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Modulation of Trabectedin (ET-743) Hepatobiliary Disposition by Multidrug Resistance-Associated Proteins (Mrps) May Prevent Hepatotoxicity

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Abstract

Trabectedin is a promising anticancer agent, but dose-limiting hepatotoxicity was observed during phase I/II clinical trials. Dexamethasone (DEX) has been shown to significantly reduce trabectedin-mediated hepatotoxicity. The current study was designed to assess the capability of sandwich-cultured primary rat hepatocytes (SCRH) to predict the hepato-protective effect of DEX against trabectedin-mediated cytotoxicity. The role of multidrug resistance-associated protein 2 (Mrp2; *Abcc2*) in trabectedin hepatic disposition also was examined. In SCRH from wild-type Wistar rats, cytotoxicity was observed after 24-hr continuous exposure to trabectedin. SCRH pretreated with additional DEX (1 µM) exhibited a 2–3-fold decrease in toxicity at 100 nM and 1000 nM trabectedin. Unexpectedly, toxicity in SCRH from Mrp2-deficient (TR⁻) compared to wild-type Wistar rats was markedly reduced. Depletion of glutathione from SCRH using buthionine sulfoximine (BSO) mitigated trabectedin toxicity associated with 100 nM and 1000 nM trabectedin. Western blot analysis demonstrated increased levels of CYP3A1/2 and Mrp2 in SCRH pretreated with DEX; interestingly, Mrp4 expression was increased in SCRH after BSO exposure. Trabectedin biliary recovery in isolated perfused livers from TR⁻ rats was decreased by ~75% compared to wild-type livers. In conclusion, SCRH represent a useful *in vitro* model to predict the hepatotoxicity of trabectedin observed *in vivo*. The protection by DEX against trabectedin-mediated cytotoxicity may be attributed, in part, to enhanced Mrp2 biliary excretion and increased metabolism by CYP3A1/2. Decreased trabectedin toxicity in SCRH from TR⁻ rats, and in SCRH pretreated with BSO, may be due to increased basolateral excretion of trabectedin by Mrp3 and/or Mrp4.

Keywords

Trabectedin; Dexamethasone; Sandwich-cultured primary rat hepatocytes; Multidrug resistance-associated proteins

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Introduction

Trabectedin (Ecteinascidin 743, Yondelis™) is a DNA alkylating agent extracted from the marine tunicate *Ecteinascidia turbinate* that exhibits potent antineoplastic activity against a variety of tumor cells at nanomolar concentrations (Izbicka et al., 1998). Trabectedin is currently under phase II/III evaluation in advanced gastrointestinal stromal tumors, pretreated soft tissue sarcoma, ovarian cancer and breast cancer (Laverdiere et al., 2003; Blay et al., 2004; Yovine et al., 2004; Zelek et al., 2006).

Trabectedin is metabolized primarily by CYP 3A4; 2C9, 2C19, 2D6 and 2E1, and to a lesser extent conjugated by the phase II enzymes uridine diphosphoglucuronosyl transferase (UGT) and glutathione-S-transferase (GST) (Reid et al., 2002; Brandon et al., 2006). Extensive metabolism of trabectedin was demonstrated in a mass balance study conducted in patients with advanced cancer (Beumer et al., 2007b). Following i.v. [^{14}C]trabectedin administration to patients, ~55.5% and ~5.9% of the dose was recovered in feces and urine, respectively, suggesting that trabectedin and its metabolites undergo biliary excretion (Beumer et al., 2005). However, the involvement of transport proteins in hepatobiliary disposition of trabectedin remains to be elucidated.

During phase I/II clinical trials, the most prevalent adverse effect associated with trabectedin treatment was dose-limiting hepatotoxicity secondary to increases in blood alkaline phosphatase and/or bilirubin levels (Delaloge et al., 2001; Ryan et al., 2001; Taamma et al., 2001). An *in vitro* cytotoxicity study using HepG2 cells showed increased trabectedin-mediated toxicity after 1-hr preincubation with inhibitors of cytochrome P450 (CYP) enzymes involved in trabectedin metabolism; CYP inducers and phase II enzyme inhibitors did not significantly affect trabectedin-mediated toxicity, suggesting that trabectedin metabolites may be less toxic compared to the parent compound (Brandon et al., 2005). Pretreatment of female Fisher rats with DEX has been shown to ameliorate the hepatotoxic effects of trabectedin while coadministration of DEX with trabectedin did not reduce toxicity (Donald et al., 2003). Consistent with the protective effect of DEX on trabectedin-mediated toxicity in rats, DEX premedication markedly reduced trabectedin-mediated hepatotoxicity in patients with advanced sarcoma (Grosso et al., 2006). However, this hepato-protective effect of DEX could not be mimicked *in vitro* using primary rat hepatocytes cultured on a fibronectin matrix (Donald et al., 2004).

Significant induction of Mrp2 mRNA and protein by DEX treatment has been reported in primary rat and mouse hepatocytes (Kast et al., 2002; Luttringer et al., 2002; Turncliff et al., 2004). Mrp2, a transport protein localized on the hepatic canalicular plasma membrane, is responsible for the biliary excretion of organic anions as well as numerous drugs including pravastatin, vincristine and etoposide (Cui et al., 1999; Kawabe et al., 1999; Konig et al., 1999; Sasaki et al., 2002). Animals with hereditary conjugated hyperbilirubinemia [Mrp2-deficient rats (TR⁻) and Eisai hyperbilirubinemic rats (EHBR)] have been used extensively to examine the substrate specificity and *in vivo* function of Mrp2 (Westley et al., 2006; Zamek-Gliszczynski et al., 2006). Patients with Dubin-Johnson syndrome lack MRP2 and hence suffer from hereditary conjugated hyperbilirubinemia (Paulusma et al., 1997). In the absence of Mrp2/MRP2, rats and humans exhibit increased expression of basolateral Mrp3/MRP3 as a compensatory mechanism that enables the excretion of Mrp2/MRP2 substrates into the systemic circulation, thus avoiding excessive accumulation of organic anions in the hepatocyte (Kiuchi et al., 1998; Hirohashi et al., 1999; Johnson et al., 2006; Nies and Keppler, 2007).

Drug-induced liver injury has emerged as the most common reason for the withdrawal of Food and Drug Administration-approved drugs from the market (Kaplowitz, 2005; FDA, 2007). Covalent adduct formation, immunotoxicity, and disruption of cellular bioenergetics have been

considered previously as mechanisms underlying hepatotoxicity, but more recent data suggest that hepatic transport proteins may be an important site of toxic interactions (Fattinger et al., 2001; Funk et al., 2001; Leslie et al., 2007). The objective of this study was to evaluate the capability of sandwich-cultured primary rat hepatocytes (SCRH), which more closely mimic the metabolic and transport capabilities of the intact liver, to predict the hepato-protective effect of DEX against trabectedin-mediated hepatotoxicity. An additional objective was to examine the role of Mrp2 in trabectedin hepatobiliary disposition.

Materials and Methods

Materials

Trabectedin (Ecteinascidin 743, Yondelis™, NSC 648766) was supplied by the National Cancer Institute, Developmental Therapeutics Program Repository (http://dtp.nci.nih.gov/branches/dscb/repo_open.html) and was prepared as a 10 mM stock solution in DMSO. DEX and BSO were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), human recombinant insulin, modified essential media non-essential amino acids, L-glutamine, penicillin and streptomycin were purchased from GIBCO (Invitrogen Corporation, Grand Island, NY). ITS$^+$ (insulin, transferin, and selenium) was purchased from BD Biosciences (Bedford, MA).

Animals

Male wild-type Wistar rats (220–300 g; Charles River Laboratories, Raleigh, NC) and male TR$^-$ Wistar rats (220–300 g; in-house breeding colony originally obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were used for hepatocyte isolation and as liver donors for isolated perfused liver experiments. Male wild-type Wistar rats (>400 g) were used as blood donors for isolated perfused liver experiments. Rats were anesthetized with ketamine/xylazine (60/12 mg/kg intraperitoneally) before surgical manipulation. The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all procedures.

Hepatocyte Isolation and Culture

Hepatocytes were isolated from male Wistar or TR$^-$ rats (220–300 g) by a modification of the two-step collagenase digestion method as described previously (Liu et al., 1998). Hepatocyte viability was >85% as determined by trypan blue exclusion. Hepatocytes were plated at a density of ~1.5×10^6 cells/well in 6-well plates coated with 0.1 ml of rat tail collagen Type I solution (1.5 mg/ml, pH 7.4) in 1.5 ml of plating medium (phenol red-free DMEM containing 5% fetal bovine serum, 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml human recombinant insulin and 1 µM DEX), and allowed to attach for 2–3 hr at 37□ in a humidified incubator with 95%O$_2$/5%CO$_2$. After attachment, medium was replaced to remove dead/unattached cells. To achieve a sandwich configuration, medium was aspirated and cells were overlaid with 0.1 ml of rat tail collagen Type I solution (1.5 mg/ml, pH 7.4) at 24 hr (day 1). Collagen was allowed to gel for 1 hr in a 37□ humidified incubator 95%O$_2$/5% CO$_2$, and then culture medium was added (1.5 ml/well; phenol red-free DMEM supplemented with 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1% ITS$^+$ and 0.1 µM DEX). Subsequently, the culture medium was changed daily until the conduct of experiments.

DEX and BSO Pretreatment in SCRH

From day 2 to day4, additional DEX (1 µM) or BSO (500 µM) was added to the culture medium containing 0.1 µM DEX. On day 3, SCRH were incubated for 24 hr in culture medium containing a range of trabectedin concentrations (1–1000 nM) to compare trabectedin-mediated cytotoxicity between SCRH pretreated with vehicle (0.1 µM DEX) and additional

DEX (1 µM) or BSO (500 µM). DEX and trabectedin were prepared in DMSO, and BSO was solubilized in water. Stock solutions were diluted in culture medium for treatment. The final concentration of DMSO was <0.2% and had no effect on untreated controls.

Determination of Cell Viability Using LDH Release

LDH leakage into the culture medium was measured at the end of the 24 hr exposure using the Cytotoxicity Detection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. The degree of LDH release was expressed as a percentage of the maximum cellular LDH release, which was measured by adding 2% Triton X-100 to SCRH.

$$\text{Cytotoxicity (\% of control)} = \frac{\text{LDH}_{sample} - \text{LDH}_{blank}}{\text{LDH}_{Triton\ X-100} - \text{LDH}_{blank}}$$

Western Blot Analysis of Mrp2, 3, 4 and CYP3A1/2 Proteins

On day 4, the medium was removed and cells were washed twice with ice-cold phosphate-buffered saline (PBS). Subsequently, cells were lysed with 400 µl of lysis buffer [1 mM EDTA, 1% sodium dodecyl sulfate (SDS), pH 8, with Complete™ protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany)] in each well of a 6-well plate. Lysates were stored at −20□ until western blot analysis. The whole cell lysates were thawed on ice and protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Protein samples (25 µg/well) were separated by SDS-polyacrylamide gel electrophoresis, and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Blots were blocked with Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 1 hr at room temperature. Subsequently, the membrane was incubated with appropriate primary antibodies for 2 hr at room temperature or over-night at 4□ and then rinsed three times at 10-min intervals with TBS-T. Mrp2, 3 and 4 were detected using monoclonal antibodies M_2III-6, M_3II-9 and M_4I-10, respectively, at a 1:1000 dilution (Alexis Biochemicals, San Diego, CA). CYP3A1/2 protein expression was determined with a polyclonal antibody against rat CYP3A1/2 at a 1:1500 dilution (Xenotech, Kansas City, KS). Protein loading was normalized to β-actin expression using a β-actin antibody at a dilution of 1:5000 (C4; Chemicon, San Fransisco, CA). Immunoreactive protein bands were detected by chemilumiscence using a Bio-Rad VersaDoc imaging system and densitometry analysis was achieved using the Quantity One software package v.4.1 (Bio-Rad Laboratories, Hercules, CA).

Isolated Perfused Liver Study

Recirculating isolated perfused liver (IPL) studies were performed with male Wistar wild-type and TR⁻ rat livers as described previously (Brouwer and Thurman, 1996). Briefly, livers were perfused *in situ* with oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4) after cannulation of the bile duct and portal vein. Livers were removed from the body cavity and placed into a humidified perfusion chamber at 37□. Perfusion was continued with oxygenated buffer containing 20% (v/v) heparinized male rat blood at a flow rate of 20 ml/min. Livers were allowed to equilibrate for 10–15 min prior to bolus administration of 24 nmol of trabectedin (preliminary recovery studies in the perfusion system without the liver indicated that the actual dose delivered to the IPL was decreased by 50% due to nonspecific binding/degradation in blood-containing perfusate). Liver viability was assessed by monitoring portal pressure (<15 cm of H_2O), observing gross morphology, and measuring bile flow in perfused livers from control and TR⁻ rats (>0.8 and >0.3 µl/min/g liver, respectively). Taurocholate (0.5 µmol/min, in saline) was infused to maintain bile flow. Bile was collected *in toto* every 10 min, and the volume was determined gravimetrically (specific gravity 1.0). Perfusate samples (0.5 ml) were immediately centrifuged following collection to obtain plasma for analysis.

Analytical Methods

Bile and perfusate samples were analyzed by liquid chromatography with detection by tandem mass spectrometry (Applied Biosystems API 4000 triple quadrupole with TurboIonSpray interface, MDS Sciex, Concord, ON, Canada). Trabectedin and cimetidine (internal standard) were eluted from an Aquasil C18 column (d_p=5μm, 2.1×50 mm, Thermo-Electron, Waltham, MA) using a mobile phase gradient (A: water with 0.1% formic acid, B: methanol with 0.1% formic acid); 0–2.5 min hold at 95% B, a linear gradient to 10% B from 2.5–3 min, 3–4 min hold at 10% B; flow rate was 0.75 mL/min (Shimadzu solvent delivery system, Columbia, MD). Trabectedin and cimetidine were detected in positive ion mode using multiple reaction monitoring; trabectedin: $744.2 \rightarrow 495.2$ m/z, cimetidine: $250.9 \rightarrow 156.9$ m/z. Trabectedin was quantified with calibration curves prepared in the appropriate matrix using analyte:internal standard (trabectedin:cimetidine) peak area ratios. The lower limit of detection was 1 nM for all analytes and standard curves ranged from 1 to 1000 nM.

Statistical Analysis

Statistical significance was assessed using two-way analysis of variance with Bonferroni's post hoc test, except where the groups being compared had unequal variances, in which case log-transformed data were assessed. SCRH data were presented as the mean ± S.E.M. when at least three sets of independent experiments were performed in triplicate. In all cases, $p < 0.05$ was considered to be statistically significant.

Results

DEX effect on trabectedin-mediated cytotoxicity in SCRH from wild-type rats

Trabectedin-mediated cytotoxicity was defined as LDH release, relative to a Triton X-100 treated control, from hepatocytes into the culture medium. Following 2 hr of trabectedin exposure, LDH release was not increased relative to untreated SCRH (data not shown). However, cytotoxicity was observed after 24 hr of continuous trabectedin exposure to SCRH (Fig. 1). From day 2 to day 4, additional DEX (1 μM) was added to the culture medium containing 0.1 μM DEX to examine the effect of DEX on trabectedin-mediated cytotoxicity; SCRH were incubated with trabectedin for 24 hr starting on day 3. This pretreatment of SCRH with additional DEX (1 μM) decreased toxicity of trabectedin compared to vehicle-pretreated SCRH (Fig. 1).

Trabectedin-mediated cytotoxicity in SCRH from TR⁻ rats

Primary hepatocytes from Mrp2-deficient TR⁻ rats were utilized to examine the involvement of Mrp2 in trabectedin-mediated cytotoxicity at 0.1 μM DEX. Unexpectedly, cytotoxicity caused by exposure to 50 nM and 100 nM trabectedin in TR⁻ SCRH was reduced compared to that in wild-type SCRH (Fig. 2).

BSO effect on trabectedin-mediated cytotoxicity in wild-type and TR⁻ SCRH

Elevated levels of glutathione in TR⁻ compared to wild-type rat hepatocytes has been proposed as one mechanism that can protect the hepatocyte from drug-induced hepatotoxicity (Lu et al., 1996). To determine the effect of GSH depletion on trabectedin cytotoxicity, BSO (500 μM), an inhibitor of γ-glutamylcysteine synthetase which is the rate-liming step in GSH synthesis, was incubated for 24 hr prior to trabectedin exposure in SCRH from wild-type and TR⁻ rats (Griffith and Meister, 1979). Interestingly, toxicity in wild-type SCRH pretreated with BSO was reduced compared to vehicle-pretreated wild-type SCRH (Fig. 3). Trabectedin-mediated cytotoxicity in BSO-pretreated TR⁻ SCRH was not different from vehicle-pretreated TR⁻ SCRH (data not shown).

Alteration of Mrp2, 3, 4 and CYP3A1/2 protein expression by DEX and BSO

When SCRH were cultured with additional DEX (1µM), the fold increase in expression of CYP3A1/2 and Mrp2 was 5.0±2.5 and 2.5±0.6, respectively, compared to vehicle, whereas the fold increase in protein expression of Mrp3 and Mrp4 was 1.2±0.7 and 1.4±0.4, respectively (Fig. 4). Interestingly, in BSO-pretreated SCRH, the fold increase in Mrp4 expression was 3.1 ±0.7 whereas the fold increase in Mrp2, Mrp3 and CYP3A1/2 protein expression was 1.5±0.7, 1.9±0.4 and 1.2±0.9, respectively.

Hepatobiliary disposition of trabectedin in IPLs

IPLs from wild-type and TR⁻ rats were utilized to examine the involvement of Mrp2 in trabectedin hepatobiliary disposition. Trabectedin concentrations in perfusate were not different between livers from wild-type and TR⁻ rats; trabectedin was rapidly extracted by the liver and concentrations were negligible after 20 min (Fig. 5.a). Biliary recovery of trabectedin represented less than 3 % of the dose in wild-type rat livers, consistent with extensive metabolism of trabectedin in rats as reported previously (Fig. 5.b) (Reid et al., 2002). Biliary recovery of trabectedin in IPLs from TR⁻ rats was decreased by ~75% compared to wild-type IPLs.

Discussion

SCRH were selected to investigate the hepato-protective effect of DEX against trabectedin-mediated cytotoxicity because they maintain metabolic enzyme activity and develop functional bile canalicular networks with proper localization of hepatic transport proteins (Liu et al., 1998; LeCluyse et al., 1999; Liu et al., 1999; Hoffmaster et al., 2004). Previous experiments in freshly isolated rat hepatocytes cultured in a conventional configuration were unable to replicate the hepato-protective effect of DEX against trabectedin-mediated cytotoxicity when rats were pretreated orally with 20 mg/kg/day of DEX 24 hr prior to hepatocyte isolation (Donald et al., 2004). When freshly isolated hepatocytes are cultured on a single collagen substratum (conventional configuration), hepatocytes do not form bile canalicular networks, do not re-establish cell polarity, and gradually lose expression of CYP enzymes (LeCluyse et al., 1996; George et al., 1997). The loss of CYP expression over time in culture of human hepatocytes varies for each isoform (George et al., 1997); mRNA levels of some P450s such as CYP1A2 and CYP2C9 did not change over time in culture whereas CYP2E1 and CYP3A4 expression declined gradually over time in culture. Therefore, induction of CYP3A protein by DEX prior to hepatocyte isolation may be lost during culture in a conventional configuration. In contrast, incubation of SCRH with additional DEX (1 µM) increased CYP3A1/2 protein levels compared to vehicle-treated SCRH (Fig. 4.) and may provide hepato-protection from trabectedin-mediated cytotoxicity.

Vectorial transport studies in polarized cell monolayers examining cellular accumulation of [^{14}C] trabectedin in the absence and presence of P-gp inhibitors indicated that trabectedin is a substrate for P-gp (Beumer et al., 2007a). However, P-gp was only able to confer resistance to trabectedin when expressed at very high levels, suggesting that the intrinsic clearance of P-gp for trabectedin is low. Even though trabectedin seems to be more toxic than its metabolites based on previous cytotoxicity studies (Brandon et al., 2005), limited information is available on the interaction of other hepatic transport proteins with trabectedin.

The present studies are the first to demonstrate the involvement of Mrp2 in the biliary excretion of trabectedin in whole rat livers. Although the biliary excretion of unchanged trabectedin was low as a percentage of the total dose, the biliary excretion of trabectedin in IPLs from TR⁻ rats was decreased ~75% compared to wild-type IPLs (Fig. 5.b). As shown in Fig. 4, induction of Mrp2 protein expression by additional DEX (1µM) in SCRH may provide hepato-protection

from trabectedin-mediated cytotoxicity by increasing the biliary excretion of parent compound and/or derived metabolites.

Interestingly, TR⁻ SCRH were more resistant to trabectedin-mediated cytotoxicity relative to wild-type controls, despite the increased potential for trabectedin accumulation in TR⁻ hepatocytes due to impaired biliary excretion. This observation could be explained by modulation of the expression/activity of hepatic basolateral efflux transport proteins and metabolic enzymes in TR⁻ rats. Mrp2-deficient rats are well-known to exhibit higher Mrp3 expression (Hirohashi et al., 1998; Ogawa et al., 2000; Johnson et al., 2006). Mrp3 mediates hepatic basolateral efflux of substrates from hepatocytes to the blood, and is thought to compensate for impaired biliary excretion of organic anions during Mrp2 deficiency (Hirohashi et al., 2000; Xiong et al., 2000). In addition, the expression and activity of several phase I and II metabolic enzymes that may be involved in trabectedin detoxification are modulated in TR⁻ rats. For example, the total P450 content and activity of CYP1A1/2 and CYP2B1/2 were significantly higher in TR⁻ compared to wild-type rats, although testosterone 6β-hydroxylation mediated by CYP3A1/2 was lower in TR⁻ compared to wild-type rats (Newton et al., 2005; Silva et al., 2005). TR⁻ rats exhibit up-regulated UGT1a (Johnson et al., 2006). Since Mrp2 plays an important role in GSH biliary excretion, impaired function of Mrp2 in TR⁻ rats has been attributed to higher hepatocellular concentrations of GSH relative to wild-type rats (Lu et al., 1996). The important role of GSH in detoxification of the reactive metabolite of acetaminophen, N-acetyl-p-benzoquinoneimine, was demonstrated recently in TR⁻ rats (Silva et al., 2005); TR⁻ rats were more resistant to acetaminophen hepatotoxicity compared to wild-type rats, but this protection against acetaminophen hepatotoxicity in TR⁻ rats was completely reversed by BSO treatment. Previous studies in cultured rat hepatocytes have documented that 200 µM BSO reduced GSH levels by 50% after 5 hr (Sinbandhit-Tricot et al., 2003). Interestingly, in the present study, pretreatment of SCRH with BSO (500 µM) for 48 hr decreased trabectedin–mediated cytotoxicity relative to vehicle-pretreated SCRH.

A novel finding in the current study was induction of Mrp4 in SCRH associated with BSO treatment. MRP4 is a basolateral protein involved in the transport of xenobiotics and endogenous substrates such as cyclic nucleotides (cAMP and cGMP) and bile acids (van Aubel et al., 2002; Reid et al., 2003; Rius et al., 2003; Tian et al., 2006). MRP4 mediates the efflux of GSH across the basolateral membrane of hepatocytes into blood by cotransport with monoanionic bile acids (Rius et al., 2003). BSO may play a role as a GSH-depleting agent not only by inhibiting synthesis, but also by enhancing hepatic efflux of GSH out of the hepatocyte by increasing the expression of Mrp4. Whether upregulation of Mrp4 by BSO is a direct effect, or an indirect response associated with oxidative stress due to GSH depletion, requires further investigation. MRP4 mRNA and protein expression were significantly up-regulated in liver samples from patients with cholestasis, indicating a potential role of MRP4 in protecting the liver by decreasing the intracellular accumulation of detergent-like bile acids (Gradhand et al., 2007). Hence, the protective role of BSO in trabectedin-mediated cytotoxicity may be due to increased efflux of trabectedin or toxic metabolites by Mrp4, although the affinity of trabectedin for Mrp4 is unknown.

In conclusion, the hepato-protective effect of DEX on trabectedin toxicity may be due to increased metabolism by CYP3A1/2 as well as enhanced biliary excretion by Mrp2. Although the interaction of trabectedin and/or derived metabolites with basolateral transport proteins such as Mrp3 and 4 remains to be elucidated, the unexpected attenuation of trabectedin toxicity in SCRH from TR⁻ rats, and in SCRH pretreated with BSO, may be associated with increased basolateral excretion of trabectedin /metabolites by Mrp3 and/or Mrp4.

Acknowledgements

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Abbreviations

ABC, ATP binding cassette
BSO, buthionine sulfoximine
CYP, cytochrome P450
DEX, dexamethasone
DMEM, Dulbecco's modified Eagle's medium
EHBR, Eisai hyperbilirubinemic rats
GSH, glutathione
GST, glutathione-S-transferase
LDH, lactate dehydrogenase
MRP, multidrug resistance-associated protein
P-gp, P-glycoprotein
SCRH, sandwich-cultured primary rat hepatocytes
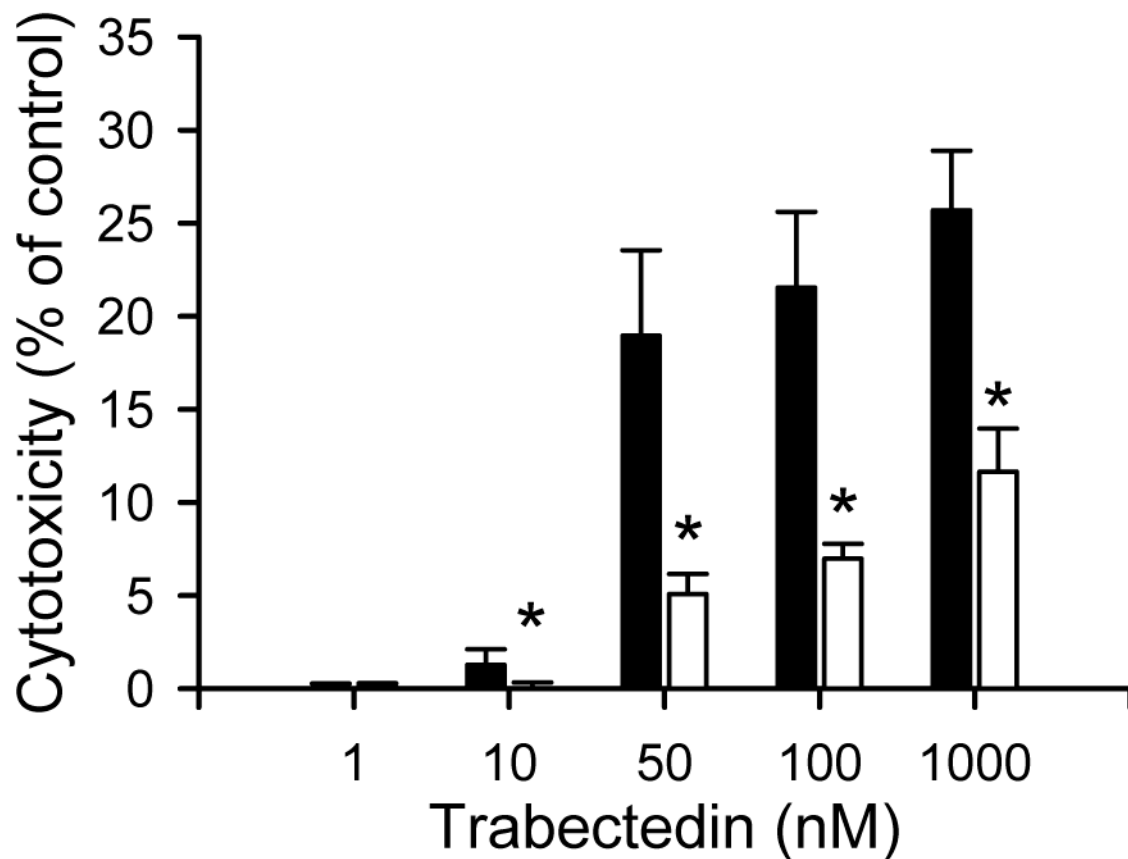UGT, uridine diphosphoglucuronosyl transferase

Fig. 1.
Effect of DEX on trabectedin-mediated cytotoxicity in SCRH isolated from wild-type Wistar rats. Cytotoxicity was determined by measuring LDH release into the culture medium after 24 hr trabectedin (1 – 1000 nM) exposure. Each bar represents the mean ± S.E.M. (n=3) for samples from vehicle- (0.1 µM DEX; solid bars) or additional DEX (1 µM; white bars); *, $p < 0.05$.

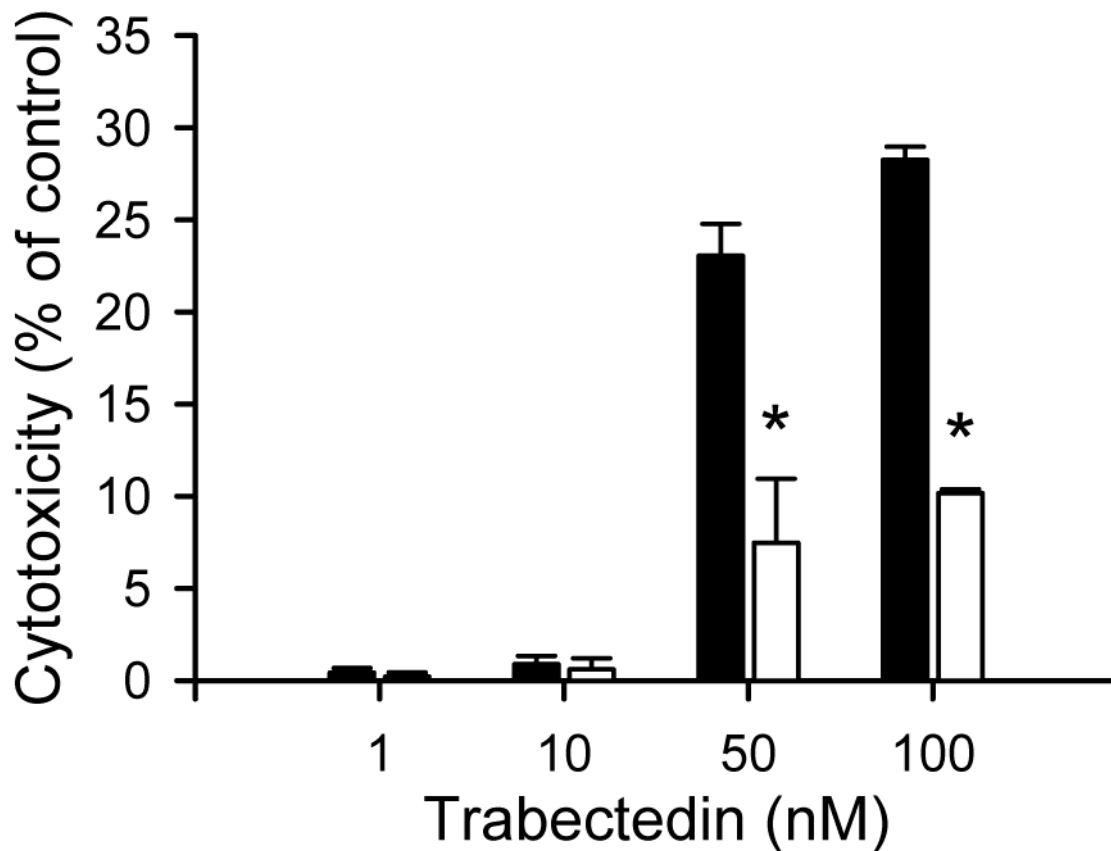**Fig. 2.**
Trabectedin-mediated cytotoxicity in SCRH from wild-type and TR⁻ rats. Cytotoxicity was determined by measuring LDH release into the culture medium after 24 hr trabectedin (1 – 100 nM) exposure. Each bar represents the mean ± S.E.M. (n=3) for samples from wild-type (solid bars) or TR⁻ (white bars) SCRH; *, $p < 0.05$.
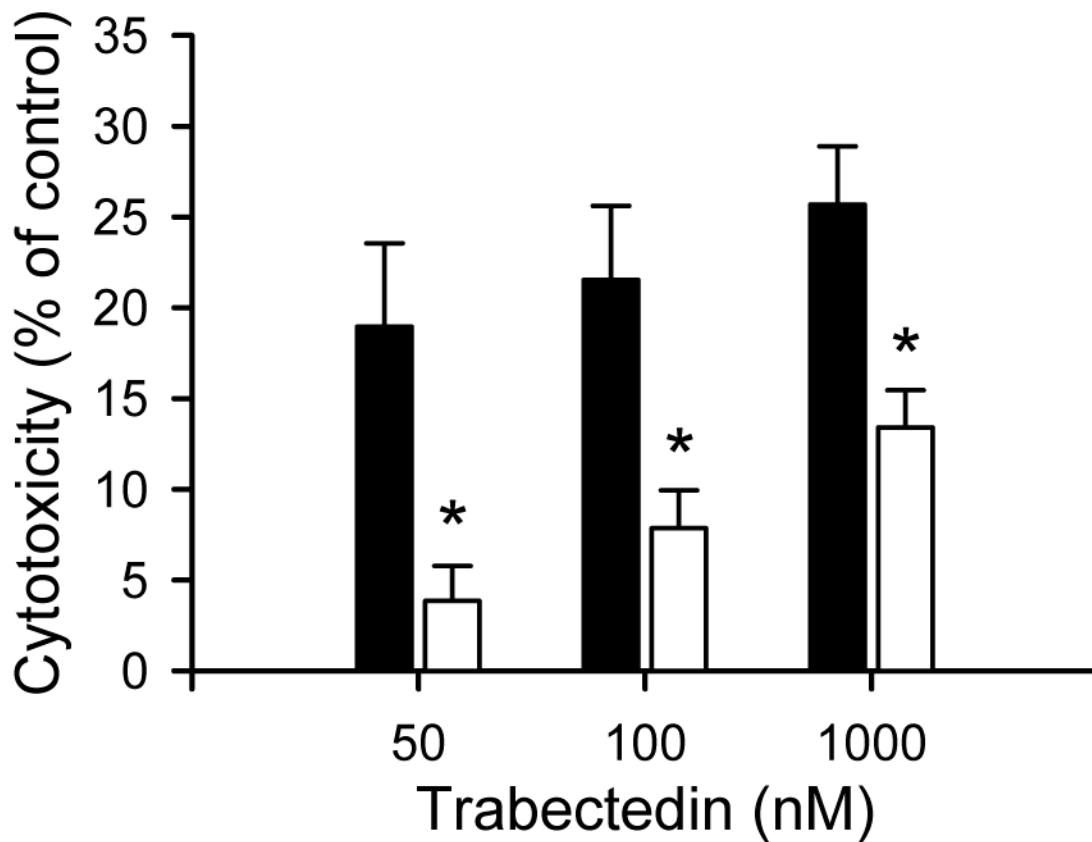
Fig. 3.
Effect of BSO on trabectedin-mediated cytotoxicity. Cytotoxicity was determined by measuring LDH release into the culture medium after 24 hr trabectedin (50, 100 and 1000 nM) exposure. BSO (500 µM) was added to the culture medium 24 h prior to trabectedin exposure. Each bar represents the mean ± S.E.M. (n=3) for samples from vehicle- (solid bars) or BSO-pretreated (white bars) SCRH; *, $p < 0.05$.
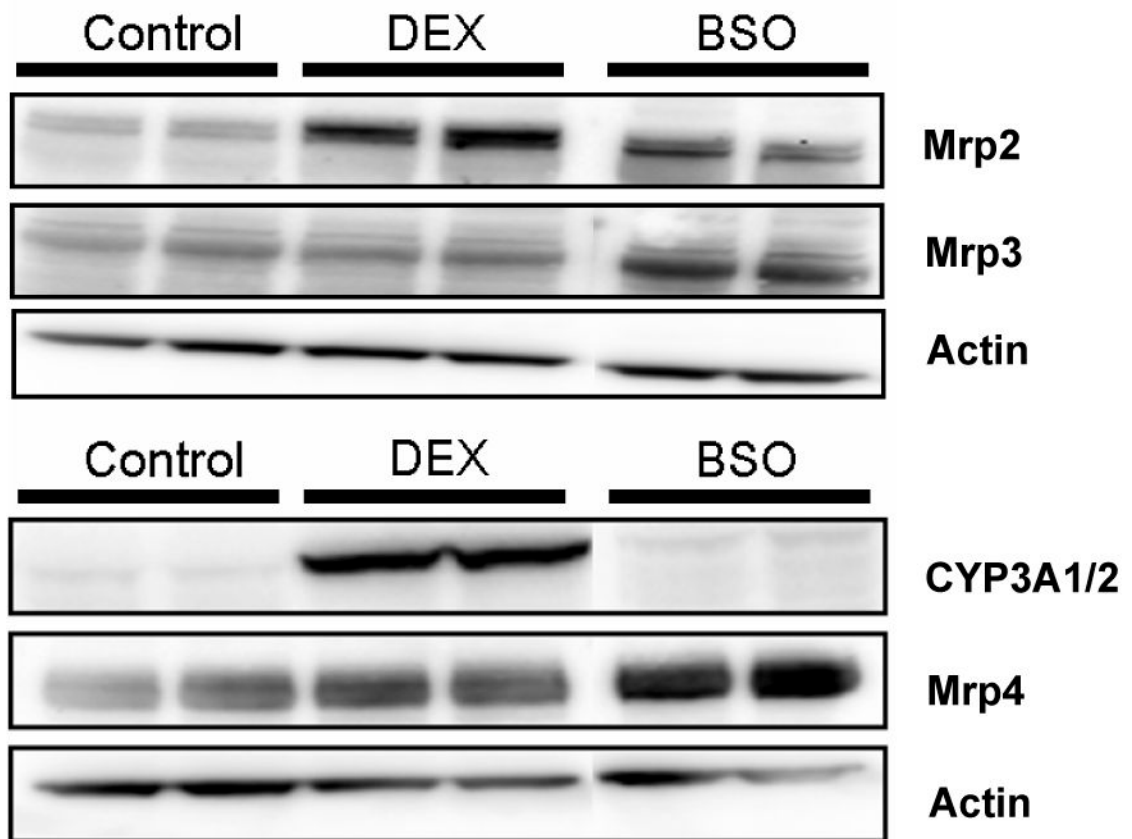
Fig. 4.
Representative Western blot from whole cell lysates for Mrp2, 3, 4 and CYP3A1/2 in SCRH pretreated with vehicle (0.1 µM DEX), additional DEX (1 µM) or BSO (500 µM) (25 µg protein/well; n=3).
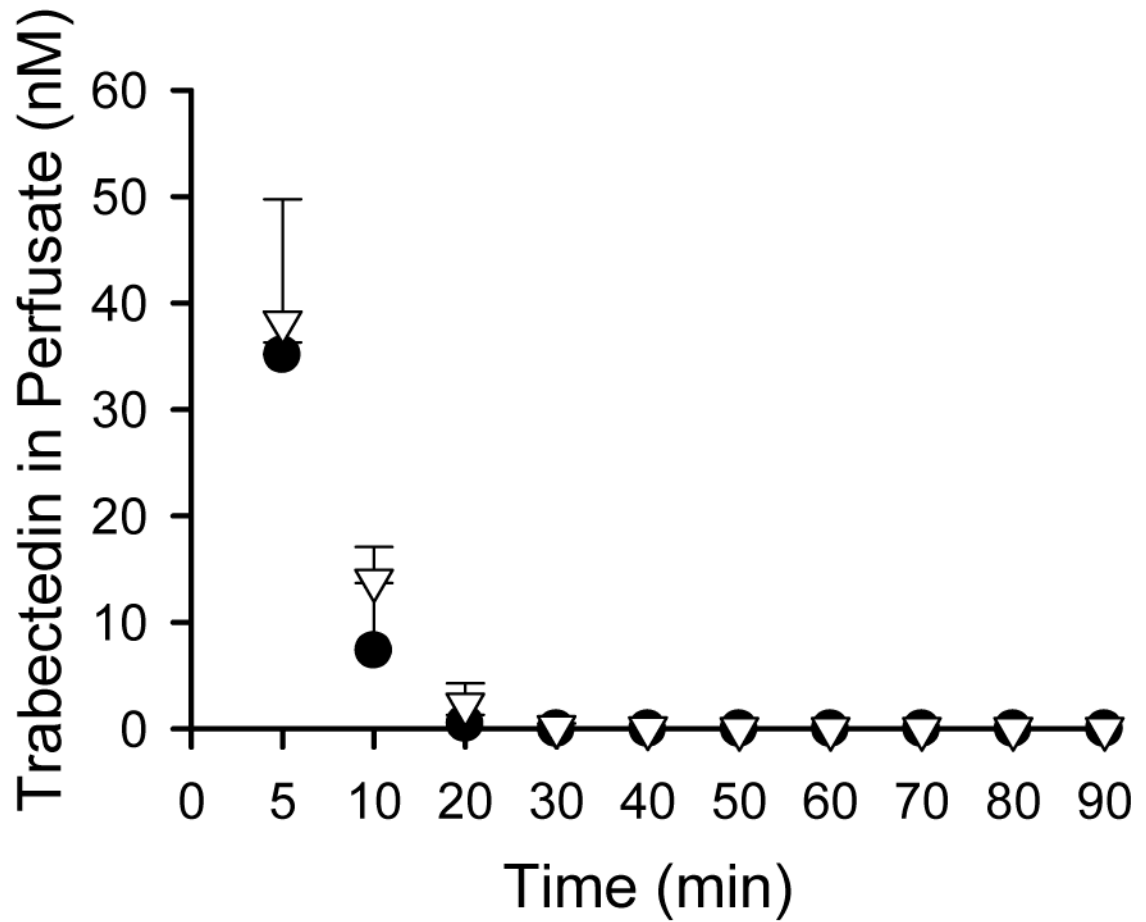
Fig. 5.
Trabectedin perfusate concentrations (Fig. 5.a) and cumulative biliary excretion (Fig. 5.b) in isolated perfused livers from wild-type (solid circle) and TR⁻ (white triangle) rats. Data are presented as mean ± S.D., n= 3/group.