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

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*(Weibke Arlt and Partha Neogi contributed equally to this work)

Abstract

Thiazolidinediones improve insulin sensitivity in type 2 diabetes mellitus by acting as peroxisome proliferator-associated receptor gamma (PPARγ) agonists, and decrease circulating androgen concentrations in polycystic ovary syndrome by unknown mechanisms. Some thiazolidinediones directly inhibit the steroidogenic enzymes P450c17 and 3β-hydroxysteroid dehydrogenase type II (3βHSDII) 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 3βHSDII 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 3βHSDII equally well, while the free cinnamic acid analog (M2) did not inhibit either enzyme. Thus, the inhibition of P450c17 and 3βHSDII by these novel thiazolidinediones reveals structure–activity relationships independent of PPARγ transactivation. PPARγ transactivation was moderate (M1), weak (M2, M3) or even absent (M4, M5). While the PPARγ 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 PPARγ agonist activity, suggests these two actions may occur by separate mechanisms.


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 (PPARγ) (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 17α-hydroxylase and 17,20-lyase activities of P450c17 and non-competitively inhibits 3β-hydroxysteroid dehydrogenase type 2 (3βHSDII) (Arlt et al. 2001). Two other TZDs, rosiglitazone and pioglitazone, also inhibited both P450c17 and 3βHSD but only at supra-pharmacologic concentrations (Arlt et al. 2001). Thus, inhibition of P450c17 and 3βHSD may be a general characteristic of TZD drugs, possibly independent of PPARγ 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 PPARγ. Thus we synthesized five novel cinnamic or phenyl propionic acid based 2,4-TZD derivatives and investigated their PPARγ agonist activity, their glucose-lowering effects in vitro and in vivo, and their ability to inhibit P450c17 or 3βHSD.

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 PPARγ agonist activity

A PPARγ2 expression vector was constructed by inserting the PPARγ2 coding region into pcDNA3·1+ vector (Invitrogen, Carlsbad, CA, USA). The luciferase reporter vector was constructed 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 × 10⁴ 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 3βHSDII, 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 17α-hydroxypregnenolone for P450c17 activities and 5 µM pregnenolone for 3βHSDII 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 3βHSDII activity, 20 000 c.p.m. of \([^{14}C]\)-progesterone (55·4 mCi/mmol) (NEN Life Science Products, Boston, MA, USA) for 17α-hydroxylase activity or 100 000 c.p.m. of \([^3H]\)-17-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 3βHSDII 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 (Tafuri 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), dexamethasone (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.
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.

PPARγ transactivation assay

Using a luciferase reporter assay, we determined PPARγ 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 µM), which had one-thirtieth the activity of rosiglitazone (EC$_{50}$ of 0.009 µM) 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 PPARγ agonist activity.

Assays of P450c17 and 3βHSD activities

P450c17 catalyzes both 17α-hydroxylase and 17,20-lyase activities, and is required for biosynthesis of human androgens (Nakajin et al. 1986, Lin et al. 1991). The 17α-hydroxylase activity of human P450c17 converts pregnenolone to 17α-OH-pregnenolone (17-Preg) and progesterone to 17α-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 17α-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). 17α-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 3β-hydroxysteroid dehydrogenase (3βHSD) 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 3βHSD 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 3βHSD 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 17α-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 3βHSD activity were somewhat different (Fig. 3). All the cinnamic or phenyl propionic acid methyl esters (M1, M3, M4 and M5) were effective 3βHSD 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 3βHSD 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 3βHSD. Furthermore, the introduction of a double bond into the conventional TZD core structure in M3 and M5 results in significantly enhanced inhibition of 17α-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
Figure 2 Effect of CLX-M1 to -M5 on the activities of human P450c17. (A) 17α-Hydroxylase activity. Microsomes prepared from yeast co-expressing human P450c17 and human P450 oxidoreductase were incubated with 1 µM [14C]progesterone (Prog) and 0–100 µM of CLX-M1 to -M5. 17α-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 µM [3H]17α-hydroxypregnenolone (17-Preg) and 0–100 µM 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 ± S.D. of triplicate determinations.
double bond in the glitazone moiety and had significantly different PPARγ 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 µM. 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 PPARγ 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 (IC50 > 15 µM) on
However, M1 was a relatively strong inhibitor of the CYP2C enzyme family (IC\textsubscript{50} values for CYP2C8, CYP2C9 and CYP2C19 were less than 5 \(\mu\)M) and it appeared to exhibit partial inhibition of all three CYP3A4 substrates tested (Kenworthy \textit{et al.} 1999). Maximum inhibition of 3A4 varied depending on the substrate. For midazolam inhibition was 50–60\% (IC\textsubscript{50} 0·60 \(\mu\)M); for nifedipine inhibition was 85–90\% (IC\textsubscript{50} 2·4 \(\mu\)M); and for testosterone inhibition was 70–80\% (IC\textsubscript{50} 1·6 \(\mu\)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\textsubscript{50} 6·9 \(\mu\)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\(\gamma\) (Schoonjans & Auwerx 2000) and exert their antidiabetic effect by improving peripheral tissue insulin sensitivity, particularly in adipose tissue (Lehmann \textit{et al.} 1995). During the last decade, an exhaustive search for novel agents based on the TZD ciglitazone (Shoda \textit{et al.} 1982) has yielded three commercially marketed drugs: pioglitazone (Mosome \textit{et al.} 1991), rosiglitazone (Cantello \textit{et al.} 1994) and troglitazone. Troglitazone also lowers the excess androgens in women with PCOS (Azziz \textit{et al.} 2001). As troglitazone directly inhibits the androgen biosynthetic enzymes P450c17 and 3\(\beta\)HSDII at therapeutically achieved concentrations, but pioglitazone and rosiglitazone do not (Arlt \textit{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 \textit{et al.} 1999), that the substituted \(\alpha\)-phenyl cinnamic acid derivative exerts a weak antihyperglycemic effect (Dey \textit{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\(\gamma\), and that such
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 3βHSDII, 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 PPARγ agonist activity.

Retention of PPARγ 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 17α-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 3β-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 PPARγ 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 PPARγ 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.

### Acknowledgements

This work was supported by NIH grant DK HD41958, and by a research grant from Calyx Therapeutics (to W.L.M.). W.A. is a Heisenberg Senior Clinical Fellow of the Deutsche Forschungsgemeinschaft (DFG; AR 310/3–1).

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

<table>
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<tr>
<th>P450</th>
<th>Substrate</th>
<th>M1 IC&lt;sub&gt;50&lt;/sub&gt;*</th>
<th>M2 IC&lt;sub&gt;50&lt;/sub&gt;*</th>
<th>Positive control IC&lt;sub&gt;50&lt;/sub&gt;*</th>
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<tr>
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*IC<sub>50</sub> values in µM; NI, not inhibited; †maximum inhibition was 50–60%; ‡maximum inhibition was 85–90%; §maximum inhibition was 70–80%.
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www.endocrinology.org

Received in final form 12 December 2003
Accepted 12 January 2004