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Troglitazone quinone formation catalyzed by human and rat CYP3A: an atypical CYP oxidation reaction☆

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Abstract

Oxidative ring opening of troglitazone (TGZ), a thiazolidine 2,4-dione derivative used for the treatment of type II diabetes mellitus, leads to the formation of a quinone metabolite. The formation of TGZ quinone was shown to be NADPH dependent and to require active microsomal enzymes. Quinone formation was not affected by co-incubation with catalase or sodium azide and was partially inhibited (25%) by superoxide dismutase (SOD). Kinetic analysis of TGZ quinone formation in human liver microsomes implied single enzyme involvement. CYP3A isoforms were characterized as the primary enzymes involved in quinone formation by several lines of evidence including: (a) troleandomycin and ketoconazole almost completely inhibited microsomal quinone formation when SOD was present, whereas other CYP inhibitors had minimal effects ($<$20%); (b) TGZ quinone formation was highly correlated with regard to both contents (r^2: 0.9374) and activities (r^2: 0.7951) of CYP3A4 in human liver microsomes (HLM); (c) baculovirus insect cell-expressed human CYP3A4 was able to catalyze TGZ quinone formation at a higher capacity ($V_{\max}/K_m$) than other human CYPs with the relative contribution of CYP3A4 in HLM estimated to be 20-fold higher than that of other CYPs; (d) TGZ quinone formation was increased by 350% in liver microsomes from rats pretreated with dexamethasone (DEX); and (e) plasma concentrations of TGZ quinone were increased by 260–680% in rats pretreated with DEX. The chemical nature of the quinone metabolite suggests an atypical CYP reaction consistent with a one-electron oxidation mechanism where an intermediate phenoxy radical combines with ferryl oxygen to subsequently form the quinone metabolite. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Troglitazone; Quinone; Metabolism; CYP3A4; One-electron oxidation

1. Introduction

TGZ (Rezulin®, CI-991) is a thiazolidine 2,4-dione derivative that is the first member of a new class of oral drugs approved for the treatment of type II diabetes mellitus [1]. Clinical studies demonstrated that TGZ effectively reduces hyperglycemia, hyperinsulinemia, and hypertriglyceridemia in patients with type II diabetes [2–4]. The mechanism of the pharmacological effects has been shown to involve increased insulin sensitivity in skeletal muscle, liver, and adipose tissue via activation of peroxisome proliferator-activated receptor-gamma (PPARγ), which results in alteration of the gene expression of key proteins involved in glucose metabolism [1,5]. As a vitamin E analog, TGZ has also been demonstrated to be an effective antioxidant that may play an important role in preventing the development of atherosclerotic disease by reducing the oxidation of lipoproteins [6,7].

The metabolism of TGZ in human subjects involves primarily conjugation reactions to form sulfate (major metabolite) and glucuronide derivatives as well as oxidation to a quinone product (Fig. 1) [8,9]. The mechanism of the oxidation reaction has not been fully elucidated. Previous studies indicated that one-electron oxidation of TGZ with ROS resulted in opening of the chroman ring, the vitamin E portion of the molecule, to form the quinone metabolite [10]. Further studies suggested that TGZ radicals or an intermediate hydroperoxydienone were formed in the reac-

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Abbreviations: CL_{int}, intrinsic clearance; CYP, cytochrome P450; DEX, dexamethasone; FMO, flavin-containing monooxygenase; β-NF, β-naphthoflavone; PB, phenobarbital; ROS, reactive oxygen species; SOD, superoxide dismutase; and TGZ, troglitazone.

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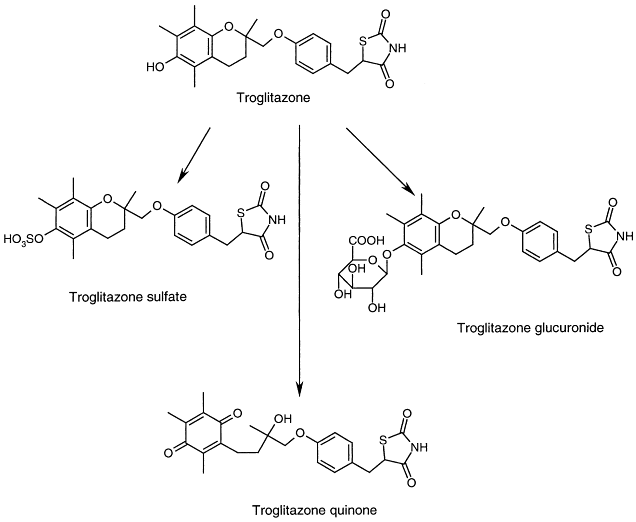

Fig. 1. Scheme of primary metabolic pathways involved in TGZ metabolism.

tion of TGZ with ROS or by photooxidation [10]. However, it is not clear if the reaction involving ROS is the primary mechanism for the formation of TGZ quinone in biological systems. Peroxidases and CYPs are also able to catalyze one-electron oxidation of several compounds such as dihydropyridines and alkylamines [11–14]. The present study was carried out to assess the potential for the involvement of CYP reaction in the formation of TGZ quinone.

2. Materials and methods

2.1. Chemicals

TGZ and PD 166793 [2-(4′-bromo-biphenyl-4-sulfonylamino)-3-methyl-butyric acid] were synthesized at Parke-Davis Pharmaceutical Research. NADPH, catalase, SOD, DEX, PB, pyridine, β-NF, quinidine, troleandomycin, and ketoconazole were purchased from the Sigma Chemical Co. Diethyldithiocarbamate, sulphaphenazole, diallylsulfoxide, and furafylline were purchased from the GENTEST Corp.

2.2. Liver microsomes and human CYPs 3A4, 2D6, 2B6, or 2C19 supersomes

Human liver microsomes and human CYPs 3A4, 2D6, 2B6, or 2C19 supersomes were purchased from the GENTEST Corp. Pooled human liver microsomes comprised a mixture of liver microsomes from six different individual donors. Human CYP supersomes comprised baculovirus insect cell-expressed human CYP and co-expressed CYP-NADPH reductase. Activities and contents of CYPs, CYP-NADPH reductase, cytochrome b_5, and FMO in the pooled liver microsomes and in liver microsomes from twelve individuals were provided by the GENTEST Corp. Phenacetin *O*-deethylation, coumarin 7-hydroxylation, (*S*)-mephenytoin *N*-demethylation, paclitaxel 6α-hydroxylation, diclofenac 4′-hydroxylation, (*S*)-mephenytoin 4′-hydroxylation, bufuralol 1′-hydroxylation, chlorzoxazone 6-hydroxylation, testosterone 6β-hydroxylation, lauric acid 12-hydroxylation, and methyl *p*-tolyl sulfide oxidation were used for assessing the activities of CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 4A, and FMO, respectively.

Rat liver microsomes were prepared by differential centrifugation from male Fischer 344 rats (161–190 g; Harlan Sprague-Dawley) treated i.p. with β-NF (80 mg/kg), PB (80 mg/kg), pyridine (100 mg/kg), or DEX (100 mg/kg) for 3–4 days, respectively. Total P450 content was measured according to the method of Omura and Sato [15].

2.3. Incubation of TGZ with liver microsomes and human CYPs 3A4, 2D6, 2B6, or 2C19 supersomes

TGZ was incubated with human liver microsomes (0.2 mg protein/mL), rat liver microsomes (0.1 nmol P450/mL), or human CYP supersomes (0.05 P450 nmol/mL) at various concentrations indicated in 50 mM potassium phosphate buffer (pH 7.4) containing 30 mM $MgCl_2$ for 15 min at 37°. The reaction was started with the addition of NADPH to give a final concentration of 1 mM, and stopped with the addition of an equal volume of cooled methanol. For inhibitory studies, CYP inhibitors at the concentrations indicated were preincubated with human liver microsomes for 5 min in the presence of 1 mM NADPH at 37° [16,17]. For kinetic studies, SOD (0.1 U/mL) was included in the incubation systems. Catalase (1000 U/mL) or sodium azide (1 mM) was included in liver microsomal incubates in the experiments designed for investigating the involvement of H_2O_2 in the formation of TGZ quinone. The reagent concentrations indicated are the final concentrations in the incubation systems.

2.4. Incubation of TGZ with human hepatocytes

Cryopreserved human hepatocytes (lot 69) were obtained from In Vitro Technologies. They were thawed according to the instructions provided by the vendor. The human hepatocytes (1 million cells/mL) were preincubated with or without ketoconazole at the final concentrations indicated for 5 min and then incubated with TGZ at a final concentration of 10 μM in hepatocyte culture medium (In Vitro Technologies) for 15 min at 37°. Reactions were stopped by the addition of an equal volume of cooled methanol.

2.5. TGZ metabolism in DEX-pretreated rats

TGZ (10 mg/kg, amorphous form) was administered orally to male Wistar rats (275–304 g) pretreated with DEX (150 mg/kg, i.p.) for 4 days. Blood samples were taken at the time points indicated. Heparin was used as the anticoagulant.

2.6. Assays for determination of TGZ quinone

TGZ quinone was quantified by LC-MS/MS. Sample analyses were carried out on a Micromass Quattro II tandem quadrupole mass spectrometer coupled with a Perkin Elmer Series 200 HPLC System. Sample introduction and ionization were achieved by electrospray ionization in the negative ionization mode. Parent-to-daughter ion transitions were established to be 440.0 → 397.1 for TGZ, 519.9 → 440.1 for TGZ sulfate, 456.0 → 179.0 for TGZ quinone, and 409.8 → 78.8 for the internal standard PD 166793 through direct infusion of the compounds into the mass spectrometer. Sensitivity was optimized by varying cone voltage and collision energy in the multiple reaction monitoring (MRM) mode and maximizing ion intensity. TGZ, TGZ metabolites, and internal standard were separated on a Discovery RP Amide C16 column (2.1 × 50 mm, 5 μm) (Supelco) utilizing a mobile phase containing acetonitrile (60%), 2 mM ammonium acetate (40%), and acetic acid (0.2%) at a flow rate of 0.2 mL/min. Samples were prepared by adding cooled methanol at a ratio of 2:1 (methanol:sample). Protein was precipitated by centrifugation at 6500 *g* for 15 min at room temperature. The supernatant was taken and evaporated to dryness at 40° under nitrogen. Finally, samples were reconstituted in a 200-μL mobile phase for injection.

2.7. Others

Michaelis–Menten parameters were determined following Eadie–Hofstee graphical analysis of the kinetic data. CL_{int} was calculated as follows: $CL_{int} = V_{max}/K_m$. Relative CL_{int} of CYP in human liver microsomes was calculated as follows: relative CL_{int} of CYP $= CL_{int} \times$ concentration of specific CYP in human liver microsomes. Statistical analysis of the data was carried out using Sigma Plot. Results for the experiments using pooled human liver microsomes or supersomes were expressed as means of at least two experiments.

3. Results

3.1. Formation of TGZ quinone in liver microsomes

TGZ quinone formed in liver microsomes was identified by LC-MS/MS using authentic TGZ quinone as a reference. The formation of TGZ quinone in liver microsomes was found to be NADPH dependent and to require active microsomal enzymes (Fig. 2). Only a trace amount of TGZ quinone was formed in water, various buffers at a pH of 7.4 to 7.5 (data not shown), heat-denatured enzyme, or the enzyme system minus the cofactor NADPH when compared with the functional enzyme system. Co-incubation of the rat liver microsomes with catalase or the catalase inhibitor sodium azide had almost no effect on TGZ quinone formation (Fig. 2). However, TGZ quinone formation was decreased in rat and human liver microsomes when co-incubated with SOD (Figs. 2 and 3).

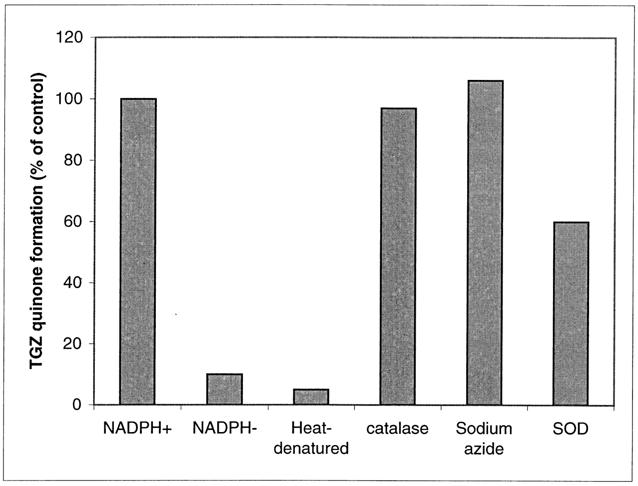

Fig. 2. Effects of NADPH, denaturing of microsomal proteins, catalase, sodium azide, and SOD on the formation of TGZ quinone in liver microsomes from rats pretreated with DEX. TGZ was incubated with liver microsomes (0.1 nmol P450/mL) in the presence or absence of 1 mM NADPH, 1000 U/mL of catalase, 1 mM sodium azide, or 1 U/mL of SOD at 37° for 10 min. Denatured liver microsomes were prepared by heating the microsomal solution at 100° for 5 min. Results are expressed as the means of two experiments. The control value (100%) was 3.4 nmol/min/nmol of P450.

3.2. Effects of CYP inhibitors on TGZ quinone in human liver microsomes and hepatocytes

Results from screening the effects of several CYP selective inhibitors on TGZ quinone formation in human liver microsomes are shown in Fig. 4. The CYP3A4 inhibitor troleandomycin was found to inhibit TGZ quinone formation by approximately 60%, while 10–20% inhibitions were observed for furafylline (1A2), sulphaphenazole (2C9), and diethyldithiocarbamate (2A6, 2B6, and 2E1). The CYP2E1 inhibitor diallylsulfoxide had no effect. Quinidine, a selective 2D6 inhibitor and substrate of CYP3A4, increased the formation of TGZ quinone in human liver microsomes (Fig. 4). Further studies showed that approximately 75% inhibition was the maximum inhibition achieved by either troleandomycin or ketoconazole (Fig. 3, upper panel). Approximately 25% of TGZ quinone formation was estimated to be due to the superoxide reaction as evidenced by the finding that this portion of the quinone metabolite was inhibited completely by SOD in human liver microsomes (Fig. 3, lower panel). Approximately 50–60% of TGZ quinone formation was inhibited in human hepatocytes by ketoconazole (Fig. 5).

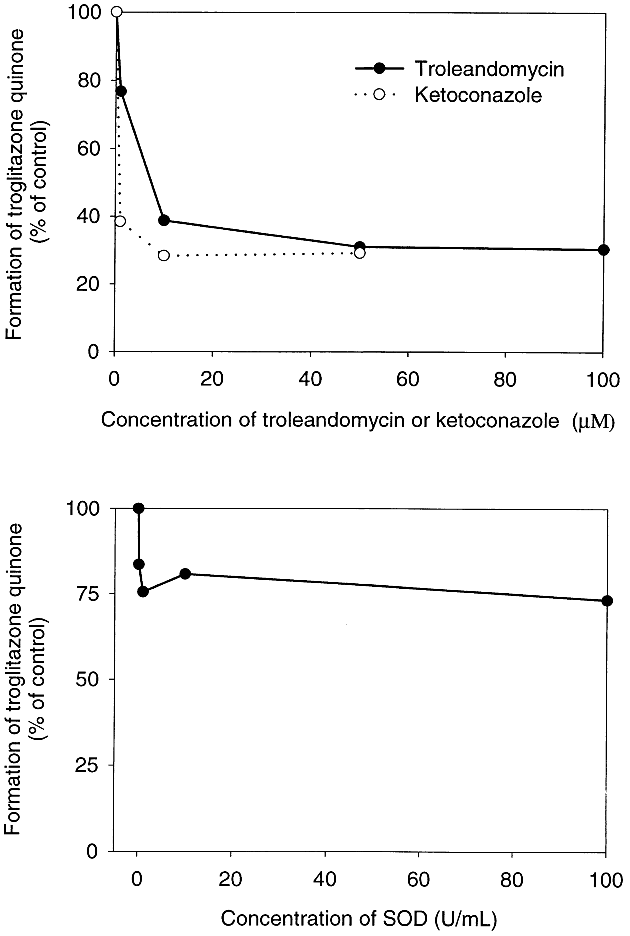

Fig. 3. Effects of troleandomycin or ketoconazole (upper panel) and SOD (lower panel) on the formation of TGZ quinone in human liver microsomes. Pooled human liver microsomes (0.2 mg protein/mL) were preincubated with troleandomycin or ketoconazole for 5 min and then incubated with 10 μM TGZ at 37° for an additional 10 min. There was no preincubation for SOD. The experimental details are described in "Materials and methods." Results are expressed as the means of two experiments. The control value (100%) was 279 pmol/min/mg protein.

3.3. Kinetics of TGZ quinone formation and correlation to CYP activities in human liver microsomes

The values of K_m and $V_{\max}$ for TGZ quinone formation in pooled human liver microsomes were determined to be 6.7 μM and 459 pmol/min/mg following Eadie–Hofstee graphical analysis (Fig. 6). The correlations between TGZ quinone formation and the activities of microsomal enzymes including ten CYPs, FMO, CYP-NADPH reductase, cytochrome b_5, and the concentration of total CYP measured by reduced-carbon monoxide binding spectra were investigated using human liver microsomes from twelve individuals. TGZ quinone formation was highly correlated to the activities and contents of CYP3A4/5 in human liver microsomes, as shown with r^2 values of 0.7951 and 0.9374, respectively (Fig. 7 and Table 1). TGZ quinone formation was also highly correlated with activities of CYP2B6 (Table 1). The correlation between TGZ quinone formation and the activities of several other microsomal enzymes was poor, as evidenced by r^2 values of less than 0.5 (Table 1).

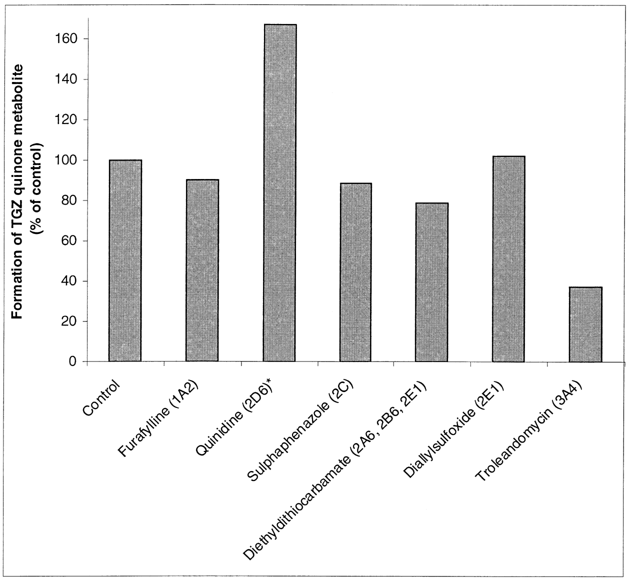

Fig. 4. Effects of CYP inhibitors on the formation of TGZ quinone in human liver microsomes. Pooled human liver microsomes (0.2 mg protein/mL) were preincubated with 5 μM furafylline, 2 μM quinidine, 10 μM sulphaphenazole, 50 μM diethyldithiocarbamate, 100 μM diallylsulfoxide, or 20 μM troleandomycin for 5 min and then incubated with 10 μM TGZ at 37° for an additional 10 min. The experimental details are described in "Materials and methods." Results are expressed as the means of two experiments. Key: (*) an approximately 67% increase of TGZ quinone formation was observed when co-incubated with quinidine. The control value (100%) was 279 pmol/min/mg protein.

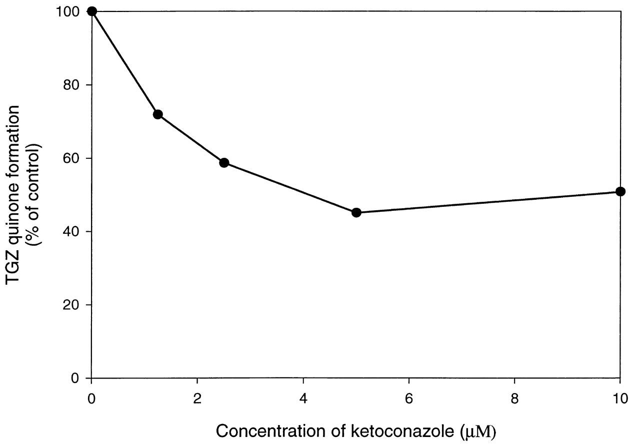

Fig. 5. Effect of ketoconazole on TGZ quinone formation in human hepatocytes. Cryopreserved human hepatocytes were preincubated with ketoconazole for 5 min and then incubated with 10 μM TGZ at 37° for an additional 10 min. The experimental details are described in "Materials and methods." Results are expressed as the means of two experiments. The control value (100%) was 123 pmol/min/million cells.

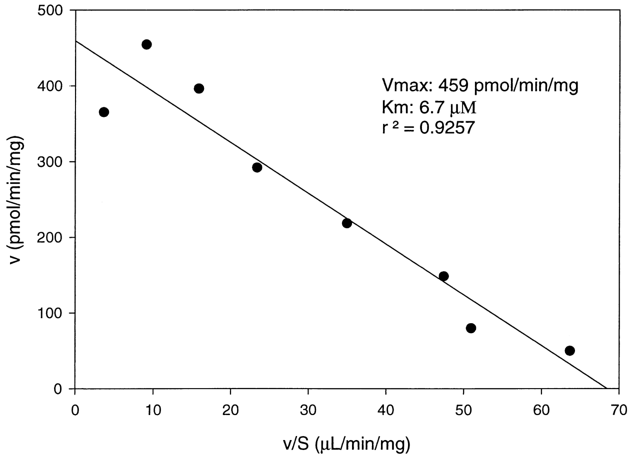

Fig. 6. Eadie–Hofstee plot for the formation of TGZ quinone in human liver microsomes. Pooled human liver microsomes (0.2 mg/mL) were incubated with TGZ at various concentrations at 37° for 10 min. The experimental details are described in “Materials and methods.” Results are expressed as the means of two experiments.

3.4. Formation of TGZ quinone in baculovirus insect cell-expressed human CYPs 3A4, 2D6, 2B6, and 2C19 and their relative contributions to TGZ quinone formation in human liver microsomes

TGZ quinone was shown to be formed with baculovirus insect cell-expressed CYP3A4 at a higher capacity than CYPs 2D6, 2C19, or 2B6. The value of CL_{int} for CYP3A4 was about 1-, 3-, and 7-fold higher than corresponding values for CYPs 2D6, 2C19, and 2B6. The relative contribution of CYPs in the formation of TGZ quinone in human liver microsomes based upon estimated specific content and CL_{int} of each enzyme is shown in Table 2.

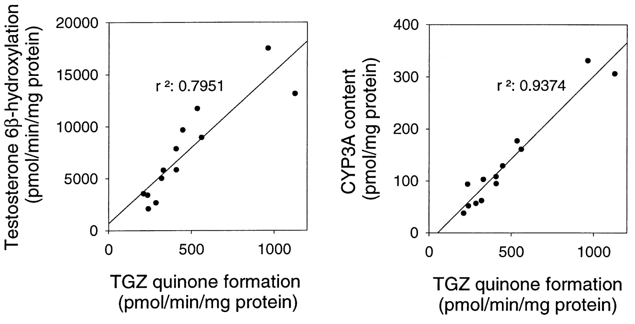

Fig. 7. Correlations between the formation of TGZ quinone and the activities (left panel) and contents (right panel) of CYP3A4 in human liver microsomes. Liver microsomes from each individual donor (0.2 mg/mL) were incubated with 10 μM TGZ at 37° for 10 min. Twelve donors were involved in this experiment. The experimental details are described in “Materials and methods.” Results are expressed as the means of two experiments.

Table 1
Correlations between formation of TGZ quinone and activities of drug-metabolizing enzymes in human liver microsomes

Enzyme	r^2 Value
Total CYP	0.4335
Cytochrome CYP-NADPH reductase	0.2574
Cytochrome b_5	0.4426
CYP1A2	0.0219
CYP2A6	0.0426
CYP2B6	0.7782
CYP2C8	0.2696
CYP2C9	0.0139
CYP2C19	0.0180
CYP2D6	0.0844
CYP2E1	0.0633
CYP3A4	0.7951
CYP4A	0.0331
FMO	0.3213

N = 12.

3.5. TGZ quinone formation in liver microsomes from rats pretreated with various CYP inducers

TGZ quinone formation was increased by 350 or 87% in rat liver microsomes from animals pretreated with DEX (CYP3A) or PB (CYPs 2B and 3A), respectively, but was decreased by 28–40% in microsomes from rats pretreated with β-NF (CYP1A) or pyridine (CYP2E1) (Fig. 8).

3.6. Formation of TGZ quinone in DEX-induced rats in vivo

Formation of TGZ quinone was increased dramatically in rats pretreated with DEX as evidenced by the approximately 260–680% increase of plasma concentrations as compared with the controls (Fig. 9). In contrast, plasma concentrations of TGZ sulfate were decreased significantly by 73–87% in rats pretreated with DEX. The plasma concentrations of TGZ were also increased by 27–74% in rats pretreated with DEX.

4. Discussion

The results presented show CYP as being able to catalyze TGZ quinone formation. First, the formation of TGZ quinone in liver microsomes was shown to be NADPH dependent, consistent with CYP. Metabolism via FMO, also NADPH dependent, is unlikely based on the known substrate specificity and the lack of correlation between TGZ quinone formation and the FMO activities in human liver microsomes [18]. Second, the likelihood of hydrogen peroxide, a byproduct of CYP reactions, being involved in TGZ quinone formation would appear remote as experiments with catalase and the catalase inhibitor sodium azide did not affect significantly quinone formation in liver microsomes. Third, superoxide anion as a byproduct of CYP

Table 2
Values of intrinsic clearance (CL_{int}) and relative contributions of human CYPs 3A4, 2D6, 2C19, and 2B6 to formation of TGZ quinone in human liver microsomes

	K_m (μM)	V_{max} (nmol/min/nmol CYP)	CL_{int}[a] (mL/min/nmol CYP)	Content in microsomes (nmol CYP/mg)	Relative CL_{int}[b] (mL/min/mg)	Relative contributions as compared with CYP3A4 (%)
CYP3A4	13.8	34.6	2.507	0.108	0.271	100
CYP2D6	10.8	12.2	1.130	0.010	0.011	4
CYP2C19	20.2	13	0.644	0.019	0.012	5
CYP2B6	33.7	10.6	0.315	0.007	0.002	1

[a] $CL_{int} = V_{max}/K_m$.

[b] Relative CL_{int} of CYP = $CL_{int} \times$ content of CYP in liver microsomes.

reaction or generated directly by CYP-NADPH reductase in the presence of NADPH was shown to contribute to less than 30% of quinone formation in human liver microsomes. However, it is not clear if this superoxide reaction plays a significant role in TGZ quinone formation *in vivo* as SOD is also present in cells and is known to rapidly dismutate superoxide anion to hydrogen peroxide. Finally, TGZ quinone formation could be inhibited by up to 75% in human liver microsomes and by approximately 50% in human hepatocytes by CYP inhibitors. The other part of quinone formation in these systems may be due to non-P450 mechanisms such as reactive oxygen species and peroxidases.

CYP3A4 was demonstrated to be the predominant CYP enzyme responsible for TGZ quinone formation in human liver microsomes and hepatocytes. Graphical analysis of an Eadie–Hofstee plot of TGZ quinone formation (Fig. 6) showed single enzyme involvement. Incubations with the CYP3A4-specific inhibitor troleanodomycin nearly completely inhibited the CYP-catalyzed quinone formation when SOD was used in the experiments to suppress superoxide-related quinone formation (Fig. 3) [19]. Increasing the concentration of ketoconazole to allow for non-specific CYP inhibition did not decrease TGZ quinone formation further (Fig. 3) [19,20], again consistent with the involvement of a single CYP enzyme. TGZ quinone formation was highly correlated with CYP3A4 levels ($r^2 = 0.9374$) and activities ($r^2 = 0.7951$) in human liver microsomes. While a correlation may appear to exist with CYP2B6 levels ($r^2 = 0.7782$), it should be pointed out that CYP2B6 is highly correlated to CYP3A4 levels in these human liver microsomes. Baculovirus insect cell-expressed human CYP3A4 was able to catalyze TGZ quinone formation at a higher capacity compared with other CYPs, including CYPs 2D6, 2C19, and 2B6 (Table 2). Finally, in human hepatocytes, the involvement of CYP3A4 in TGZ quinone formation was demonstrated by co-incubation experiments using ketoconazole (Fig. 5).

In human liver microsomes, CYPs besides CYP3A4 were shown to play a much deceased role in quinone formation as demonstrated by incubations with selective inhibitors. The correlations between the formation of the quinone and activities of CYPs 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, and 4A in human liver microsomes were also found to be poor. Given the importance and concerns of CYPs 2D6,

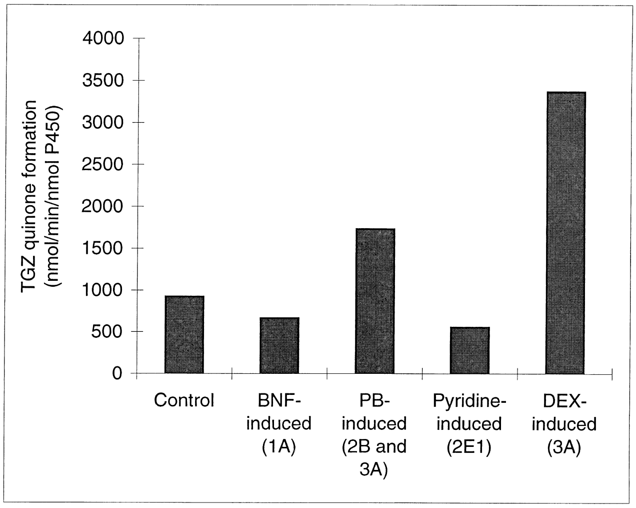

Fig. 8. Formation of TGZ quinone in liver microsomes from rats treated with CYP inducers. Rat liver microsomes (0.1 nmol/mL) were incubated with 10 μM TGZ at 37° for 10 min. The experimental details are described in "Materials and methods." Results are expressed as the means of two experiments.

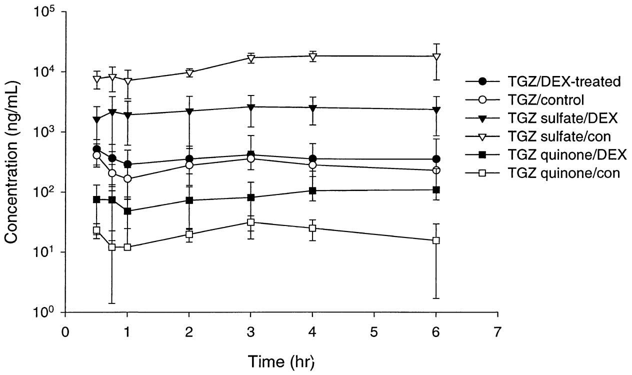

Fig. 9. Effects of the CYP3A4 inducer DEX on TGZ quinone formation in rats *in vivo*. TGZ (10 mg/kg, amorphous form) was administered orally to male Wistar rats (275–304 g) pretreated with DEX (150 mg/kg, i.p.) for 4 days. The experimental details are described in "Materials and methods." Values are means ± SD (N = 3).

2C19, and 2B6 for drug metabolism and polymorphism, we investigated their relative contributions to quinone formation versus that of CYP3A4. Our results indicate that these CYP isoforms could contribute up to 5% in TGZ quinone formation in human liver microsomes. Again, this result is consistent with that of the CYP inhibition experiments. It is interesting to point out that quinidine, while a highly selective CYP2D6 inhibitor and a substrate for CYP3A isoforms, could conceivably act as an activator of CYP 3A4 and increased TGZ quinone formation in human liver microsomes. Recently, similar observations have been reported for meloxicam and diclofenac [21,22]. The effect of quinidine on TGZ quinone formation *in vivo* is under investigation.

The formation of TGZ quinone was increased significantly in liver microsomes from rats pretreated with CYP3A inducers as compared with the other CYP inducers (Fig. 8). The involvement of CYP 3A isoforms in TGZ quinone formation was also investigated *in vivo* in rats. TGZ quinone formation was increased significantly in animals pretreated with DEX as an inducer of CYP3A enzymes (Fig. 9) [23]. Previous studies indicated that TGZ quinone formation, as assessed by plasma concentrations, was much higher in human and monkeys than in untreated mice, rats, and dogs [8,9]. This apparent species difference in TGZ quinone formation may be a result of inherent species differences in CYP3A content [24].

In conclusion, our results demonstrated that CYP3A isoforms are involved in the formation of TGZ quinone. However, the relative contribution of CYP3A4 to quinone formation in humans is uncertain given that peroxidases and ROS may be involved. The chemical nature of the chroman ring opening indicates that the CYP reaction involved is atypical. Our recent study indicated that the keto oxygen on TGZ quinone was derived from water when TGZ was converted to the quinone metabolite by CYP, implying the involvement of hydrolysis of an intermediate (quinonium cation) formed by P450 enzymes (unpublished results). The mechanism(s) for this atypical P450 reaction remains unclear.

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