

METABOLIC ACTIVATION OF TROGLITAZONE: IDENTIFICATION OF A REACTIVE METABOLITE AND MECHANISMS INVOLVED

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ABSTRACT:

Troglitazone (TGZ), the first glitazone used for the treatment of type II diabetes mellitus and removed from the market for liver toxicity, was shown to bind covalently to microsomal protein and glutathione (GSH) following activation by cytochrome P450 (P450). The covalent binding of ¹⁴C-TGZ in dexamethasone-induced rat liver microsomes was NADPH-dependent and required the active form of P450; it was completely inhibited by ketoconazole (10 μM) and GSH (4 mM). The covalent binding in P450 3A4 Supersomes (9.2 nmol of TGZ Eq/nmol P450) was greater than that with P450 1A2 (0.7), 2C8 (3.7), 2C19 (1.4), 2E1 (0.6), and 2D6 (1.1) and 3A5 (3.0). The covalent binding in liver microsomes from rats pretreated with dexamethasone (5.3 nmol of TGZ Eq bound/nmol P450) was greater than that from rats pretreated with vehicle (3.5), β-naphthoflavone (0.4), phenobarbital (1.1), or pyridine (2.5). A TGZ-GSH

adduct was detected by liquid chromatography-tandem mass spectrometry and radioactivity detection with a deprotonated quasi-molecular ion [M-H]⁻ at *m/z* 745, with fragment ions at *m/z* 438 (deprotonated TGZ moiety), and at *m/z* 306 (deprotonated GSH moiety). The TGZ-GSH adduct was determined to be 5-glutathionyl-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thiazolidine-2,4-dione based on collision-induced dissociation fragmentation, and one- and two-dimensional NMR analysis of the isolated adduct. The synthetic 5-hydroxy TGZ and the benzylidene derivative of TGZ did not react with GSH or GSH ethyl ester. The mechanisms for metabolic activation of TGZ may involve an ultimate reactive sulfonium ion which could be formed from an initial sulfoxide followed by a formal Pummerer rearrangement, or a C5 thiazolidinedione radical or a sulfur cation radical.

Troglitazone (TGZ;⁴ Rezulin, CI-991), (±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thiazolidine-2,4-dione, was the first of the glitazones used for the treatment of type II diabetes mellitus (Saltiel and Olefsky, 1996). Acting as a partial agonist for peroxisome proliferator-activated receptor-γ, TGZ alters gene expression of key proteins involved in glucose metabolism, resulting in an increase of insulin sensitivity in skeletal muscle, liver,

and adipose tissues (Lehmann et al., 1995; Saltiel and Olefsky, 1996). Although TGZ offered significant clinical benefits to many diabetic patients, it was reported to be associated with elevation of serum alanine aminotransferase in approximately 1 to 2% of patients and, in rare cases, hepatic failure (Watkins and Whitcomb, 1998). TGZ-induced liver toxicity was characterized by a delayed onset with no apparent immunological component. There was no clear association between alanine aminotransferase elevation and gender, age, dosage, and concomitant medications. As compared with drugs that cause dose-dependent intrinsic hepatotoxicity in a predictable manner, TGZ-induced hepatotoxicity is characterized as idiosyncratic and unpredictable (Lee et al., 1995; Speeg and Bay, 1995).

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⁴ Abbreviations: TGZ, troglitazone; AMX, isolated spin system CH_AH_M-CH_X; CID, collision-induced dissociation; P450, cytochrome P450; DEX, dexamethasone; FID, free induction decay; FMO, flavin-containing monooxygenase; GSH, glutathione; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MPO, myeloperoxidase; MS, mass spectrometry; β-NF, β-naphthoflavone; PB, phenobarbital; ROY-1719, 5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzylidene]-thiazolidine-2,4-dione; ROY-1993, 5-hydroxy-5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzyl]-thiazolidine-2,4-dione; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; 1D, one-dimensional; 2D, two-dimensional.

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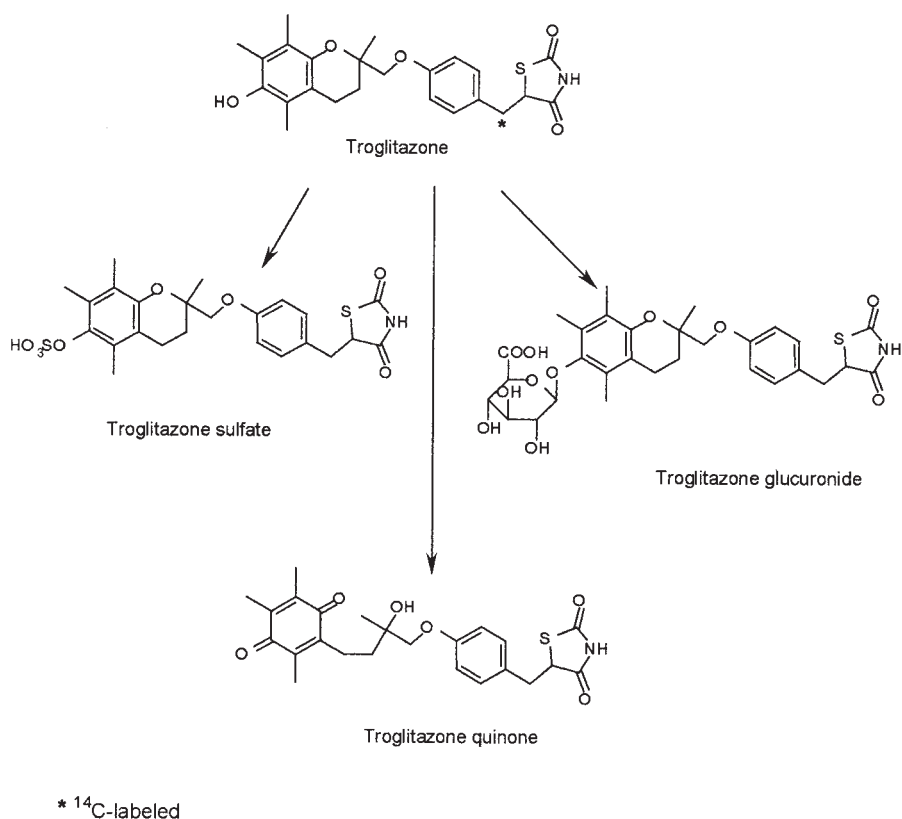


FIG. 1. Scheme of primary metabolic pathways involved in TGZ metabolism.

TGZ (M1–M5) detected in liver microsomal incubates with TGZ and in rat bile following administration of TGZ. Tettey et al. (2001) and Prabhu et al. (2002) reported a major GSH adduct of TGZ formed in rats (ML3) and in human hepatocytes (MET1/2) with a mass spectral pattern similar to that of M5, but the assignment of the position attached to GSH was different. The structure of M5 proposed by Kassahun et al. (2001) resulted from the addition of GSH to an *o*-quinone methide derivative of TGZ. However, the structures of ML3 or MET1/2 proposed by Tettey et al. (2001) and Prabhu et al. (2002) resulted from the addition of the GSH to either the thiazolidinedione ring or the adjacent methylene bridge. The exact structure of the major GSH conjugate of TGZ has yet to be determined. Due to lack of analytical standard and radiolabeled TGZ, the relative significance of the identified adducts and the covalent protein binding are unknown. As a part of our study on the mechanism involved in TGZ-induced hepatotoxicity, we investigated the formation of reactive metabolite(s) of TGZ, and the mechanisms involved, in various in vitro systems using ¹⁴C-labeled TGZ. The structure of the major GSH conjugate of TGZ was determined using tandem mass spectrometry and 1D and 2D NMR.

Materials and Methods

Chemicals. TGZ (5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzyl]-thiazolidine-2,4-dione) and ¹⁴C-TGZ were synthesized at Parke-Davis Pharmaceutical Research (Ann Arbor, MI). ROY-1719 (5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzylidene]-thiazolidine-2,4-dione) and ROY-1993 (5-hydroxy-5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzyl]-thiazolidine-2,4-dione) were synthesized at Sankyo Co. (Tokyo, Japan). Myeloperoxidase (MPO), NADPH, dexamethasone (DEX), phenobarbital (PB), pyridine, β -naphthoflavone (β -NF), ketoconazole, bovine serum albumin, GSH, GSH ethyl ester, dimethyl sulfoxide, trifluoroacetic acid (TFA), formic acid, sodium hydride, triethyl-

amine, and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO).

Liver Microsomes, Hepatocytes, and Human P450 3A4, 2D6, 2B6, or 2C19 Supersomes. Rat liver microsomes were prepared by differential centrifugation from male Fischer 344 rats (161–190 g; Harlan, Indianapolis, IN) treated i.p. with β -NF (80 mg/kg), PB (80 mg/kg), pyridine (100 mg/kg), or DEX (100 mg/kg) for 3 to 4 days, respectively. Total P450 content was measured according to the method of Omura and Sato (1964). Human liver microsomes, human P450s 1A2, 2C8, 2C19, 2D6, 2E1, 3A4, and 3A5, and human FMO₃ Supersomes were purchased from BD Gentest (Woburn, MA). Pooled human liver microsomes comprised a mixture of liver microsomes from six different individual donors. Cryopreserved human hepatocytes (lot 69) were obtained from In Vitro Technologies (Baltimore, MD).

Expression and Purification of Human P450 3A4. P450 3A4 was expressed in DH 5 α *Escherichia coli* transfected with pCW vector containing a full-length P450 3A4 cDNA (except for the deletion of codons 3–12 at the 5' end so as to modify the N terminus) (Gillam et al., 1993; He et al., 1998). The growth of the transformed *E. coli* was carried out in modified Terrific Broth, and the expression of P450 3A4 was induced by addition of 1 mM isopropyl β -D-thiogalactoside. δ -Aminolevulinic acid (0.5 mM) was added to increase heme synthesis. The membrane fraction was prepared from the bacterial cells by sonication after treatment with lysozyme and subsequently isolated from the bacterial cell homogenate by differential centrifugation. P450 3A4 was purified to homogeneity by chromatography on a DE52 column from the detergent-solubilized membranes as described previously (Gillam et al., 1993).

Isolation of NADPH-Cytochrome P450 Reductase and Cytochrome *b*₅. P450-NADPH reductase and cytochrome *b*₅ were purified from PB-induced rat liver microsomes according to methods described elsewhere (Strobel and Dignam, 1978; Waxman and Walsh, 1982).

Incubation of ¹⁴C-TGZ with Liver Microsomes, Hepatocytes, and FMO and MPO systems. ¹⁴C-TGZ (50 μ M) was incubated with rat liver microsomes (1 nmol of P450/ml), pooled human liver microsomes (0.4 mg/ml), or human P450 Supersomes (0.2 nmol/ml) in the presence or absence of NADPH in 50 mM Hepes buffer (pH 7.4) containing 30 mM MgCl₂ and 20% glycerol

at 37°C for 15 min. The reactions were started with the addition of NADPH and stopped with the addition of an equal volume of cooled methanol. Ketoconazole (10 μ M), GSH (4 mM), cysteine, or *N*-acetylcysteine (4 mM) was coincubated with liver microsomes in experiments indicated under *Results*.

¹⁴C-TGZ (50 μ M) was incubated with human hepatocytes (1 million cells/ml) in hepatocyte culture media (In Vitro Technologies) for 5, 15, 30, or 60 min at 37°C. The controls were without incubation at 37°C. The reactions were stopped by addition of an equal volume of cooled methanol.

¹⁴C-TGZ (50 μ M) was incubated with human FMO₃ Supersomes (1 mg/ml) in 0.2 M glycine buffer (pH 7.4) or 0.2 M glycine buffer (pH 9.5) containing 3.3 mM MgCl₂ in the presence or absence of 2 mM NADPH at 37°C for 15 min. The reactions were started with the addition of NADPH and stopped with the addition of an equal volume of cooled methanol.

¹⁴C-TGZ (50 μ M) was incubated with the MPO system containing purified human MPO (0.1 U/ml) in the presence or absence of H₂O₂ (2 μ M) in 0.1 M phosphate buffer (pH 7.5) at 37°C for 15 min. The reaction was started with the addition of ¹⁴C-TGZ and stopped with the addition of an equal amount of cooled methanol. GSH (4 mM) or 2 mg of bovine serum albumin (2 mg/ml) was included in the experiments to trap the reactive species.

Covalent Binding of TGZ Derivatives to Liver Microsomal Proteins.

Covalent binding was assessed according to a method described elsewhere (Chan et al., 1993). After incubation, microsomal proteins were precipitated in 10-fold cooled methanol containing 5% sulfuric acid, along with bovine serum albumin (final concentration 20 mg/ml) added at the end of the incubation as a carrier protein. The protein pellet was washed with the same solution until no radioactivity was detected in the supernatant. Finally, the pellet was dissolved in 100 μ l of 1 N NaOH at 60°C. An aliquot was neutralized with an equal amount of 1 N HCl before scintillation counting.

Preparation and Purification of TGZ-GSH Adduct. TGZ-GSH adduct was generated in P450 reaction systems with DEX-induced rat liver microsomes or reconstituted P450 3A4 system. The GSH adduct formed in these two systems was shown to be identical based on MS and NMR analyses. The experimental conditions for liver microsomes were described above. P450 3A4 (0.5 nmol/ml) was reconstituted with a mixture (1:1:1) of *L*- α -dilauroyl- and *L*- α -dioleoyl-*sn*-glycero-3-phosphocholines and phosphatidyl serine (20 μ g/ml), cholic acid (200 μ g/ml), NADPH reductase (1 nmol/ml), and cytochrome *b*₅ (0.5 nmol) in 50 mM Hepes buffer (pH 7.5) containing catalase (500 U/ml), GSH (3 mM), MgCl₂ (30 mM), EDTA (0.5 mM), and 20% glycerol. The reconstituted system was incubated with 100 μ M TGZ at 37°C for 60 min. Reactions were initiated by addition of 1 mM NADPH. At the end of the incubation, the incubation mixture was placed directly on a C18-solid phase extraction column. The loaded columns were washed with water (2 column volumes) and eluted with methanol (1 column volume). The methanol eluates were dried under N₂.

TGZ-GSH adduct was purified on a μ BONDPAK C18 column (Waters, Milford, MA; P/N84176) eluted with solvent A (0.1% formic acid) and solvent B (acetonitrile) in a stepwise linear gradient of 30% B for 5 min, 30% to 50% B for 60 min, 50% to 100% B for 5 min, and 100% B for 5 min. The eluate was monitored by UV detection at 230 nm. Fractions containing GSH adduct were detected with LC-MS/MS and were concentrated to dryness under N₂. GSH-adduct eluted at approximately 25 to 26 min under these HPLC conditions.

LC-MS/MS Determination of GSH or Cysteine Adduct with TGZ. GSH or cysteine adducts with TGZ or TGZ derivatives were determined on a Micromass Quattro-II triple quadrupole mass spectrometer (Micromass, Manchester, UK; SN# 6260) coupled with a PerkinElmer Series 200 HPLC System (PerkinElmer Life and Analytical Sciences, Boston, MA), an IN/US β -RAM radioactive detector (IN/US, Tampa, FL), and a Waters 486 UV detector. Sample introduction and ionization were achieved by electrospray ionization in the negative ion detection mode. Ionization parameters include source cone voltages of 50 V, capillary voltages of 3.0 kV, and source temperature of 120°C. The initial scan rate was 1.6 s/decade over a mass range of 50 to 800 atomic mass units. Scan data were acquired using MassLynx multitasking operating system v. 3.0. The analytical standard and the samples were monitored in MS scanning and product precursor ion detection modes. Tandem mass spectrometry experiments were based on collision-induced dissociation (CID) occurring in the rf-only collision cell of the triple quadrupole at a collision energy of 45 eV. Argon was used as the collision gas in the

range of 2.0 to 2.3 mtorr. Both product ion and precursor ion (data not shown) CID were performed. Chromatographic separation was achieved on a C18 column (150 \times 2.0 mm, 5 μ m; MetaChem MetaSil AQ; ANSYS Technologies, Ltd., London, UK) using a solvent system containing 0.1% formic acid (A) and acetonitrile (B) in a stepwise linear gradient of 10% B for 5 min, 10 to 85% B for 30 min, and 85% B for 5 min at a flow rate of 0.25 ml/min.

¹H NMR Analysis of TGZ-GSH Adduct. All NMR spectra were acquired on a Varian Inova 600 (Varian, Inc., Palo Alto, CA) running VNMR software version 6.1B and equipped with a ¹H-¹⁵N, ¹³C} flow cell with 120- μ l active volume. The NMR spectra were generated using fast Fourier transformation of free induction decays (FIDs).

For 1D spectroscopy, FIDs were accumulations of 16 (TGZ), 16 (GSH), or 2464 (TGZ-GSH adduct) transients. A spectral sweep width of 9000 to 10,000 Hz and a total recycle time of 3.2 s (TGZ, GSH) or 2.82 s (TGZ-GSH adduct) were used. The resulting time-averaged FIDs were multiplied by an exponential decay function to enhance signal-to-noise and in some cases were treated via digital signal processing to further suppress solvent signals.

The 2D TOCSY spectra were generated using fast Fourier transformation of a 2D FID matrix. A spectral sweep width of 9000.9 Hz and a total recycle time of 1.146 s were used. The resulting time-averaged FID matrices were multiplied by shifted sinebell functions in both dimensions (sb = sbs = -0.114, sb1 = sbs1 = -0.028) to enhance signal-to-noise and minimize truncation artifacts.

Chemical Reactivity of TGZ Derivatives ROY-1719 and ROY-1993.

ROY-1719 was dissolved in dimethyl sulfoxide and activated with either triethylamine (2 Eq), TFA (2 Eq), or sodium hydride (2 Eq). This activation was followed by the addition of GSH or GSH ethyl ester (2 Eq). The reaction mixtures were sonicated for 1 h and analyzed by ¹H NMR (Varian Unity + 400 MHz) and MS after 1 h, 24 h, and 7 days. MS analysis was carried out on a Micromass Platform LC mass spectrometer using OpenLynx open access software, a Hewlett-Packard (Palo Alto, CA) HP1100 quaternary high performance liquid chromatograph, and a Gilson 215 Liquid Handler (Gilson Medical Electronics, Middleton, WI) as an autosampler, and a Micromass LCT orthogonal-acceleration time-of-flight mass spectrometer. Samples were introduced by continuous infusion of a solution using a Harvard 22 syringe pump. ROY-1993 (100 μ M) was treated with buffers at pH values ranging from 3 to 10 for up to 24 h with or without GSH (10 mM). The products were analyzed by LC-MS/MS methods as described above.

Results

Covalent Protein Binding of ¹⁴C-TGZ. As shown in Fig. 2, incubation of ¹⁴C-TGZ (50 μ M) with rat liver microsomes resulted in covalent binding to microsomal proteins. The covalent binding in liver microsomes from rats pretreated with DEX (P450 3A inducer, 5.3 nmol of TGZ Eq bound/nmol P450) was greater than that from rats pretreated with vehicle (control, 3.5), β -NF (P450 1A inducer, 0.4), PB (P450 2B inducer, 1.1), or pyridine (P450 2E1 inducer, 2.5). The covalent binding was NADPH-dependent and completely inhibited by coincubation with the P450 inhibitor ketoconazole (10 μ M) in DEX-induced rat liver microsomes. There was no significant covalent binding in human liver microsomes in the presence of NADPH compared with the minus NADPH control (1.7 versus 1.4 nmol of TGZ Eq/mg protein, respectively).

Covalent protein binding of ¹⁴C-TGZ was examined using cDNA-expressed P450s 1A2, 2C8, 2C19, 2D6, 2E1, 3A4, and 3A5 (Fig. 3). The covalent binding in P450 3A4 incubates (9.2 nmol of TGZ Eq/nmol P450) was greater than that in P450s 3A5 (3.0) 2C8 (3.7), 1A2 (0.7), 2C19 (1.4), 2D6 (1.1), or 2E1 (0.6). There was no significant covalent binding in FMO₃ or MPO incubates (data not shown).

The ¹⁴C-TGZ residues remaining in human hepatocytes after extensive washing with methanol containing 5% sulfuric acid were 0.61 nmol of TGZ Eq bound/million cells after incubation at 37°C for 15 min compared with 0.28 nmol of TGZ Eq bound/million cells for the control without incubation. The covalent binding was not increased when ¹⁴C-TGZ was incubated for 30 min or 60 min.

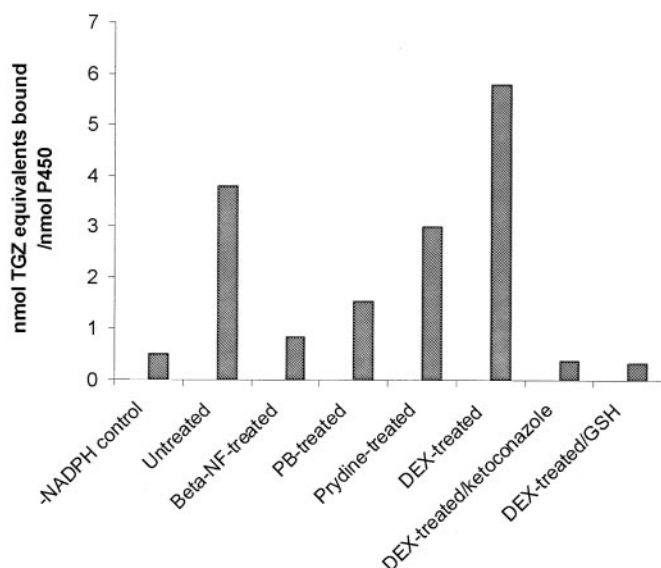


Fig. 2. Covalent binding of ¹⁴C-TGZ to liver microsomes from rats pretreated with P450 inducers.

¹⁴C-TGZ (50 μM) was incubated with liver microsomal reaction systems for 15 min at 37°C. Ketoconazole (10 μM) or GSH (4 mM) was coincubated as indicated. The results are expressed as means of two experiments.

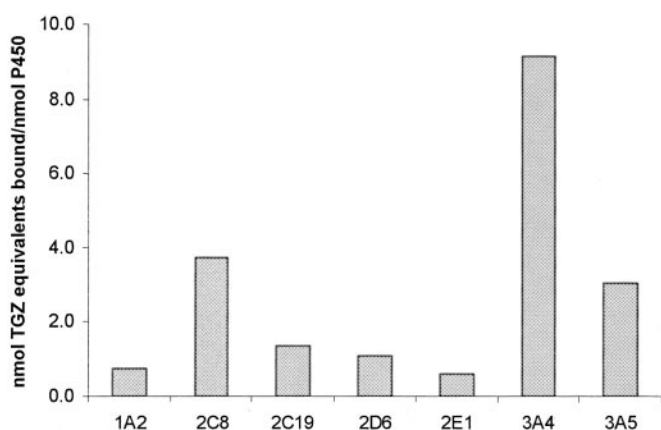


Fig. 3. Covalent protein binding of ¹⁴C-TGZ in cDNA-expressed human P450 isoforms.

¹⁴C-TGZ (50 μM) was incubated with human P450 Supersomes reaction systems for 15 min at 37°C. The results are expressed as means of two experiments.

Formation of TGZ-GSH Adduct. GSH adducts of TGZ or TGZ derivatives were detected by LC-MS/MS coupled with radioactive detection. As shown in Fig. 4, a radioactive peak with a retention time of 38.03 min was detected with a deprotonated quasi-molecular ion [M-H]⁻ at *m/z* 745, which was consistent with a TGZ-GSH adduct, in the incubates of ¹⁴C-TGZ with DEX-induced rat liver microsomes in the presence of GSH. The radioactive peak was analyzed by electrospray MS/MS in negative ion mode, and showed diagnostic fragment ions [M-306-H]⁻ at *m/z* 438 (deprotonated TGZ moiety) and [M-438-H]⁻ at *m/z* 306 (deprotonated GSH moiety). The cysteine or *N*-acetylcysteine adduct of TGZ was also detected when TGZ was incubated with DEX-induced rat liver microsomes in the presence of cysteine or *N*-acetylcysteine (data not shown). The same GSH adduct was formed in human liver microsomes and hepatocytes. Possible GSH adducts of TGZ quinone or any monooxidized TGZ derivatives were not detected in DEX-induced rat liver microsomes under the

present conditions, although approximately 60% of TGZ was converted to TGZ quinone (Fig. 4).

The GSH adduct of TGZ was not detected, nor was any GSH adduct with TGZ quinone or other monooxidized TGZ derivatives with deprotonated molecular ion at *m/z* 761 [M-H]⁻ when TGZ was incubated with the MPO system in the presence of GSH (data not shown).

Structural Elucidation of TGZ-GSH Adduct by Tandem MS and ¹H NMR. The CID spectrum of the isolated TGZ-GSH adduct is shown in Fig. 5. As illustrated, MS/MS product ions at *m/z* 143, 128, and 74 are the characteristic fragmentations derived from the GSH moiety. The ions at *m/z* 472 and 272 are formed by cleavage of the thiol bond with the sulfur atom attached to TGZ. Loss of the intact chroman moiety from the ion at *m/z* 472 results in an ion at *m/z* 254. Whereas loss of CONH₂ from *m/z* 254 results in an ion at *m/z* 210, the loss of the phenoxy moiety from *m/z* 254 affords a unique and diagnostic ion at *m/z* 160. The product ion at *m/z* 179 results from the cleavage of the unchanged chroman ring. This fragmentation pattern is only consistent with attachment on the thiazolidinedione moiety of TGZ, either at the 5-position as illustrated, or at the adjacent benzylic position.

The 600-MHz ¹H NMR spectrum of the TGZ-GSH adduct is shown in Fig. 6. The spectrum is characterized by two aromatic doublets at 7.2 and 6.85 ppm that correspond to the 1,4-substituted phenyl ring in TGZ, a multiplet at 4.62 ppm, and several multiplets in the region between 1 and 4.2 ppm. The 2D TOCSY spectrum was recorded to elaborate the scalar couple spin systems in the adduct (Fig. 7). The multiplet at 4.62 is coupled with two peaks at 2.92 and 2.70 ppm. This AMX (CH_ACH_MCH_X)₂-type spin system is consistent with the cysteine residue in GSH. Additional correlation to the glutamine residue of GSH is indicated in Fig. 7. Both TGZ and cysteine have AMX spin systems in which the methine signals resonate at 4.45 and 4.62 ppm, respectively; however, the TGZ-GSH adduct has only one methine signal at 4.62 ppm. The possibility that this methine is upfield-shifted to coincide with the H₂O peak at 4.25 ppm is ruled out by the 2D spectrum showing no cross peaks from the 4.25 ppm solvent peak to the 3.0- to 3.5-ppm region (Fig. 7). All of the evidence indicates that the TGZ-GSH adduct loses the TGZ AMX system because of the attachment of the glutathionyl moiety to the C5 position, which is entirely consistent with the mass fragmentation pattern. Based on the MS and NMR spectra, the TGZ-GSH adduct was determined to be 5-glutathionyl-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thiazolidine-2,4-dione.

Reactivity of TGZ Derivatives. The characteristic vinylic proton and molecular weight of ROY-1719 (Fig. 8) remained unchanged after treatment with triethylamine, TFA, or sodium hydride in the presence of GSH or GSH ethyl ester. ROY-1719 (Fig. 8) remained unchanged after treated with buffer at pH values ranging from 3 to 10. No adduct between GSH and ROY-1719 or ROY-1993 was detected with LC-MS/MS or NMR.

Discussion

Our results demonstrated that TGZ undergoes metabolic activation by P450 enzymes, in particular, the P450 3A4 isoform, to form reactive metabolites that bind covalently to proteins and nucleophiles such as GSH and cysteine. The major TGZ-GSH adduct was isolated, and its structure was determined by tandem mass spectrometry and NMR.

P450 enzymes were shown to catalyze the metabolic activation of TGZ. Covalent protein binding of TGZ was observed only in P450 systems, and not in the human MPO functional system or in human FMO₃ Supersomes. The covalent protein binding in liver microsomes

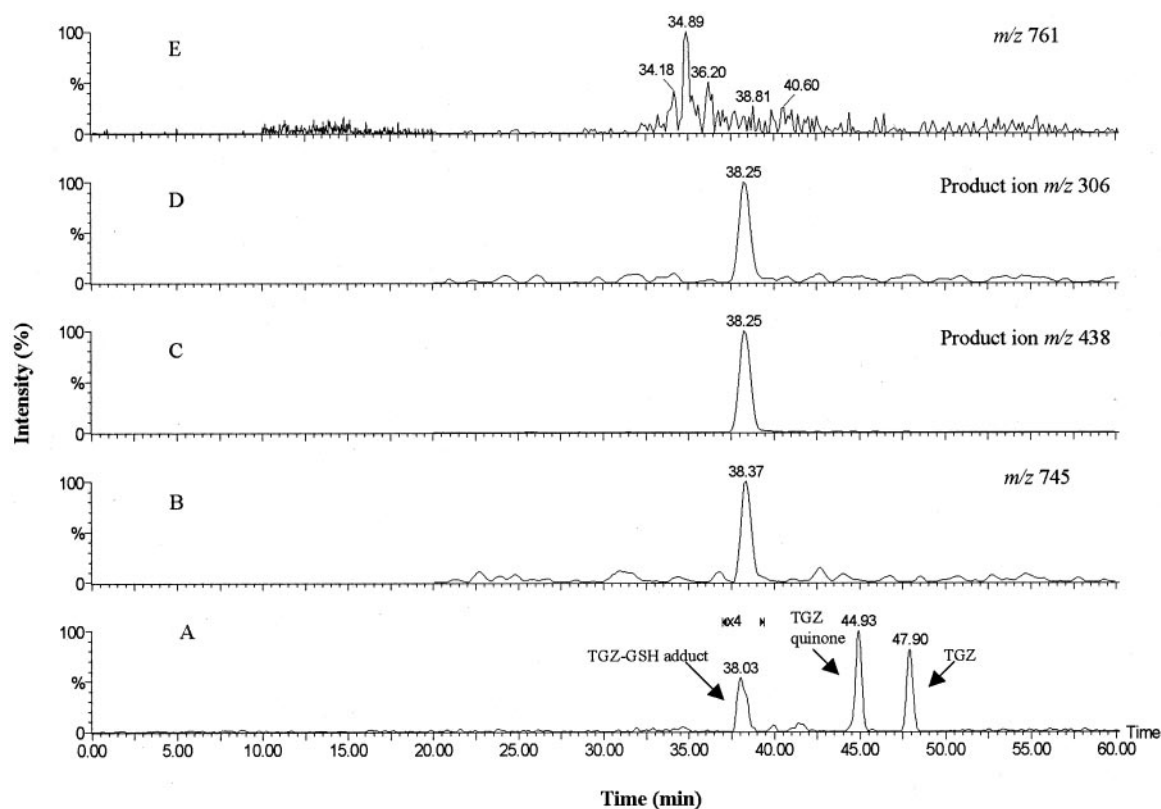


FIG. 4. Representative HPLC profiles for ^{14}C -TGZ-GSH adduct formed in DEX-induced rat liver microsomes.

The eluate was monitored with radioactive detection (A) and MS/MS in negative ion mode. Selected ion chromatograms are shown for the precursor ion at m/z 745 of the deprotonated molecular ion of TGZ-GSH adduct (B), the product ion of deprotonated TGZ moiety at m/z 438 (C), and the product ion at m/z 306 of deprotonated GSH moiety (D). E, ion chromatogram for the precursor ion at m/z 761, as would be expected with TGZ quinone-GSH adduct.

was NADPH-dependent and required the active forms of the enzymes, and was completely inhibited by coincubation with the P450 inhibitor ketoconazole (10 μM). Among several human P450s, P450 3A4 showed higher activity than other isoforms in the metabolic activation of TGZ. The covalent protein binding with P450 3A4 was greater than that with several other major human P450 isoforms. Consistently, the covalent binding in liver microsomes from rats pretreated with the P450 3A inducer DEX was also greater than that from rats pretreated with other P450 inducers. Human P450 2C8 and rat 2C11 (the predominant constitutive P450 isoform in untreated male rat liver microsomes) were shown to catalyze metabolic activation of TGZ but were considerably less active than P450 3A isoforms. In addition, the covalent binding in liver microsomes from rats pretreated with the P450 1A inducer β -NF and the P450 2B inducer PB was lower than that from rats pretreated with vehicle (control), suggesting that P450 1A and 2B isoforms are less active in catalyzing formation of TGZ reactive metabolites than P450 2C11, whose expression is known to be repressed by treatment with the P450 inducers (Correia, 1995). The lower covalent binding of ^{14}C -TGZ in human liver microsomes was probably due to the lower specific activity of P450 3A isoforms compared with DEX-induced rat liver microsomes or cDNA-expressed P450 3A4 Supersomes. In human hepatocytes, the covalent protein binding of ^{14}C -TGZ could be prevented by endogenous GSH or other nucleophiles. Collectively, these results indicate that the metabolism of TGZ by P450 enzymes, in particular, P450 3A isoforms, leads to formation of reactive metabolites that bind covalently to microsomal proteins.

Reactive metabolites of TGZ were able to react with GSH. The covalent binding of ^{14}C -TGZ to microsomal protein was completely

prevented by coincubation with 4 mM GSH in DEX-induced rat liver microsomes, indicating that TGZ reactive species were trapped with GSH (Fig. 2). A TGZ-GSH adduct was detected by LC-MS/MS coupled with radioactive detection (Fig. 4). Consistent with the covalent binding results, the GSH adduct was formed only in P450 reaction systems, providing additional evidence for the involvement of P450 enzymes in metabolic activation of TGZ. TGZ reactive metabolites appear relatively stable and are able to diffuse from the active site of P450 to react with GSH. These features are different from typical mechanism-based inactivators that form highly reactive intermediates bound covalently at the enzyme active site in such a way that GSH could not completely prevent covalent binding (Walsh, 1984; He et al., 1999).

The location of the glutathionyl moiety in the molecule of the TGZ-GSH adduct was determined to be the C5 position of the thiazolidinedione ring by LC-MS/MS and NMR. As illustrated in Fig. 5, the fragments at m/z 472, which are a result of the cleavage of the thiol bond, are crucial for identification of the location of the glutathionyl moiety. The loss of the intact chroman moiety from the ion at m/z 472 results in an ion at m/z 254. A product ion at m/z 160, which is due to loss of the phenoxy moiety from the fragment at m/z 254, is the unique and diagnostic fragment ion that indicates the sulfur atom is attached to either the C5 position or the benzylic position. The precise position was further determined by 1D and 2D NMR. As shown in Figs. 6 and 7, only one single methine resonance at 4.62 ppm was obtained from the isolated GSH adduct, although both TGZ and cysteine have AMX spin systems in which the methine signals resonate at 4.45 and 4.62 ppm, respectively. The multiplet at 4.62 is coupled with two peaks at 2.92 and 2.70 ppm in an AMX-type spin

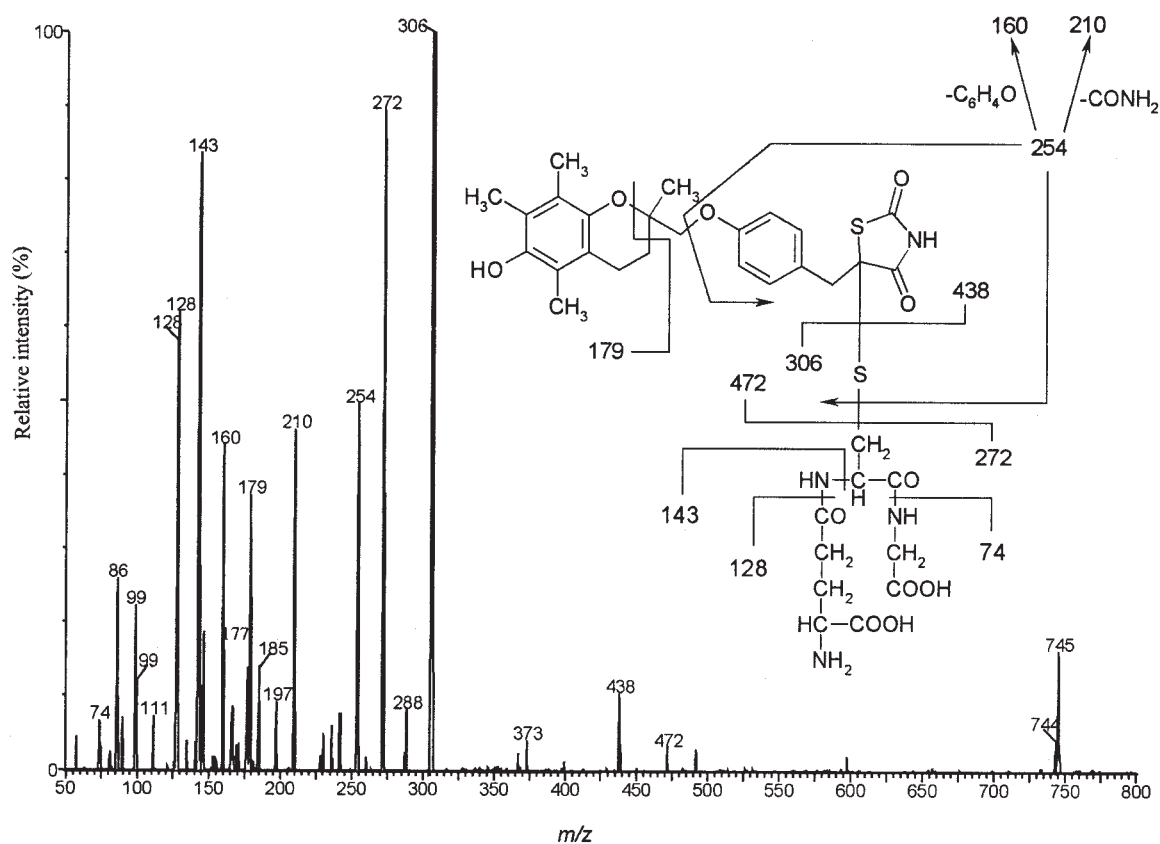


Fig. 5. Representative CID spectrum of the TGZ-GSH adduct by electrospray ionization-MS/MS (negative ion mode).

The TGZ-GSH adduct was formed in P450 reaction systems including liver microsomes or the P450 3A4 reconstituted system.

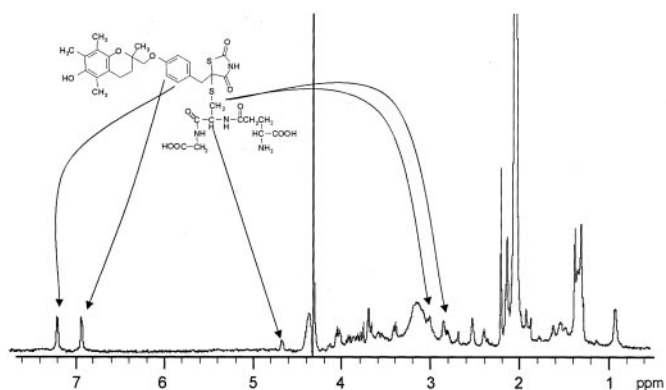


Fig. 6. ^1H NMR spectrum of the isolated TGZ-GSH adduct.

Only one methine signal at 4.62 ppm is observed with the adduct, although both TGZ and cysteine have AMX spin systems in which the methine signals resonate at 4.45 and 4.62 ppm, respectively. The loss of the AMX spin system of TGZ is due to the attachment of the glutathionyl group to the C5 position of the thiazolidinedione ring.

system, which is consistent with the cysteine residue of GSH. The lack of the methine signal at 4.45 ppm indicates that the TGZ-GSH adduct loses the TGZ AMX group due to the attachment of the glutathionyl moiety to the C5 position of the thiazolidinedione ring. If the glutathionyl moiety were attached to the benzylic methylene adjacent to the C5 position of TGZ, there would be three methine signals with expected chemical shifts in the range from 4.4 to 4.8 ppm. However, the attachment at the C5 position should lead to only one such methine proton, which is exactly what was observed. Of the possibilities consistent with MS analysis, the NMR spectral patterns can only be explained by the

attachment of the glutathionyl moiety at the C5 position of the thiazolidinedione ring as illustrated in Fig. 5. This adduct may be the same as ML3 or MET1/2, which was reported to be the major adduct formed in rats or in human hepatocytes, as judged based on the similarity of the MS fragmentation pattern (Tetty et al., 2001; Prabhu et al., 2002). Collectively, the major TGZ-GSH adduct is determined to be 5-glutathionyl-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thiazolidine-2,4-dione.

There are several possible mechanisms for P450-mediated activation of TGZ **1**, leading to the formation of the observed GSH adduct **4** (Fig. 8). An initial sulfoxide **2** could be formed by P450 via two-electron oxidation or initiated by abstraction of an electron from the sulfur atom (radical **5**). The sulfoxide could be a reactive intermediate as demonstrated in metabolic activation of thiophenes (Valadon et al., 1996). However, compared with thiophenes, sulfoxidation on the thiazolidinedione ring would be more difficult because of the electron-withdrawing carbonyl groups. The sulfoxide **2** could undergo a formal Pummerer rearrangement, which could involve formation of a hydroxysulfonium species by protonation, to form an ultimate reactive species **3**, possibly by loss of water (House, 1972). The sulfonium ion **3** should be prone to nucleophilic attack by GSH on the C5 position.

However, the sulfoxide is not the only intermediate that could form the ultimate reactive species **3**. The sulfonium ion **3** could be formed directly from radical cation **5** by loss of a proton, or from radical **6** by loss of an electron. The pathways appear more favored than the sulfoxide pathway because there is no intervening intermediate required for the transition. Radical **6** would be expected to be a considerably stable radical and could be formed by hydrogen abstraction

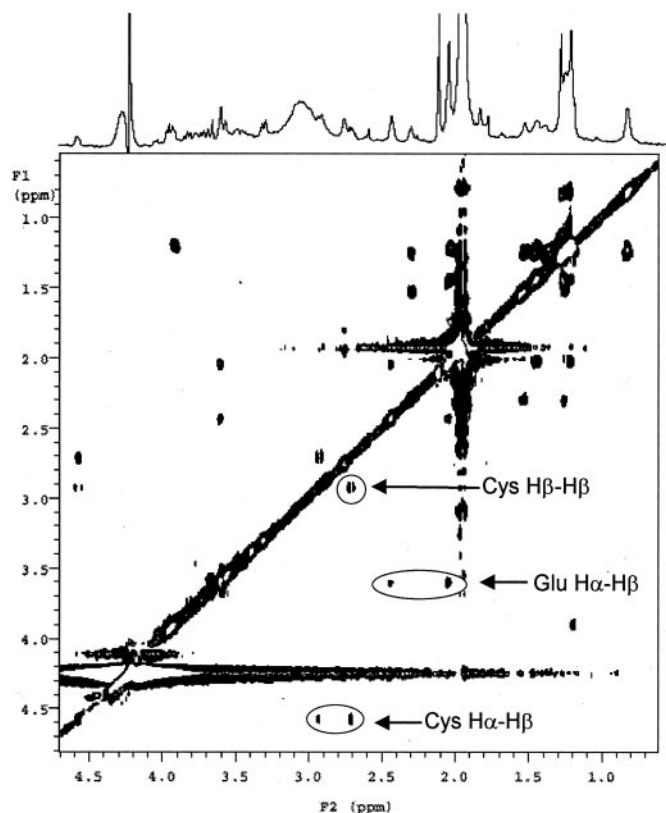


FIG. 7. 2D TOCSY spectrum of the isolated TGZ-GSH adduct.

The multiplet at 4.62 ppm is coupled with cysteine peaks at 2.92 and 2.70 ppm. The confirmation that only one methine signal belongs to the cysteine in the adduct indicates that the TGZ-GSH adduct loses the AMX spin system of TGZ because of the attachment of the glutathionyl group to the C5 position of the thiazolidinedione ring.

on the C5 position, or produced from **5** by loss of a proton. Radical **6** could be a precursor for a hemithioacetal **7** that could be a potential precursor for **4**. However, the electron-withdrawing carbonyl groups make the required elimination of the hydroxyl group difficult and less likely. Consistent with this hypothesis, there was no observable adduct **4** when the synthetic **7** (ROY-1993) was exposed to GSH at pH values from 3 to 10, suggesting that **7** is not an intermediate for the formation of **4**. Another potential product from **6** is a benzylidene **8**, which might be expected to form a Michael adduct with GSH to produce **9**. To investigate this possibility, the synthetic **8** (ROY-1719) was treated with GSH in the presence of various catalysts, such as triethylamine, sodium hydride, and trifluoroacetic acid under conditions which should have allowed equilibrium formation of adduct **9**. But, in no case was any Michael adduct detected by either NMR or MS techniques over a time period of a few minutes to 1 week. By ruling out some possible pathways, we postulate that a sulfonium ion intermediate (**3**) is the ultimate reactive species that is prone to nucleophilic attack by GSH at the C5 position of the thiazolidinedione ring. The ultimate sulfonium ion intermediate could be formed from an initial sulfoxide (**2**) or a relatively stable C5 thiazolidinedione radical (**6**) or a sulfur cation radical (**5**).

In summary, our results indicate that the thiazolidinedione portion of TGZ undergoes metabolic activation by P450 enzymes, in particular, P450 3A isoforms, to form reactive intermediates covalently bound to microsomal proteins and GSH. The structure of the major GSH-adduct formed in liver microsomes and in the P450 3A4 reconstituted system was determined to be 5-glutathionyl-5-[4-(6-hydroxy-

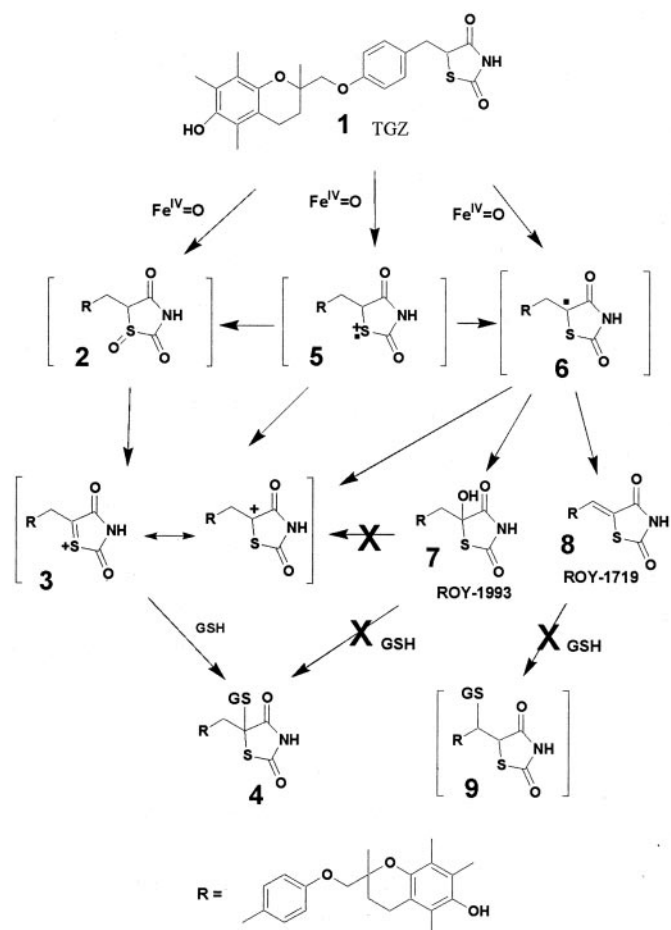


FIG. 8. Postulated mechanism for bioactivation of TGZ and formation of the major GSH adduct *in vitro*.

2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thiazolidine-2,4-dione. The mechanisms for P450-mediated metabolic activation of TGZ may involve the formation of an ultimate reactive sulfonium which could be derived from an initial sulfoxide followed by a formal Pummerer rearrangement, or a C5 thiazolidinedione radical or a sulfur cation radical. Because reactive metabolites have been demonstrated in several cases to be associated with idiosyncratic adverse reactions, there is a hypothesis about the potential roles of the reactive metabolites of TGZ in the cause of the idiosyncratic liver toxicity associated to TGZ. Other potential mechanisms for the liver toxicity involve interference of the bile salt export pump and the organic anion-transporting polypeptide transporters by the sulfate conjugate of TGZ, and potential cell death due to activation of c-Jun N-terminal protein kinase (Funk et al., 2001; Bae and Song, 2003; Nozawa et al., 2004). However, it would be difficult to test those hypotheses in a manner that would have a clinical relevance because of the idiosyncratic nature of the liver toxicity associated with TGZ.

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