assessed as the conversion rate of Preg to progesterone (Prog). Each data point represents the mean $\pm$ S.D. of triplicate determinations.

double bond in the glitazone moiety and had significantly different PPAR γ agonist activities, but had minimal differences in their ability to induce glucose uptake (no statistically significant differences by ANOVA). Compound M5, with only one double bond joined to the TZD ring, and the doubly reduced product M4 were devoid of activity at $0.1\ \mu\text{M}$. Thus, the absence of the double bond joined to the TZD ring and the presence of the cinnamic acid double bond seem to be important for increased glucose uptake in this system.

Effects of M1 in mouse models of type 2 diabetes

Because the *in vitro* assays described above indicated that compound M1 exerts the greatest PPAR γ agonist activity and the greatest activity to promote glucose uptake while exerting low inhibitory activity against steroidogenic enzymes, we sought to evaluate compound M1 in mouse models of type 2 diabetes. The genetically obese, diabetic *ob/ob* mouse, which is defective in leptin synthesis, and the genetically hyperinsulinemic diabetic *db/db* mouse, which is defective in the leptin receptor, are well-established models of type 2 diabetes (Primer 2000). A single oral dose of 50 mg/kg of compound M1 lowered blood glucose in *db/db* mice in a time-dependent fashion, reaching a nadir of 23% of the control value after 24 h (data not shown). When

administered orally once daily (50 mg/kg per day) for 14 days to *db/db* mice, compound M1 and rosiglitazone exhibited equivalent effects to lower blood glucose (Fig. 4A) but neither compound affected body weight (Fig. 4B). Similar results are seen in *ob/ob* mice, in which M-1 (10 mg/kg per day) and rosiglitazone (10 mg/kg per day) lowered blood glucose by 47% and 53% respectively after 8 days, compared with vehicle.

Effects of M1 and M2 on hepatic cytochrome P450 enzymes

Due to severe idiosyncratic hepatocellular injury, troglitazone has been withdrawn from the market. This toxicity may originate from inhibition of hepatic cytochrome P450 enzymes by troglitazone and its two metabolites (Yamazaki *et al.* 2000). Thus, we studied the potential effects of M1 and its free acid form M2 on hepatic cytochrome P450 enzymes. M2 is the primary metabolite of M1 in most species including humans. The metabolism of about 90% of the clinically important pharmaceutical drugs is carried out by only eight cytochrome P450 enzymes: CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Rendic and Di Carlo 1997, Obach 1999). Compound M1 did not inhibit CYP1A2, and it had a low inhibitory effect ($\text{IC}_{50} > 15\ \mu\text{M}$) on

Figure 4 Effects of 2 weeks of treatment in 8-week-old male *db/db* mice with 50 mg/kg of compound M1 and rosiglitazone on blood glucose (A) and body weight (B) as compared with empty vehicle. The number of animals per group was five. **P* value < 0.05.

CYP2A6, CYP2D6 and CYP2E1 (Table 2). However, M1 was a relatively strong inhibitor of the CYP2C enzyme family (IC_{50} values for CYP2C8, CYP2C9 and CYP2C19 were less than

5 μ M) and it appeared to exhibit partial inhibition of all three CYP3A4 substrates tested (Kenworthy *et al.* 1999). Maximum inhibition of 3A4 varied depending on the substrate. For midazolam inhibition was 50–60% (IC_{50} 0.60 μ M); for nifedipine inhibition was 85–90% (IC_{50} 2.4 μ M); and for testosterone inhibition was 70–80% (IC_{50} 1.6 μ M). Compared with M1, M2 was a less potent inhibitor of all the hepatic P450 enzymes examined (Table 2). In particular, there was little if any inhibition of the CYP2C enzymes other than CYP2C8, which was moderately inhibited by M2 (IC_{50} 6.9 μ M). M2 was 20-fold less potent at inhibiting CYP2C8 and at least 14-fold less potent at inhibiting CYP3A4 compared with M1. This suggests an important role in the methylester moiety in hepatic P450 inhibition.

Discussion

TZDs act as agonists of PPAR γ (Schoonjans & Auwerx 2000) and exert their antidiabetic effect by improving peripheral tissue insulin sensitivity, particularly in adipose tissue (Lehmann *et al.* 1995). During the last decade, an exhaustive search for novel agents based on the TZD ciglitazone (Shoda *et al.* 1982) has yielded three commercially marketed drugs: pioglitazone (Mosome *et al.* 1991), rosiglitazone (Cantello *et al.* 1994) and troglitazone. Troglitazone also lowers the excess androgens in women with PCOS (Azziz *et al.* 2001). As troglitazone directly inhibits the androgen biosynthetic enzymes P450c17 and 3 β HSDII at therapeutically achieved concentrations, but pioglitazone and rosiglitazone do not (Arlt *et al.* 2001), it is not yet apparent whether pioglitazone, rosiglitazone and other TZD drugs will exert a similar effect on the hyperandrogenism of PCOS. Therefore, we have sought to synthesize novel TZD drugs and examine the potential roles of different portions of the molecule on various physiologic variables.

We have previously shown, using various animal models (Dey *et al.* 1999), that the substituted α -phenyl cinnamic acid derivative exerts a weak antihyperglycemic effect (Dey *et al.* 2001). Therefore, we postulated that linking this molecule with a 2,4-TZD moiety could provide compounds retaining the original glucose-lowering activity and, additionally, an affinity for PPAR γ , and that such

Table 2 *In vitro* inhibition studies on hepatic P450 enzymes

P450	Substrate	M1 IC ₅₀ [*]	M2 IC ₅₀ [*]	Positive control IC ₅₀ [*]
CYP1A2	Phenacetin	NI	NI	2.8
CYP2A6	Coumarin	>50	>50	0.41
CYP2C8	Paclitaxel	0.34	6.9	1.4
CYP2C9	Diclofenac	0.88	>50	0.69
CYP2C19	Mephenytoin	2.8	>50	11.2
CYP2D6	Dextromethorphan	>50	NI	0.035
CYP2E1	Chlozoxazone	>20	>50	3.6
CYP3A4	Midazolam	0.6†	8.6	0.082
CYP3A4	Nifedipine	2.43	NI	0.17
CYP3A4	Testosterone	1.6	>50	0.3

^{*}IC₅₀ values in μ M; NI, not inhibited; †maximum inhibition was 50–60%; ‡maximum inhibition was 85–90%; §maximum inhibition was 70–80%.

compounds would be useful for treating type 2 diabetes. Our analysis of the five compounds synthesized suggests that compound M1 may hold therapeutic promise. Troglitazone, the first commercial TZD drug, was withdrawn from the market because of idiosyncratic hepatocellular injury due to inhibition of hepatic P450 enzymes by the drug and two of its metabolites (Yamazaki *et al.* 2000). Because troglitazone also inhibits steroidogenic P450c17 (Arlt *et al.* 2001), we examined the effects of these five compounds on P450c17 and 3 β HSDII, and the effects of M1 on hepatic cytochrome P450 enzymes. These data showed that M1 was a poor inhibitor of P450c17 but a moderate inhibitor of some hepatic P450 enzymes tested, while exerting strong PPAR γ agonist activity.

Retention of PPAR γ agonist activity, which is essential for the anti-diabetic actions of TZD drugs, required the presence of the cinnamic acid double bond (see activities of M1, 2 and 3 compared with M4 and M5). Both the nature of the scaffold or TZD side group and modifications to the TZD core itself can influence the actions of TZDs to inhibit steroidogenic enzymes, but changes in the scaffold are more important. This is seen with the differences between M3 and M5 compared with M2 and M4, and is consistent with the previously observed differences among tro-, pio- and rosiglitazone (Arlt *et al.* 2001), which contain the same TZD core but different side groups. When TZDs inhibit steroidogenic activities, the 17 α -hydroxylase and 17,20-lyase activities are inhibited similarly, as would be expected by the common active site for

both reactions (Auchus & Miller 1999) and the competitive mode of inhibition by TZD drugs (Arlt *et al.* 2001). By contrast, the inhibition of these two activities is not proportional to the inhibition of 3 β -hydroxysteroid dehydrogenase activity, which is consistent with this activity being catalyzed by a different enzyme that is inhibited non-competitively by TZD drugs (Arlt *et al.* 2001).

Finally, the most remarkable aspect of these new TZD drugs is evidenced by a comparison of the data *in vivo* and *in vitro*. The compound M1 had only 3% of PPAR γ agonist activity of rosiglitazone in the transcriptional assay, yet both M1 and rosiglitazone exerted equivalent glucose-lowering activity in animal models of diabetes. This suggests that M1 is exerting some of its glucose-lowering effects by mechanisms independent of PPAR γ activation. Based on our previous studies (Dey *et al.* 1999, 2001) we would suggest that this may be due to interaction of the cinnamic acid moiety with the insulin receptor. This observation indicates that bifunctional drugs can be designed that will elicit glucose-lowering effects at more than one cellular target.

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