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Cholestatic Potential of Troglitazone as a Possible Factor Contributing to Troglitazone-Induced Hepatotoxicity: In Vivo and in Vitro Interaction at the Canalicular Bile Salt Export Pump (Bsep) in the Rat

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ABSTRACT

Troglitazone is a thiazolidinedione insulin sensitizer drug for the treatment of type 2 non–insulin-dependent diabetes mellitus (NIDDM). Based on an increasing number of reports on troglitazone-associated liver toxicity, the cholestatic potential of troglitazone has been investigated. Rapid and dose-dependent increases in the plasma bile acid concentrations were observed in rats after a single intravenous administration of troglitazone. A radiolabeled taurocholic acid tracer accumulated in liver tissue, indicating an interference with the hepatobiliary export of bile acids. In isolated canalicular rat liver plasma membrane preparations, troglitazone competitively inhibited the ATP-dependent taurocholate transport (apparent K_i value, 1.3 μM), mediated by the canalicular bile salt export pump (Bsep). Troglitazone sulfate, the main troglitazone metabolite eliminated into bile, also showed competitive Bsep inhibition with an apparent K_i value of 0.23 μM. A comparable inhibition was observed for both compounds in canalicular plasma membrane vesicles prepared from Mrp2-deficient (TR^-) rats, suggesting a direct (*cis*-) inhibition of Bsep by troglitazone and troglitazone sulfate. A high accumulation potential was observed for troglitazone sulfate in rat liver tissue, indicating that the hepatobiliary export of this conjugated metabolite might represent a rate-limiting step in the overall elimination process of troglitazone. This accumulation in combination with the high Bsep inhibition potential suggested that mainly troglitazone sulfate was responsible for the interaction with the hepatobiliary export of bile acids at the level of the canalicular Bsep in rats. Such an interaction might lead to a troglitazone-induced intrahepatic cholestasis in humans as well, contributing to the formation of a troglitazone-induced liver toxicity.

Troglitazone is an insulin sensitizer of the thiazolidinedione class for the treatment of type 2 non–insulin-dependent diabetes mellitus (Chen 1998). In clinical trials, elevations in liver enzyme levels were observed; since its market introduction in early 1997, several cases of fulminant hepatic failure were reported, leading to withdrawal of this compound from the market in March 2000 (Gitlin et al., 1998; Neuschwander-Tetri et al., 1998; Shibuya et al., 1998; Herrine and Choudhary, 1999). The mechanism(s) underlying the troglitazone-associated hepatotoxicity are unclear at present. Troglitazone is extensively metabolized in the liver mainly by sulfation, glucuronidation, and oxidation (Loi et al., 1999). The main metabolite, troglitazone sulfate, undergoes biliary excretion and accounted for up to 60% of the dose in rats (Kawai et al., 1997). A strong reduction of the bile flow has been observed in isolated perfused rat liver (Preininger et al., 1999) and, in some patients, indications for a drug-induced cholestasis were described (Gitlin et al., 1998).

An intrahepatic cholestasis can be induced by an interference with the vectorial transport of biliary constituents from blood to bile resulting in an intracellular accumulation of bile salts (Meier-Abt, 1990; Erlinger, 1997; Trauner et al., 1998). High intracellular bile salt levels have been reported to induce cellular necrosis and mitochondrial dysfunction because of their intrinsic detergent activity (Delzenne et al., 1992; Desmet, 1995; Gores et al., 1998). The vectorial transport of both endobiotics (bile acids, steroids, bilirubin-glucuronide, and other metabolic products) and xenobiotics and their metabolites from plasma to bile is facilitated by several transport systems (Zimniak et al., 1999). The export across the canalicular membrane, where the greatest uphill concentration gradient has to be overcome, often represents the rate-limiting step in this excretion process (Kadmon et al., 1993; Arrese et al., 1998). For bile acids, this step is catalyzed by a primary active, ATP-dependent transporter of the ATP-binding cassette protein family, the canalicular bile salt export pump (Bsep) localized in the canalicular liver plasma membrane (Gerloff et al., 1997). For several cholestatic com-

ABBREVIATIONS: Bsep, canalicular bile salt export pump; HPLC high-performance liquid chromatography; cLPMV, canalicular liver plasma membrane vesicles; Mrp2, rat canalicular multidrug resistance protein 2.

pounds, interactions with the export of bile acids were found at the level of the canalicular ATP-dependent Bsep (Stieger et al., 2000). The immune-suppressive agent cyclosporin A was found to inhibit the canalicular Bsep in vitro (Kadmon et al., 1993). A similar mechanism was recently postulated for the NSAID sulindac (Bolder et al., 1999), for rifamycin, rifampicin, and glibenclamide (Stieger et al., 2000).

The cholestatic potential of troglitazone has been studied using an in vivo rat model, established with several cholestatic reference compounds. A rapid, dose-dependent increase in the bile acid plasma concentration was observed after a single intravenous administration of troglitazone. Mechanistic in vitro studies indicated that troglitazone and, to a much greater extent, troglitazone sulfate, competitively inhibited the ATP-dependent taurocholate transport, catalyzed by the ATP-dependent Bsep. This inhibition of the hepatobiliary export of bile salts by troglitazone and troglitazone sulfate may lead to a drug-induced intrahepatic cholestasis in humans, contributing as one possible factor to the hepatotoxicity of troglitazone.

Materials and Methods

Substances. All buffer salts (HEPES, Tris, $NaHCO_3$, KNO_3, $Mg(NO_3)_2$, $CaCl_2$, and $MgSO_4$), taurocholic acid, ATP, creatine phosphate, and creatine phosphokinase were from Fluka AG (Buchs, Switzerland). Sucrose was obtained from E. Merck (Darmstadt, Germany). Radiolabeled [^{3}H]taurocholic acid was purchased from DuPont NEN (Boston, MA) at a specific activity of 128.4 GBq/mmol. Radiolabeled [^{14}C]taurocholic acid was purchased from DuPont NEN at a specific activity of 1.7 GBq/mmol. Compounds tested as inhibitors: troglitazone was synthesized at Roche Diagnostics (Mannheim, Germany); glibenclamide (Ro 06–9036) was obtained from the Roche compound repository, and cyclosporin A was obtained from Fluka AG. Water was prepared with a MilliQ-plus apparatus (Millipore, Bedford, MA). All other chemicals and solvents were of the highest purity available from commercial sources where not otherwise stated.

Male Wistar rats were obtained from RCC, Ltd. (Füllinsdorf, Switzerland) and had free access to food and water. Male Mrp2-mutant Wistar (TR^-) rats were supplied by Dr. P.J. Oude Elferink (Academic Medical Center, Amsterdam, Netherlands) and were kept under quarantine (4 weeks) at RCC, Ltd., before initializing the studies.

In Vivo Cholestatic Effect in Rats. The in vivo cholestatic potential of troglitazone was assessed in rats. Male Wistar rats were treated intravenously with troglitazone at doses of 1 to 50 mg/kg. The compound was dissolved in glycofurol and administered as a bolus via the tail vein or a jugular vein catheter (0.5–1 ml/kg). Control rats were treated with the same volume of glycofurol. Radiolabeled [^{14}C]taurocholate tracer was added 8 min before the end of the experiments when animals were sacrificed. The tracer (4 μCi/kg, 86 nmol/kg) was given as a solution in physiologic saline (0.8 ml/kg) via the jugular vein catheter. Blood samples were taken by retro-orbital puncture or the jugular vein catheter before and at indicated time points after troglitazone application. At the end of the experiments, the animals were anesthetized and sacrificed by exsanguination and livers were frozen at −20°C. Plasma was prepared (stabilized by EDTA/NaF) and frozen at −20°C until analysis. Bile acid plasma concentrations were determined enzymatically using a commercially available test kit (Sigma 450-A) after the recommended procedure. Remaining plasma samples and the liver tissue were used for HPLC analysis and determination of radioactive concentrations by liquid scintillation counting. The relative changes in plasma bile acid concentrations were determined by subtracting the basal bile acid concentration before treatment and ED_{50} doses were calculated by nonlinear fitting of these results with Origin (MicroCal Software, Northampton, MA).

Preparation of Rat Liver Canalicular Membrane Vesicles (cLPMV). cLPMV were prepared and purified on sucrose density step gradients as outlined elsewhere (Boyer et al., 1983; Meier et al., 1984; Fricker et al., 1989; Wolters et al., 1991). Livers from male Wistar rats or the Mrp2-mutant TR^- rats were used. Briefly, the mixed plasma membrane vesicles were prepared from 10 livers (in two batches of five livers each) using a loose-fitting Dounce homogenizer. The crude membranes were purified on a sucrose density gradient, at the 44%/36.5% sucrose (w/w) interphase after separation for 150 min at 95,000*g*/4°C (Kontron TST 28.38 rotor; Kontron Instruments, Watford, Herts, UK). The plasma membranes were vesiculated (tight Dounce homogenizer, 50 strokes) and shock frozen in liquid nitrogen. Plasma membranes from two batches were thawed, combined, vesiculated as before, and purified on a second sucrose density step gradient [38%/34%/31% (w/w) sucrose] centrifuged at 195,700*g* for 180 min at 4°C (Kontron TST 41.14 rotor). The separated canalicular and basolateral plasma membrane vesicles were washed once and frozen in membrane suspension buffer (10 mM Tris/HEPES, pH 7.5, 250 mM sucrose). Marker enzymes [ATPase (Scharschmidt et al., 1979), alkaline phosphatase (Keefe et al., 1979), leucine aminopeptidase (Goldbarg and Rutenburg, 1957)] and total protein concentration (Smith et al., 1985) were determined at each purification step.

Incubation of cLPMV. The uptake of [^{3}H]taurocholic acid into liver plasma membrane vesicles was measured following a described method (Stieger et al., 1992; Wolters et al., 1992) with slight modifications. The incubations contained 10 mM Tris/HEPES, pH 7.4, 250 mM sucrose, 10 mM KNO_3, 10 mM $Mg(NO_3)_2$, an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, and 100 μg/ml creatine phosphokinase) and [^{3}H]taurocholate (typically 1 μM, 3.47 μCi/nmol) in a total volume of 100 μl. The ATP was replaced by AMP or omitted in blank incubations. Inhibitors were added from dimethyl sulfoxide stock solutions (50× concentration) and the same amount of solvent was added to the control incubations. The uptake was started by the addition of the resuspended liver plasma membranes (20 μg per assay) followed by incubation at 37°C (typically 2 min). The reaction was stopped by addition of 2 ml of ice-cold assay buffer and subjected to rapid filtration (Meier et al., 1987) using a rapid filtration manifold (Millipore, Bedford, MA) equipped with 0.45-μm mixed nitrate/acetate cellulose filters (Millipore HAWP, Bedford, MA). The filters were equilibrated by filtration of 1 ml of 1 mM taurocholate in the assay buffer before rapid filtration to reduce nonspecific binding of the labeled taurocholate. The vesicles were filtered and the membranes were rinsed twice with 2 ml of ice-cold assay buffer. The filters were dissolved (0.5 ml of acetone) and the radioactivity was determined by liquid scintillation counting. Active, ATP-dependent transport was determined as the difference between the uptake in presence of ATP and the control incubation without ATP. The IC_{50} and apparent K_i concentrations were calculated by nonlinear fitting of these results with Origin (Microcal) and Grafit (Erithacus Software, Horley, Surrey, UK), respectively.

HPLC Analysis of Troglitazone and Troglitazone Sulfate. For the analysis of troglitazone and related metabolites in rat plasma samples, aliquots (1 ml, stabilized with EDTA/NaF) were treated with an equal volume of acetonitrile and the precipitated protein was removed by centrifugation (13,000*g*, 10 min). The supernatant was evaporated to dryness under vacuum (speedvac) and dissolved in 500 μl HPLC phase A containing 20% acetonitrile. After centrifugation (13,000*g*, 10 min), an aliquot (400 μl) of the supernatant was analyzed by HPLC. Rat livers were homogenized in an equal amount (w/v) of HPLC phase A using a polytron mixer at maximal speed. The resulting liver homogenate was further treated as outlined for rat plasma. HPLC analyses were performed on a Shimadzu LC-10 gradient HPLC system, with UV detection (255 nm). The stationary phase was a Superspher 60 RP Select B 250 × 4 mm (Merck) with a corresponding precolumn (4 × 4 mm). As

mobile phases ammonium acetate (50 mM, pH 6.0 by means of trifluoroacetic acid; phase A) and acetonitrile (phase B) were mixed in a linear gradient from 20 to 70%B in 40 min. The flow rate was 1 ml/min. A troglitazone standard curve in blank rat plasma was prepared for external calibration. The concentrations of troglitazone and troglitazone sulfate were estimated based on their UV-absorption and expressed in nanomoles per milliliter or nanomoles per gram of liver tissue.

Isolation and Characterization of Troglitazone Sulfate. Troglitazone sulfate was purified from rat liver homogenate, by solid phase extraction (Oasis solid phase extraction cartridges; Waters, Milford, MA), followed by preparative HPLC using the chromatographic conditions outlined above. After elution of troglitazone sulfate, an additional HPLC fraction was collected to be used as a control for the in vitro incubations. The purified troglitazone sulfate has been analyzed by online liquid chromatography/mass spectrometry using an HPLC system (L-7100; Hitachi, Tokyo, Japan) coupled to an API 150 quadrupole mass spectrometer (PerkinElmer Sciex, Ontario, Canada). The HPLC gradient system was adapted with the same stationary and mobile phases as outlined above, using a 2-mm column with a flow of 400 μl/min. An atmospheric pressure interface with turbo Ion spray was used as electrospray ionization method in positive ion mode. The ion source was set to 5000 V and 450°C, the orifice tension was 10 V, and the ring electrode was set to 160 V.

Results

Cholestatic Potential of Troglitazone and Two Cholestatic Drugs in Rats. The cholestatic potential of troglitazone was investigated in an in vivo rat model established to detect interactions with the hepatic excretion of bile acids. The time course of the cholestatic effect of troglitazone has been studied in rats with jugular vein catheters treated with single intravenous doses of 25 mg/kg (Fig. 1A). The plasma bile acid levels increased rapidly, within 5 to 10 min after troglitazone treatment, and remained at elevated levels of 50 to 60 μM above the basal concentration for up to 60 min. No significant elevations were observed upon vehicle treatment. The relatively high variability observed in the plasma concentration values from single animals was probably caused by different sensitivities of the individual animals toward troglitazone-induced cholestasis. The effect of increasing doses on the plasma bile acid concentrations was studied using nonoperated, naive rats. Based on previous results the plasma bile acid concentrations were determined at two time points, 10 and 30 min after intravenous administration of troglitazone to two animals per dose level. The increases in plasma bile acid levels relative to the predose bile acid plasma concentrations were used to determine the cholestatic effect and the dose at which 50% of the maximal effect was reached, the ED_{50} dose (Fig. 1B). Troglitazone elicited a very strong, dose-dependent increase in plasma bile acids, with maximal concentrations reaching 53 μM above baseline level. The ED_{50} dose was estimated to be around 7.7 mg/kg. Two drugs with known cholestatic side effects, cyclosporin A and glibenclamide, were studied in this model to compare the relative effects observed. Cyclosporin A exhibited a stronger response ($\Delta_{10\ min}$ = 115 μM), whereas glibenclamide produced a much weaker effect ($\Delta_{10\ min}$ = 10 μM) on plasma bile

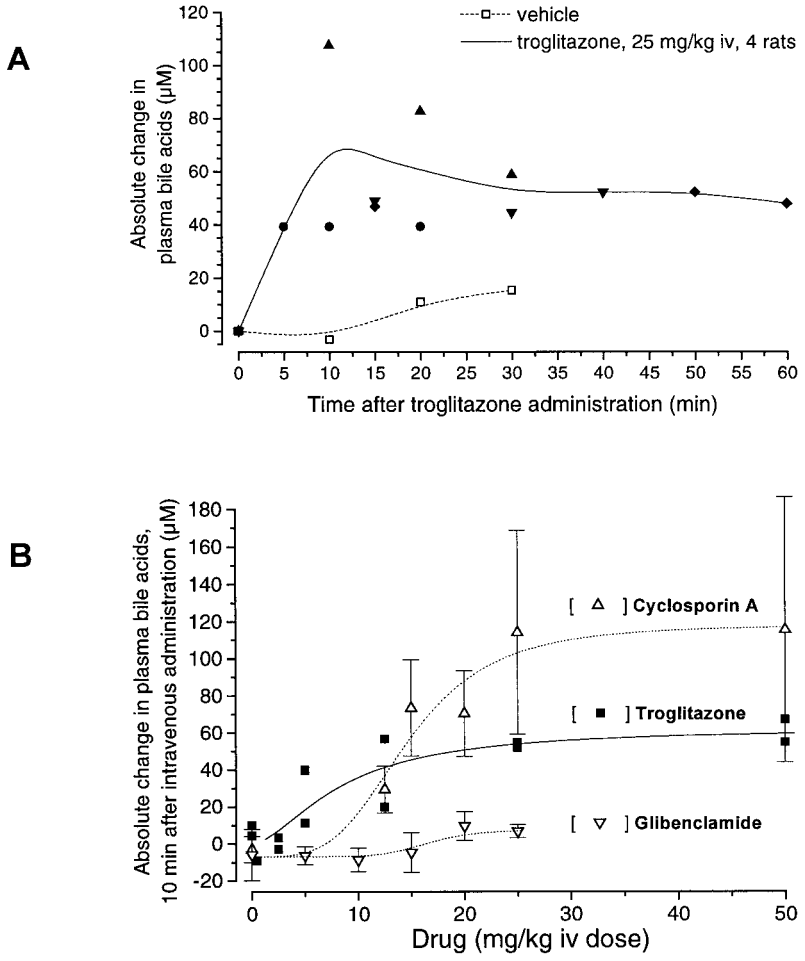

Fig. 1. Acute cholestatic potential of troglitazone, cyclosporin A, and glibenclamide and in male rats. A, increase of the plasma bile acid concentrations relative to the basal concentrations in four troglitazone-treated jugular vein-cannulated rats (intravenous doses of 25 mg/kg; different symbol for each animal) and one vehicle-treated animal. B, increase of the plasma bile acid concentration during the first 10 min after intravenous administration of increasing doses to naïve rats. For cyclosporin A and glibenclamide, the mean values and S.D. of three to four rats were calculated, whereas for troglitazone the plasma concentrations of two individual animals are shown.

acids (Table 1). The estimated ED_{50} doses (14 and 17 mg/kg, respectively; Table 1) were both higher for these two compounds compared with troglitazone.

The interference of troglitazone with the hepatic excretion of bile acids was further studied using trace amounts (4 μCi/kg, 86 nmol/kg) of radiolabeled taurocholate that was administered at different time points after troglitazone treatment, always 8 min before sacrifice of the animals (Fig. 2A). No significant levels of radiolabeled taurocholate were found in liver tissue at 30 min after vehicle treatment. However, most of the radiolabeled taurocholate tracer applied was recovered from liver tissue of troglitazone-treated animals (25 mg/kg intravenous) over the whole experimental period of 60 min.

TABLE 1

Cholestatic potential of different compounds in the in vivo rat model and corresponding in vitro inhibitory effects on Bsep

Compound	In vivo effect on plasma bile acids after intravenous administration to rats			In vitro inhibition of taurocholate transport in cLPMV
	ED_{50}	$\Delta_{10\ min}$	$\Delta_{30\ min}$	
	mg/kg	*μM*		*μM*
Cyclosporin A	14	115	100	IC_{50} = 0.8 ± 0.23
Glibenclamide	17	10	5	IC_{50} = 8.6 ± 1.9
Troglitazone	7.7	53	60	K_i = 1.3 ± 0.3
Troglitazone sulfate				K_i = 0.23 ± 0.09

Metabolism of Troglitazone in rats. The concentrations of troglitazone and the main metabolite troglitazone sulfate in rat plasma and in homogenized liver tissue were determined by HPLC chromatography. Rats containing jugular vein catheters were treated with single intravenous troglitazone doses of 25 mg/kg (Table 2, Fig. 2B). Troglitazone reached equal levels in plasma and liver tissue (~ 13 nmol per milliliter of plasma or per gram of tissue), whereas troglitazone sulfate reached much higher concentrations in plasma (~ 110 nmol/ml) and in liver tissue (~ 260 nmol/g) 30 min after administration (Table 2). The high concentration of troglitazone sulfate in liver tissue was observed over the whole experimental period, from 20 to 60 min (Fig. 2B).

Troglitazone sulfate was isolated from homogenized rat liver tissue by solid phase extraction, followed by preparative HPLC purification and the purified troglitazone sulfate was analyzed by liquid chromatography/mass spectrometry. The UV and total ion current signals indicated the presence of a single metabolite (Fig. 3A). Under the conditions of the positive ionization mode troglitazone sulfate (*m/z* ratio of 522.3 of the H^+ ion), decomposed partially with a loss of 80 Da, characteristic for sulfate, to troglitazone (*m/z* ratio of 442.3 of

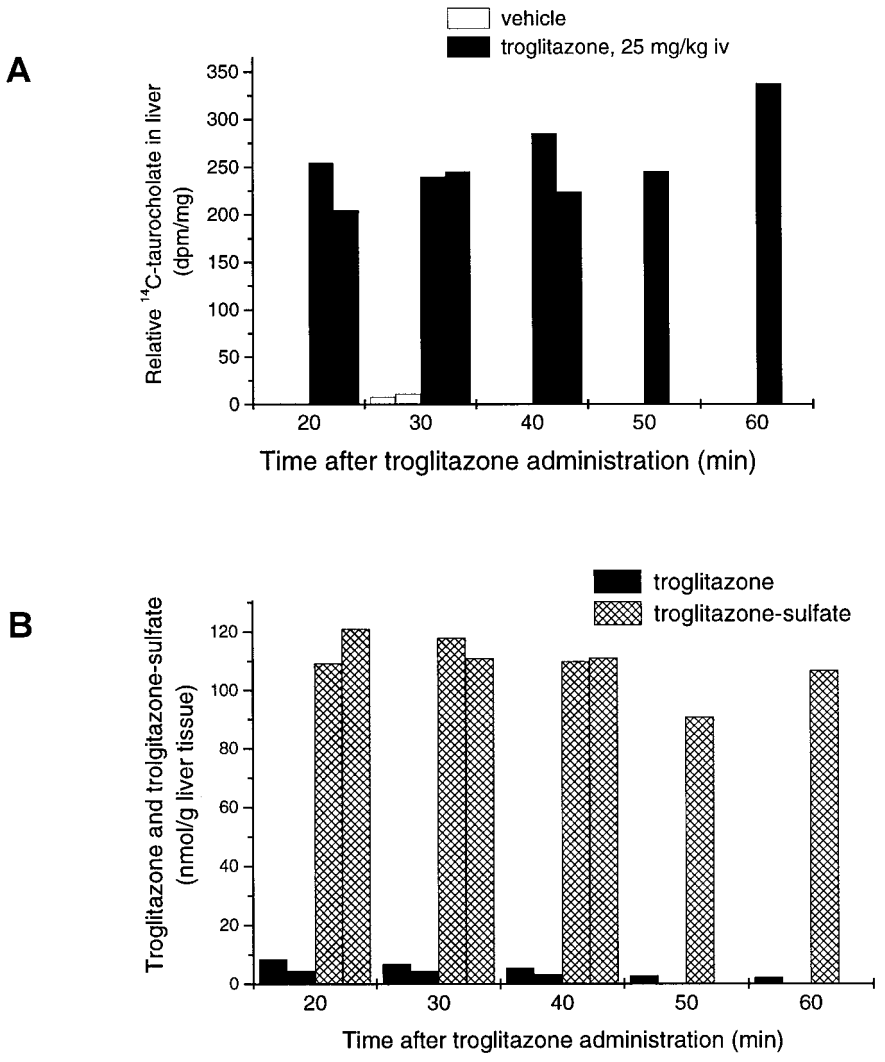

Fig. 2. Acute cholestatic potential of troglitazone after intravenous doses of 25 mg/kg to jugular vein-cannulated male rats. A, relative liver tissue levels of [^{14}C]taurocholate, administered as a tracer 8 min before sacrifice of the animals at the indicated time points after troglitazone- or vehicle-treatment for individual animals. B, troglitazone and troglitazone sulfate liver tissue concentrations for individual animals.

the H^+ ion; Fig. 3B). In addition, ammonium and sodium adducts of both compounds were formed. Troglitazone eluted at a retention time of 29.7 min under these chromatographic conditions.

Characterization of taurocholate transport into cLPMV. For mechanistic in vitro studies, cLPMV were prepared by sucrose density step gradient centrifugation. Specific marker enzymes (as outlined in *Materials and Methods*) were measured in the different membrane fractions obtained for their characterization and to determine the enrichment in the individual purification steps. The purity of the canalicular membrane vesicles was estimated by the absence of measurable Na^+/K^+-ATPase activity relative to the Mg^{2+}-ATPase activity (Meier et al., 1984). The ATP-dependent [^{3}H]taurocholate uptake was determined in canalicular membrane vesicles by a rapid filtration method. A time-dependent uptake of radiolabeled taurocholate was observed in presence of ATP (Fig. 4A). The kinetic properties of this uptake, catalyzed by Bsep were studied (Fig. 4B). The difference between the uptake in presence of ATP and the nonspecific binding in absence of ATP, representing the ATP-dependent transport rate, was used to calculate the enzyme kinetic parameters. The affinity (K_m = 2.2 ± 0.7 μM) and the maximal transport rate were in good agreement with published values (Stieger et al., 1992; Wolters et al., 1992).

TABLE 2

Comparison of plasma and liver concentrations for troglitazone and its main metabolite troglitazone-sulfate with the in vitro inhibitory effect on the Bsep

Experimental system	Troglitazone	Troglitazone sulfate
Therapeutic range in man (400–600 mg/day)[a]		
Plasma concentration	~1–2 μg/ml[a] (2–4 nmol/ml)	—
Estimated liver and plasma concentrations 30 min after IV administration of troglitazone (25 mg/kg) in rats (in vivo)		
Plasma concentration	~13 nmol/ml	~110 nmol/ml
Liver concentration	~13 nmol/g	~260 nmol/g
Inhibition of Bsep into rat cLPMV (in vitro)		
K_i	1.3 ± 0.3 nmol/ml	0.23 ± 0.09 nmol/ml

[a] Loi et al. (1999).

In Vitro Inhibition Studies Involving the Canalicular Bsep. The inhibition of the hepatobiliary transport of taurocholate by troglitazone and its main liver metabolite, troglitazone sulfate, was studied along with the two cholestatic compounds cyclosporin A and glibenclamide. All compounds inhibited the ATP-dependent transport of radiolabeled taurocholate into cLPMV (Fig. 5). For cyclosporin A, an IC_{50} value of

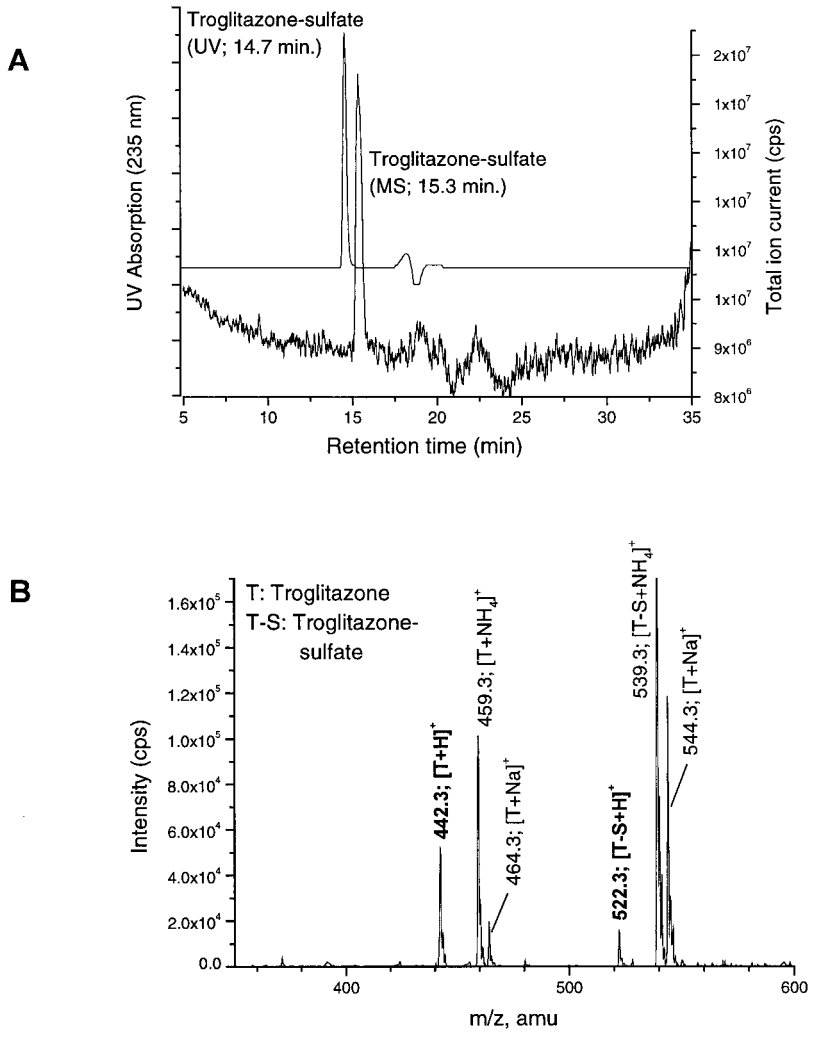

Fig. 3. Liquid chromatography/mass spectrometry analysis of troglitazone sulfate isolated and purified from rat liver tissue. A, UV chromatogram and total ion current. B, mass spectrum of troglitazone sulfate eluting at 15.3 min. Under the conditions used troglitazone sulfate and the cleavage product troglitazone were observed as $M+H^+$ ions and as NH_4^+ and Na^+ adducts.

0.8 μM was found in good agreement with affinity values in the literature (K_i = 0.3 μM) (Keppler et al., 1992). Glibenclamide showed a much weaker inhibition, with an IC_{50} value of 8.6 μM, consistent with a recent literature value (K_i ~5.7 μM) (Stieger et al., 2000). These in vitro results were consistent with the cholestatic potential observed for these two compounds in vivo, in the rat cholestasis model.

Troglitazone showed a moderate inhibition of the in vitro taurocholate transport, with an IC_{50} value of 3.9 μM. However, the main metabolite, troglitazone sulfate, which was isolated from rat liver tissue by preparative HPLC, inhibited the ATP-dependent taurocholate transport very potently. An IC_{50} value of 0.4 μM was observed, and a corresponding amount of a HPLC control fraction (eluting after troglitazone sulfate) showed no significant inhibition (result not shown). The mechanism of inhibition has been further studied for troglitazone and troglitazone sulfate using a different membrane preparation. Both compounds showed a competitive inhibition of the ATP-dependent taurocholate transport into cLPMV, suggesting a specific interaction with Bsep (Fig. 6). The kinetic parameters for taurocholate transport (K_m and V_{max}), calculated by nonlinear fitting of these data sets, were in good agreement with previous data (data not shown). For troglitazone, an apparent inhibition constant K_i of 1.3 ± 0.3 μM was estimated, while for troglitazone sulfate the apparent K_i was 0.23 ± 0.09 μM, in good agreement with the previously determined IC_{50} values for both compounds (3.9 and 0.4 μM; Fig. 5), using a different cLPMV preparation.

The involvement of Mrp2 in the inhibition of Bsep by troglitazone and troglitazone sulfate in cLPMV was studied using membrane vesicles prepared from TR^- rats, expressing a nonfunctional Mrp2 protein (Paulusma et al., 1996). Using cLPMV preparations from TR^- rats, troglitazone and troglitazone sulfate inhibited the Bsep activity with IC_{50} values of 12.0 ± 3.8 and 1.3 ± 0.3 μM, respectively (Fig. 7). For both compounds, the apparent Bsep inhibition was lower in this cLPMV preparation compared with the inhibition in vesicles from normal rats with IC_{50} values of 3.9 ± 0.6 and 0.4 ± 0.06 μM, respectively. This difference in apparent inhibition might be associated with different protein expression levels and related differences in nonspecific binding in the two preparations. In both vesicle preparations, however, troglitazone sulfate showed Bsep inhibition ~10 times stronger than that of troglitazone. This result supported a direct (*cis*-) inhibition of Bsep by troglitazone and troglitazone sulfate, without the necessity of Mrp2-mediated export into the canalicular lumen.

Discussion

Cholestatic Potential of Troglitazone in Rats by an Interaction with the Hepatobiliary Export of Bile Acids. As a marker for the cholestatic potential of several

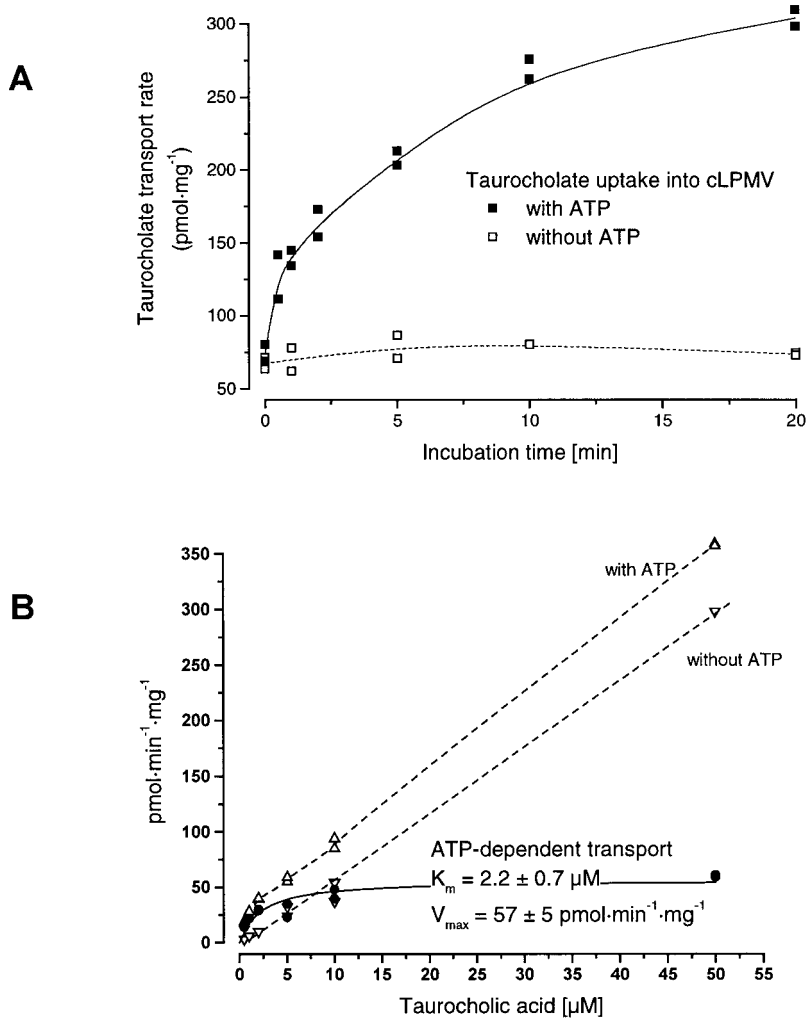

Fig. 4. ATP-dependent transport of taurocholate into cLPMV. A, time course of the taurocholate uptake in presence and absence of ATP. B, enzyme kinetics of the ATP-dependent taurocholate transport into cLPMV.

xenobiotics, the changes in plasma bile acid concentrations were studied in rats (Kadmon et al., 1993; Boehme et al., 1994). An interference with the vectorial transport of biliary constituents from plasma to bile resulted in a rapid and transient bile acid increase in plasma because of a reduction in the overall bile acid secretion. Maximal plasma bile acid levels were observed 5 to 10 min after intravenous administration of troglitazone (25 mg/kg) and remained at similar levels of 50 to 60 μM above baseline for more than 60 min (Fig. 1A). The strong interaction potential of troglitazone with the hepatobiliary elimination of bile acids, with a dose to reach a half-maximal effect (ED_{50}) of 7.7 mg/kg, was comparable with the effect of cyclosporin A (Fig. 1B). This compound with cholestatic side effects has been shown to interfere with the hepatic excretion of bile acids at the level of their hepatobiliary export (Kadmon et al., 1993; Boehme et al., 1994). A similar mechanism of inhibition has recently been described for glibenclamide (Stieger et al., 2000), known to induce cholestasis in a few cases in man (Krivoy et al., 1996). For this compound, only a weak response was observed in the rat cholestasis model, in agreement with the weak in vitro Bsep inhibition (Fig. 1B, Table 1).

The interference of troglitazone with the hepatobiliary export of bile salts was further studied using a radiolabeled taurocholate tracer. In troglitazone-treated rats, radiolabeled taurocholate accumulated in the liver tissue, pointing toward an interference of troglitazone with the hepatobiliary export of bile acids into bile at the canalicular pole of the

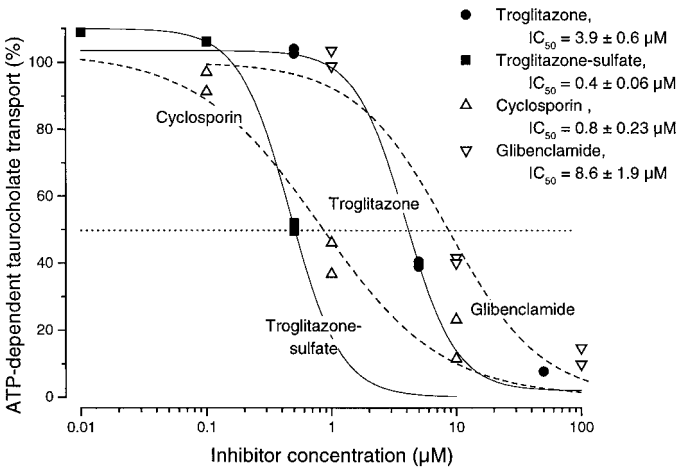

Fig. 5. In vitro interaction of several drugs with the ATP-dependent transport of taurocholate into cLPMV. Determination of IC_{50} values were performed at a substrate (taurocholate) concentration of 1 μM.

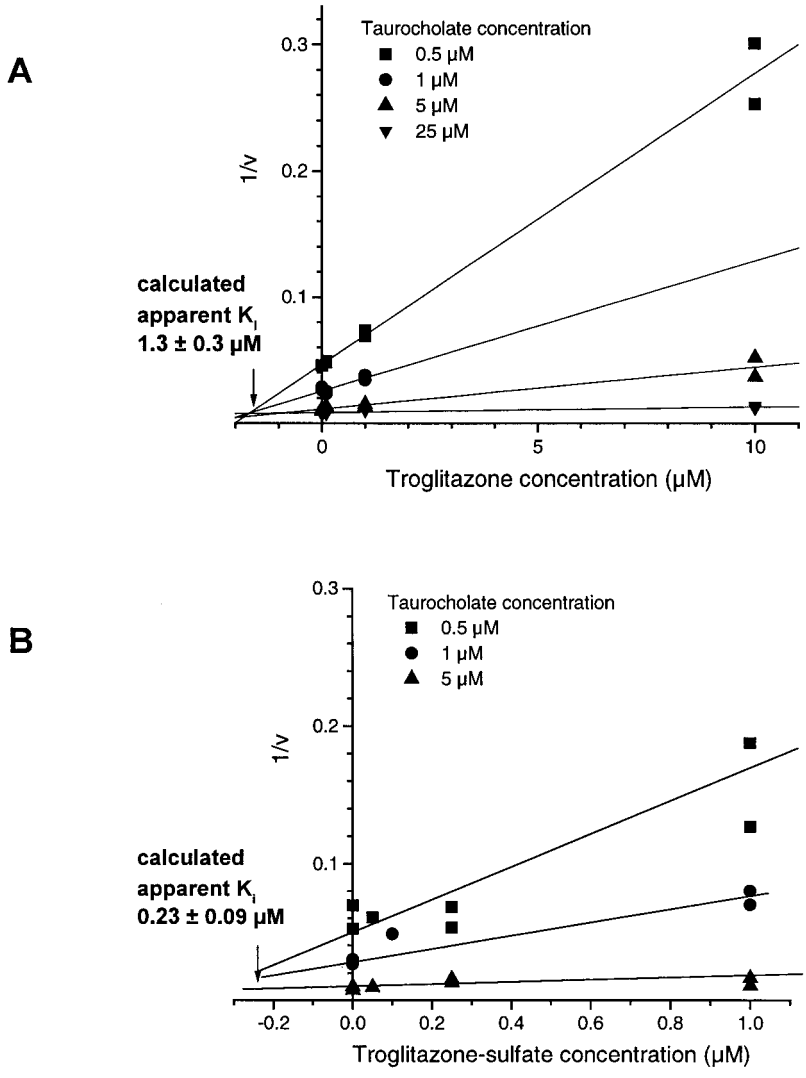

Fig. 6. Competitive inhibition of the ATP-dependent taurocholate transport (Bsep) into rat cLPMV by troglitazone (A) and troglitazone sulfate (B). The apparent inhibitor constants and apparent K_i values were calculated by nonlinear fitting of the entire data sets.

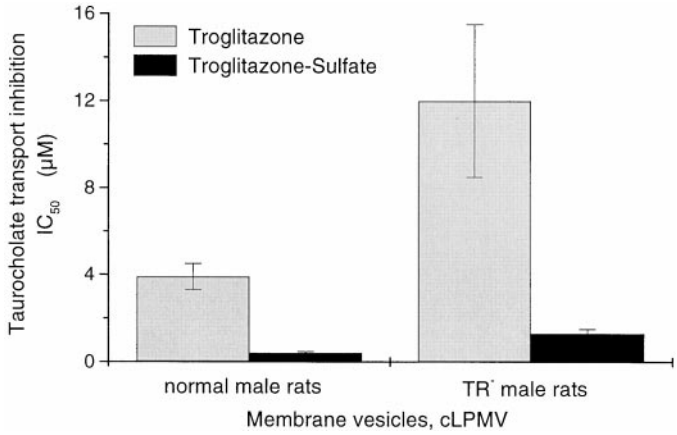

Fig. 7. Determination of the inhibitory effect of troglitazone and troglitazone sulfate on the ATP-dependent taurocholate transport (Bsep) into rat cLPMV prepared from normal male rats and male TR$^-$ rats deficient in Mrp2.

hepatocyte (Fig. 2A). Only a small amount of radiolabeled taurocholate was found in liver tissue of vehicle-treated animals. The effect of troglitazone on the plasma bile acid levels and the accumulation of radiolabeled taurocholate tracer in liver tissue were observed over the whole experimental period (60 min). This time course correlated well with the accumulation and presence of the main troglitazone metabolite, troglitazone sulfate, within the liver tissue (Fig. 2B). Indeed, troglitazone sulfate was reported as the main drug-related metabolite excreted into bile in several animal species (Kawai et al., 1997). Furthermore, a strong interaction of troglitazone or related material with the hepatobiliary excretion of bile acids was supported by a study with isolated perfused rat livers, where a rapid decrease in bile flow (67% within 60 min) was observed after troglitazone infusion (Preininger et al., 1999).

In Vitro Interaction of Troglitazone and Troglitazone-Sulfate with Bsep. Based on the apparent interference of troglitazone with the hepatobiliary export of bile salts, mechanistic in vitro studies were performed using isolated cLPMV. For cyclosporin A, glibenclamide and troglitazone the in vitro potential to inhibit the ATP-dependent transport of taurocholate into cLPMV, catalyzed by the canalicular Bsep, correlated well with the cholestatic potential observed in vivo (Fig. 5, Table 1). The mechanism of inhibition was further studied for troglitazone and its metabolite troglitazone sulfate. Both compounds competitively inhibited the canalicular Bsep with apparent K_i values of 1.3 and 0.23 μM, respectively (Fig. 6). A different ATP-dependent canalicular transporter, Mrp2, is involved in the hepatobiliary export of organic anions, including many drug-conjugates (Müller and Jansen, 1998). For some conjugated drug metabolites, such as ethinylestradiol-17β-glucuronide, Mrp2-mediated export into the canalicular lumen was required for in vitro Bsep (*trans*-) inhibition (Stieger et al., 2000). For troglitazone and troglitazone sulfate, an equal inhibition of the ATP-dependent taurocholate transport was also observed in cLPMV prepared from Mrp2 deficient TR$^-$ rats. Therefore, both compounds directly inhibit Bsep on the cytoplasmic side of the canalicular membrane (*cis*-inhibition; Fig. 7). A similar inhibition pattern has been described for rifamycin, rifampicin, and glibenclamide (Stieger et al., 2000). However, the conjugated ethinylestradiol-17β-glucuronide did not inhibit taurocholate transport in this in vitro system because of a lack of Mrp2-mediated transport into the canalicular lumen, indicating a *trans*-inhibition of Bsep (Stieger et al., 2000).

The canalicular bile salt export pump, Bsep, has lagged behind other ATP-binding cassette transporters of the canalicular liver plasma membrane with respect to its characterization and molecular cloning (Gerloff et al., 1997). The available data indicate that this transporter is involved in the excretion of conjugated bile acids (taurine- and glycine-amidates and acyl-glucuronides) (Oude Elferink et al., 1989) and related, nonglucuronidated nonsulfated bile salts (Keppler et al., 1992). It is mainly responsible for the bile salt-dependent fraction of the overall bile flow. An involvement of Bsep in the transport of xenobiotics was suggested for nonconjugated organic anions (Hofmann, 1992). Based on its expression pattern, a broader substrate specificity of Bsep has been postulated in analogy to other multidrug resistance proteins (Török et al., 1999). It might be speculated that the canalicular Bsep might have a similar function in the hepatobiliary export of troglitazone and troglitazone sulfate, a hypothesis supported by the competitive mechanism of Bsep inhibition by both compounds.

Elimination of Troglitazone by an Interplay of Drug Metabolism and Drug Transport. Several lines of evidence suggested that the hepatobiliary export of troglitazone and related material might represent a rate-limiting step in the overall elimination of troglitazone. In patients with hepatic impairment, troglitazone sulfate was found to accumulate about 4-fold in plasma, with a 3-fold-increased half-life (Ott et al., 1998). In addition, troglitazone sulfate accumulated as the major drug-related metabolite in rat liver tissue (Fig. 2B), increasing the likelihood for drug-interactions induced by troglitazone sulfate. The inhibition of the canalicular Bsep by troglitazone sulfate might result in an interference with the hepatic export of bile salts, leading to an intrahepatic cholestasis. Such a mechanism of interaction leading to a drug-induced intrahepatic cholestasis has recently been described for several other drugs (Bolder et al., 1999; Stieger et al., 2000).

In rats, which were treated with 25 mg/kg troglitazone to produce a maximal cholestatic response, the troglitazone plasma concentration was in the range of ~13 nmol/ml (Table 2). In liver tissue a similar troglitazone concentration was observed, while troglitazone sulfate reached a concentration of ~ 260 nmol/g of liver tissue (Table 2). These levels of troglitazone and troglitazone sulfate in rat liver tissue were about 1 and 3 orders of magnitude above the respective K_i values for in vitro Bsep inhibition. Consequently, an inhibition of the hepatobiliary bile salt export in vivo at the level of Bsep by troglitazone sulfate is probable and this metabolite might be mainly responsible for the cholestatic effect of troglitazone in rats. An accumulation of troglitazone and related material in liver tissue has also been reported based on whole body autoradiography studies in rats (Kawai et al., 1997).

Typical daily troglitazone doses in man are in the range of 400 to 600 mg, producing troglitazone plasma concentrations of 1 to 2 μg/ml (2 to 4 nmol/ml) (Table 2) (Loi et al., 1999). A quantitative extrapolation of the cholestatic effect observed in rats to man is not possible, because of species-specific differences in the process of bile formation and in the disposition of troglitazone. However, the processes involved in the

hepatobiliary metabolism and export of the absorbed fraction of troglitazone, represent potential targets for drug-interactions. Therefore, in combination with other cholestatic drugs, other diseases or pharmacogenetic liabilities, troglitazone might induce an intrahepatic cholestasis and thereby contribute to the hepatotoxicity observed in some patients treated with this compound.

Cholestatic signs have been described for one patient with a troglitazone-induced hepatic toxicity (Gitlin et al., 1998). In addition, several potentially cholestatic drugs were comedicated with troglitazone in patients who developed signs of liver toxicity and might have contributed to the development of cholestasis. Simvastatin (Herrine and Choudhary, 1999) and lisinopril (Gitlin et al., 1998), both drugs known to induce cholestatic side effects, were comedicated with troglitazone in patients developing signs of liver toxicity. One report described three of four cases of troglitazone-induced fulminant hepatitis in which glibenclamide has been comedicated with troglitazone. An interaction of the two drugs was suspected without further elaborating on a possible mechanism (Shibuya et al., 1998).

In conclusion, several lines of evidence suggested that troglitazone is eliminated by closely interrelated drug metabolizing and drug transporting processes. The hepatobiliary elimination of the main metabolite, troglitazone sulfate, seems to be a rate-limiting step in the overall disposition of troglitazone. Accordingly, troglitazone sulfate was found to accumulate in rat liver tissue and was reported to accumulate in plasma of patients with hepatic impairment. Troglitazone, and to a greater extent troglitazone sulfate, were found to interfere with the hepatobiliary elimination of bile salts by a competitive inhibition of Bsep in the rat. Potentially this inhibition may lead to an intrahepatic cholestasis also in man, contributing to the formation of troglitazone-induced liver toxicity.

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