ABCC2, ABCC3, and ABCB1, but not CYP3A, Protect against Trabectedin-Mediated Hepatotoxicity

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Abstract

Purpose: Trabectedin (Yondelis, ET-743) is a novel anticancer drug with potent activity against various tumors. However, dose-limiting hepatotoxicity was observed during clinical trials. Because recent reports have suggested that cytochrome P450 3A (CYP3A), as well as the drug transporters ABCB1, ABCC2, and ABCC3 might protect against trabectedin-mediated hepatotoxicity, we investigated the individual and combined roles of these detoxifying systems.

Experimental Design: Madin-Darby canine kidney cells expressing ABCC2 and ABCC3 were used to study in vitro trabectedin transport. We investigated the hepatotoxicity of trabectedin, and the plasma and liver levels of this drug and its metabolites in mice deficient for CYP3A, Abcb1a/1b, Abcc2, and/or Abcc3 after i.v. trabectedin administration.

Results: Trabectedin was transported by ABCC2 but only modestly by ABCC3. Contrary to our expectation, absence of CYP3A resulted in only a marginal increase in hepatotoxicity. Some hepatotoxicity was observed in Abcc2⁻/⁻ mice, but very little in Abcb1a/1b⁻/⁻ and Abcc3⁻/⁻ mice. Strikingly, severe hepatotoxicity was found in Abcb1a/1b⁻/⁻ and Abcc2/Abcc3⁻/⁻ mice. However, hepatotoxicity was drastically decreased in Cyp3a/Abcb1a/1b/Abcc2⁻/⁻ compared with Abcb1a/1b/Abcc2⁻/⁻ mice. This suggests that the formation of CYP3A-specific metabolites is an important prerequisite for trabectedin-mediated hepatotoxicity. Further studies revealed that there is increased accumulation of metabolites of trabectedin, but not of trabectedin itself, in the livers of mice that lack Abcc2 but are CYP3A proficient.

Conclusions: Our data show that ABCB1, ABCC2, and ABCC3 have a profound and partially redundant function in protection from trabectedin-mediated hepatotoxicity, presumably by clearing the liver from hepatotoxic trabectedin metabolites that are primarily formed by CYP3A.

Trabectedin is an anticancer drug isolated from a marine tunicate that has recently been approved for the treatment of soft tissue carcinoma. In addition, several phases II and III clinical trials of trabectedin for treatment of other cancer types have been published or are still ongoing, including trials for breast (1), ovarian (2, 3), and prostate cancer (4). Although trabectedin has impressive antitumor activity, dose-limiting hepatotoxicity became apparent during several clinical trials (reviewed in ref. 5).

Mass balance studies have revealed that trabectedin is extensively metabolized, and virtually all trabectedin is excreted in its metabolite form into bile. The chemical structure of trabectedin provides numerous sites for metabolic conversion. Indeed, a large number of (unknown) metabolites, without clearly predominating metabolites, could be observed in radiochromatograms after i.v. administration of (¹⁴C)trabectedin to patients (6). No metabolites in plasma have been structurally identified so far and only a few metabolites from feces (6). The low dose and high volume of distribution, and long terminal half-life of trabectedin result in very low concentrations of trabectedin and its metabolites in plasma, urine, and feces, hampering the structural elucidation of the trabectedin metabolites (6, 7). Consequently, there is also only very limited evidence on whether the trabectedin metabolites are pharmacodynamically active or could in part be responsible for the trabectedin-mediated hepatotoxicity.

In vitro studies have indicated that cytochrome P450 3A (CYP3A) enzymes are primarily responsible for the metabolic degradation of trabectedin (8, 9). CYP3A enzymes are known to metabolize ~50% of the currently marketed drugs, including many anticancer drugs (10). Importantly, interactions at the CYP3A level are often the cause of drug-drug interactions. This is especially relevant in the field of oncology, in which patients often receive multiple drug treatments and with drugs that are potentially highly toxic (11).
CYP3A and Drug Transporters Determine Trabectedin Toxicity

Translational Relevance

In this study we found that metabolites of trabectedin that are specifically formed by the drug-metabolizing enzyme CYP3A are an important determinant of trabectedin-mediated hepatotoxicity. In particular, when the drug efflux transporter Abcc2 is absent, these trabectedin metabolites accumulate in the liver, which results in hepatotoxicity. This trabectedin-mediated hepatotoxicity becomes especially severe when, in addition to Abcc2, the drug transporter Abcb1a/1b or Abcc3 is also absent. Therefore, patients with reduced Abcc2 (and ABCB1 or ABCC3) activity, due to polymorphisms or due to inhibition by comedication and/or food constituents, might be at increased risk for severe hepatotoxicity during trabectedin treatment. These hepatotoxic effects caused by trabectedin could be even more severe in patients in whom low ABC2 activity is accompanied by high CYP3A activity.

Interestingly, pretreatment with the glucocorticoid dexamethasone, 24 hours before trabectedin administration, reduces the hepatotoxic effects of trabectedin, whereas direct dexamethasone coadministration has no effect (12, 13). The mechanism of how dexamethasone reduces the hepatotoxicity is not understood, but it has been suggested that its protective effect could be due to increased metabolism by CYP3A enzymes (14, 15). Indeed, dexamethasone is a well-known inducer of CYP3A expression (e.g., ref. 16). In addition, it has been suggested that the anti-inflammatory properties of dexamethasone could be responsible for the protection from trabectedin-mediated hepatotoxicity (5).

Recent reports have suggested that in addition to CYP3A, drug transporters of the ATP-binding cassette family are also involved in the disposition of trabectedin. For example, trabectedin was shown to be a substrate for human ABCB1 (P-glycoprotein/MDR1) as well as for mouse Abcb1a in vitro (17). In addition, it was recently shown that there was altered toxicity in hepatocytes from ABCC2 (MRP2)-deficient (TR -) rats, indicating a possible role for ABCC2 in the hepatotoxicity of trabectedin (14). It was also hypothesized that the basolaterally located drug transporter ABCC3 (MRP3) could affect the trabectedin-mediated hepatotoxicity (14). Little in vivo evidence, however, is currently available about whether any of these drug transporters affects the disposition, and possibly also the hepatotoxicity, of trabectedin.

In this study we aimed to investigate the individual and combined roles of CYP3A, and the drug transporters ABCB1, ABCC2, and ABCC3 in the trabectedin-mediated hepatotoxicity. To this end, we gave trabectedin to several mouse strains that were lacking CYP3A, Abcb1a/1b, Abcc2, and/or Abcc3. Based on the available literature, we expected a strong protective role for CYP3A in the trabectedin-mediated hepatotoxicity. However, our data revealed that in an Abcc2-deficient situation, CYP3A has a toxifying rather than a detoxifying role. These unexpected findings qualify the role of CYP3A in the detoxification of trabectedin and indicate a protective role for drug transporters, especially for ABC2. The findings presented in this paper could have important implications for the clinical use of trabectedin.

Materials and Methods

Chemicals and reagents. Trabectedin (Yondelis, eceitansidin 743, ET-743) was obtained from the National Cancer Institute Developmental Therapeutics Program Repository. 3H (3H)-trabectedin was kindly provided by Dr. Luis Lopez-Lazaro (PharmaMar, Madrid, Spain). Methoxyflurane (Metofane) was obtained from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). All other chemicals were of analytical grade and obtained from standard commercial suppliers unless mentioned otherwise.

Animals. All mice used in this study were housed and handled according to institutional guidelines complying with the Dutch national law. All mice were constantly kept in a temperature-controlled environment, where a 12:12-hour light/dark cycle was maintained. The animals were given a standard AM-III diet from Hope Farms (Woerden, the Netherlands) and acidified water ad libitum. The different mouse strains used in this study consisted of wild-type (FVB), Cyp3a knockout (Cyp3a−/−; ref. 18), Abcb1a/1b knockout (Abcb1a/1b−/−; ref. 19), Abcc2 knockout (Abcc2−/−; ref. 20), Abcc3 knockout (Abcc3−/−; ref. 21), Abcb1a/1b/Abcc2 knockout (Abcb1a/1b/Abcc2−/−; ref. 22), Abcc2/Abcc3 knockout (Abcc2/Abcc3−/−; ref. 23), and Cyp3a/Abcb1a/1b/Abcc2 knockout (Cyp3a/Abcb1a/1b/Abcc2−/−) mice. All knockouts were on a 99% FVB background. Only 8-to-15-wk-old male mice were used throughout the study. All experiments were approved by the local committee for animal experiments.

Cell lines and tissue culture. The polarized Madin-Darby canine kidney (MDCK-II) cell line was used in transport studies. Human ABCC2-transduced and ABCC3-transduced, as well as mouse Abcc2-transduced MDCK-II subclones were described previously (24–26). The MDCK-II cells and transduced subclones were cultured in DMEM supplied with Glutamax (Invitrogen, Carlsbad, CA), and supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% (volume for volume) fetal calf serum (Invitrogen) at 37°C in the presence of 5% CO2. The cells were trypsinized every 3 to 4 d for subculturing.

In vitro transport studies. Transport assays were done as reported previously with some minor modifications (17, 27). Briefly, cells were seeded on microporous polycarbonate membrane filters (Transwell 3414; Costar, Corning, NY) at a density of 1.0 × 106 cells per well in 2 mL of complete medium and subsequently grown for 3 d. Two hours before the start of the experiment, the complete medium in the apical chamber was removed and replaced with Opti-MEM medium with (14C)-trabectedin (166 ng/mL) in the appropriate compartment. This was done in the presence of 5 μmol/L elaidic (to inhibit any endogenous P-glycoprotein activity). At t = 0 h, the experiment was started by replacing the medium with fresh Opti-MEM medium with (14C)-trabectedin (166 ng/mL) in the appropriate compartment. Transport was considered the fraction of drug found in the acceptor compartment relative to the total amount added to the donor compartment at the beginning of the experiment. Transport was given as mean percent (SD = 3). Membrane tightness was assessed with the use of (1H) inulin, which was added to the donor compartment. Leakage was not allowed to be >1% of the total added radioactivity/h.

In vivo study of hepatotoxicity caused by trabectedin. Wild-type, Cyp3a−/−, Abcb1a/1b−/−, Abcc2−/−, Abcc3−/−, Abcc2/Abcc3−/−, Abcb1a/1b/ Abcc2−/−, and Cyp3a/Abcb1a/1b/Abcc2−/− mice were used. Before drug administration blood was collected by tail sampling. After i.v. injection of trabectedin (100 μg/kg) in ethanol and KH2PO4 (50 mmol/L, pH 4;
lated. Livers were weighed and directly dissolved in 4 mL Solvable tis-

\[ P < 0.01; \text{and} \] ***, and ***, respectively.

\[ \text{Hepatotoxicity caused by trabectedin} \]
\[ \text{indicated fairly efficient (apically directed) transport by ABCC2/} \]
\[ \text{and ABCC3 (Supplementary Data 1).} \]

\[ \text{Previous studies in mice indicated that according to these} \]
\[ \text{studies with trabectedin in mice (28). Blood was collected in heparin-} \]
\[ \text{with the use of liquid chromatography with tandem mass} \]
\[ \text{in vivo} \]
\[ \text{To investigate the role of CYP3A and several drug transporters in the pharmacokinetics of trabectedin, wild-type, Cyp3a/} \]
\[ \text{To determine levels of total biliru-} \]
\[ \text{and} \] ***, and ***, respectively.

\[ \text{Determination of trabectedin levels in plasma and liver.} \]
\[ \text{To investigate the role of CYP3A and several drug transporters in the pharmacokinet-} \]
\[ \text{in} \]
\[ \text{in vitro} \]
\[ \text{in vitro} \]
\[ \text{in vitro} \]

\[ \text{Statistical analysis.} \]
\[ \text{For data comparisons, Student's unpaired, two-} \]
\[ \text{1:100), blood was drawn by tail sampling after 2, 3, and 4 d. All samples} \]
\[ \text{were collected in heparinized tubes, and plasma was obtained after cen-} \]
\[ \text{in vitro} \]
\[ \text{in vivo} \]
\[ \text{in vitro} \]

\[ \text{Results} \]
\[ \text{In vitro transport of trabectedin by ABCB2 and ABCC3.} \]
\[ \text{Previous in vitro studies have shown that trabectedin can be transport-} \]
\[ \text{in vitro} \]
\[ \text{in vitro} \]

\[ \text{Role of CYP3A, Abcb1a/1b, and Abcc2 in the trabectedin-mediated hepatotoxicity.} \]
\[ \text{Hepatotoxicity caused by trabectedin has been reported based on elevated blood levels of several} \]
\[ \text{in vivo} \]

\[ \text{Determination of trabectedin levels in plasma and liver.} \]
\[ \text{To get more insight into the trabectedin metabolism, (14C)-trabectedin (200 μg/kg) was given i.v. to wild-type, Cyp3a/} \]

1:100), blood was drawn by tail sampling after 2, 3, and 4 d. All samples were collected in heparinized tubes, and plasma was obtained after centrifugation at 8,000 rpm for 6 min. An aliquot of 50 μL of plasma was diluted 3-fold with saline, and analyzed for alanine aminotransaminase, aspartate aminotransaminase, alkaline phosphatase, and bilirubin levels. At every time point, the loss of body weight of individual mice was determined and did not exceed 20% during the experiment. Four days after drug administration, the mice were sacrificed by cardiac puncture under methoxyflurane anesthesia followed by cervical dislocation.

Clinical-chemical analysis of plasma. Standard clinical chemistry analyses on mouse plasma were done on a Roche Hitachi 917 analyzer (Roche Diagnostics, Basel, Switzerland) to determine levels of total bilirubin, alkaline phosphatase, aspartate aminotransaminase, alanine aminotransaminase, γ-glutamyl transferase, lactate dehydrogenase, creatinine, and urea.

Determination of trabectedin levels in plasma and liver. To investigate the role of CYP3A and several drug transporters in the pharmacokinetics of trabectedin, wild-type, Cyp3a/Abcb1a/1b/Abcc2/Abcc1a/1b/Abcc2/Abcc1a/1b/Abcc2/Abcc1a/1b/Abcc2 mice were used in this study. At \( t = 0 \), trabectedin (200 μg/kg) was given i.v. in ethanol and KH₃PO₄ (50 mmol/L, pH 4; 1:50) was given i.v. After 40 min, cardiac puncture was done under anesthesia with methoxyflurane for three mice per time point. Forty minutes was selected based on previous pharmacokinetic studies with trabectedin in mice (28). Blood was collected in heparinized syringes, and plasma was obtained after centrifugation at 2,100 × g for 6 min. Mice were sacrificed by cervical dislocation and livers were isolated. Plasma samples and liver homogenates were analyzed for trabectedin with the use of liquid chromatography with tandem mass spectrometry, according to Rosing et al. (29).

To get insight into the trabectedin metabolism, (14C)-trabectedin (200 μg/kg) was given i.v. to wild-type, Cyp3a/Abcb1a/1b/Abcc2/Abcc1a/1b/Abcc2/Abcc1a/1b/Abcc2/Abcc1a/1b/Abcc2/Abcc1a/1b/Abcc2/Abcc1a/1b/Abcc2 mice. After 20 min, blood was collected in heparinized tubes through tail sampling. After 40 min, a cardiac puncture was done under anesthesia with methoxyflurane. After centrifugation of the blood samples (2,100 × g for 6 min), plasma was obtained at both time points. Mice were sacrificed by cervical dislocation and livers were isolated. Livers were weighed and directly dissolved in 4 mL solvable tissue solubilizer from Packard (Groningen, the Netherlands). Plasma and liver samples were quantified for radioactivity.

Statistical analysis. For data comparisons, Student's unpaired, two-tailed \( t \) test was used. Data are presented as means ± SD (*, \( P < 0.05; **, \) \( P < 0.01; \) and ***, \( P < 0.001 \)).

Results

In vitro transport of trabectedin by ABCB2 and ABCC3. Previous in vitro studies have shown that trabectedin can be transported by human and mouse ABCB1/Abcb1a (17). Analysis of in vitro transport of trabectedin by either human ABCC2 or ABCC3, or mouse Abcc2 in transduced polarized MDCK-II cells indicated fairly efficient (apically directed) transport by ABCC2/Abcc2, but only very modest (basolaterally directed) transport by ABCC3 (Supplementary Data 1).

Role of CYP3A, Abcb1a/1b, and Abcc2 in the trabectedin-mediated hepatotoxicity. Hepatotoxicity caused by trabectedin has been reported based on elevated blood levels of several markers, including alanine aminotransaminase, aspartate aminotransaminase, alkaline phosphatase, and bilirubin (5). Previous studies in mice indicated that according to these markers, the hepatotoxicity is maximal 3 days after trabectedin administration (28). CYP3A has been suggested to efficiently metabolize trabectedin in the liver, thereby reducing the hepatotoxic effects (8, 9, 12). To get more insights into the in vitro protective role of CYP3A in trabectedin-mediated hepatotoxicity, we gave trabectedin (100 μg/kg i.v.) to Cyp3a/−/− and wild-type mice. Alanine aminotransaminase and bilirubin levels were unchanged 3 days after administration when compared with wild-type mice (Fig. 1A and D). A modest increase (<2-fold) was observed in aspartate aminotransaminase and alkaline phosphatase levels for Cyp3a/−/− mice compared with wild-type mice, representing a very mild hepatotoxic profile (Fig. 1B and C). Pathologic examination also did not reveal any signs of hepatotoxicity (Fig. 2). Nonetheless, 4 days after drug administration the alanine aminotransaminase, aspartate aminotransaminase, and alkaline phosphatase levels were slightly increased in Cyp3a/−/− mice (Supplementary Data 2), perhaps suggesting a very modest late hepatotoxicity in these mice compared with wild-type mice.

Trabectedin can be transported by ABCB1/Abcb1a and ABCC2/Abcc2 in vitro (see ref. 17 and Supplementary Data 1). To address the in vivo importance of these drug transporters in the protection against trabectedin-mediated hepatotoxicity, we gave trabectedin to Abcb1a/1b/Abcc2/−/− mice. Strikingly, severe hepatotoxicity was observed in Abcb1a/1b/Abcc2/−/− mice, with levels of alanine aminotransaminase and aspartate aminotransaminase that were each elevated >100-fold in comparison with wild-type (Fig. 1). Alkaline phosphatase levels were also increased ~6-fold at this time point (Fig. 1). In addition, whereas levels of bilirubin were below the detection limit in wild-type mice (<6 units/L), they reached levels >100 units/L in Abcb1a/1b/Abcc2/−/− mice (Fig. 1D). Histopathologic examination also revealed severe hepatotoxicity (Fig. 2). The parenchymal cells of the liver from Abcb1a/1b/Abcc2/−/− mice underwent massive degeneration and necrosis, and collapse of the lobular structures. The hepatocytes showed vacuolization and eosinophilic changes of the cytoplasm, and pyknosis and breakdown of the nuclei. In marked contrast, the livers from wild-type and Cyp3a/−/− mice showed hardly any pathologic alterations after trabectedin treatment (Fig. 2).

Overall, these data indicate that in contrast to wild-type and Cyp3a/−/− mice, severe hepatotoxicity was observed in Abcb1a/1b/Abcc2/−/− mice. We note that besides the hepatotoxicity markers, no other clinical chemistry variables (see Materials & Methods) were significantly different in these mice (data not shown).

To investigate the effect of the combined loss of both CYP3A and the drug transporters Abcb1a/1b and Abcc2 on the trabectedin-mediated hepatotoxicity, we gave trabectedin to Cyp3a/Abcb1a/1b/Abcc2/−/− mice. Surprisingly, in contrast to the severe toxicity in Abcb1a/1b/Abcc2/−/− mice, only very mild hepatotoxicity was observed in Cyp3a/Abcb1a/1b/Abcc2/−/− mice, with vastly lower alanine aminotransaminase, aspartate aminotransaminase, alkaline phosphatase, and bilirubin levels than in Abcb1a/1b/Abcc2/−/− mice (Fig. 1). Accordingly, histopathologic analysis of Cyp3a/Abcb1a/1b/Abcc2/−/− livers revealed hardly any pathologic alterations (Fig. 2). These data point to an important role for CYP3A-generated metabolites in the hepatotoxicity of trabectedin.

Determination of trabectedin levels in plasma and liver. To get more insight into how CYP3A, Abcb1a/1b, and Abcc2 determine the trabectedin-mediated hepatotoxicity we evaluated levels of trabectedin in plasma and liver 40 minutes after i.v. administration. In addition, we coadministered (14C)-trabectedin to assess the amount of metabolites present in plasma and liver.
Consistent with the proposed important role of CYP3A in trabectedin metabolism, trabectedin levels in plasma and liver of Cyp3a-/- mice were significantly higher (8.3-fold and 2.6-fold, respectively) than in wild-type (Fig. 3, white bars). Note that from the toxicity data above, we did not find severe hepatotoxicity in Cyp3a-/- but rather in Abcb1a/1b/Abcc2-/- mice. Nevertheless, trabectedin levels in the plasma of Abcb1a/1b/Abcc2-/- mice were only slightly higher (2.1-fold) whereas trabectedin levels in the liver were even significantly lower (2.5-fold) compared with wild-type, and both were much lower than in Cyp3a-/- mice (Fig. 3, white bars). This suggests that trabectedin itself is not a major hepatotoxicity-determining factor in these mouse strains, as its liver level seems inversely correlated with the amount of hepatotoxicity. Interestingly, however, levels of trabectedin metabolites [(14C)-label] were highly elevated in both plasma and liver (5.2-fold and 2.4-fold, respectively) of Abcb1a/1b/Abcc2-/- mice, and were the highest among all strains investigated (Fig. 3, gray bars). Note that in all strains the amount of trabectedin metabolites in liver was far higher than that of the parent trabectedin.

In Cyp3a/Abcb1a/1b/Abcc2-/- mice, consistent with the absence of CYP3A, we found higher trabectedin levels in plasma and liver compared with wild-type (Fig. 3, white bars). However, whereas [(14C)]-levels were also higher in plasma of Cyp3a/Abcb1a/1b/Abcc2-/- mice, they were not significantly different in the liver when compared with wild-type (Fig. 3, gray bars). Overall, these data show that there is substantial accumulation of trabectedin metabolites, but not of trabectedin itself, in the livers (and plasma) of Abcb1a/1b/Abcc2-/- mice. Together with the toxicity data above, this suggests that CYP3A-generated metabolites, rather than trabectedin itself, are primarily responsible for the severe hepatotoxicity in Abcb1a/1b/Abcc2-/- mice.

Role of drug transporters in trabectedin-mediated hepatotoxicity. Given the severe hepatotoxicity in Abcb1a/1b/Abcc2-/- mice after trabectedin administration, we wanted to further investigate the individual roles of Abcb1a/1b and Abcc2 in the trabectedin-mediated hepatotoxicity. When trabectedin was given to Abcb1a/1b-/- mice, no significant hepatotoxicity was observed during the experiment (Fig. 4; Supplementary Data 3). In contrast, moderate hepatotoxicity was apparent in Abcc2-/- mice according to the elevated alanine aminotransaminase (13-fold) and aspartate aminotransaminase (7.1-fold) levels when compared with wild-type mice (Fig. 4). Alkaline phosphatase and bilirubin levels were also significantly increased (Fig. 4). In combined Abcb1a/1b/Abcc2-/- mice, however, the hepatotoxicity profile was far more severe (Fig. 4). These data indicate that Abcc2 plays an important role in protection from trabectedin-mediated hepatotoxicity and that this role becomes especially apparent in the absence of Abcb1a/1b function.

It has recently been suggested that, in addition to ABCB1 and ABCC2, the basolateral drug transporter ABCC3 could also play a (protective) role in the trabectedin-mediated hepatotoxicity.

**Fig. 1.** Levels of alanine aminotransaminase (ALAT; A), aspartate aminotransaminase (ASAT; B), alkaline phosphatase (Alk. Phos.; C), and total (unconjugated plus conjugated) bilirubin (D) in plasma of wild-type, Cyp3a-/-, Abcb1a/1b/Abcc2-/-, Cyp3a/Abcb1a/1b/Abcc2-/- mice before and 3 d after i.v. administration of 100 μg/kg trabectedin. Values, means ± SD; n = 6 to 9 male mice for each strain. Significance is in comparison with wild-type. Note break in Y-axes of the ALAT and ASAT levels.
Although our in vitro studies indicated that trabectedin itself is not a very good substrate for ABCC3 (Supplementary Data 1), ABCC3 might transport some trabectedin metabolites more efficiently. To investigate the in vivo role of ABCC3 in the trabectedin-mediated hepatotoxicity, we gave trabectedin to Abcc3-/- as well as to Abcc2/Abcc3-/- mice (Abcb1a/1b/Abcc2-/- mice are not available in our institute and were thus not included). Compared with wild-type mice, Abcc3-/- mice showed very little hepatotoxicity during the experiment (Fig. 4; Supplementary Data 3). In contrast, severe hepatotoxicity was observed in Abcc2/Abcc3-/- mice showing highly elevated alanine aminotransaminase (348-fold) and aspartate aminotransaminase (143-fold) levels comparable with the levels seen in Abcb1a/1b/Abcc2-/- mice and far higher than in Abcc2-/- mice (Fig. 4). Alkaline phosphatase and bilirubin levels were significantly elevated (Fig. 4). These data indicate that absence of Abcc3 markedly exacerbates the consequences of Abcc2 absence on trabectedin-mediated hepatotoxicity.

We also determined the trabectedin and [14C]-levels in plasma and liver after administration of trabectedin or [14C]-trabectedin, respectively. Mice that only lack Abcb1a/1b, Abcc2, or Abcc3 did not have significantly higher trabectedin plasma levels when compared with wild-type, in contrast to Abcb1a/1b/Abcc2-/- and Abcc2/Abcc3-/- mice (Fig. 5A, white bars). In addition, Abcb1a/1b/Abcc2-/- and Abcc2/Abcc3-/- mice also had the highest levels of [14C]-label in the plasma when compared
with wild-type (Fig. 5A, gray bars). More important for hepatotoxicity considerations, the parent trabectedin levels in the liver were significantly lower in Abcb1a/1b−/− and Abcb1a/1b/Abcc2−/− mice, but were similar for all other knockout strains when compared with wild-type (Fig. 5B, white bars). Interestingly, the levels of (14C)-label in the liver were only markedly higher in those strains that lack Abcc2 (Abcc2−/−, Abcb1a/1b/Abcc2−/−, and Abcc2/Abcc3−/−; Fig. 5B gray bars). Thus, combined with the toxicity data above, these data show a clear correlation between the mice that have the highest levels of trabectedin metabolites in liver and those that have the most severe hepatotoxicity profile, although the hepatotoxicity in single Abcc2−/− was relatively modest. In contrast, the hepatic levels of parent trabectedin did not correlate with the severity of hepatotoxicity.

### Discussion

This study has yielded a number of unexpected results. With the use of Cyp3a knockout mice, we could confirm that CYP3A plays an important role in clearance of parent trabectedin, with markedly higher plasma and liver levels in the absence of CYP3A. However, the hepatotoxicity of trabectedin was only marginally increased in Cyp3a−/− mice, suggesting that CYP3A activity is not a major factor in protection from trabectedin-mediated hepatotoxicity. In contrast, simultaneous loss of Abcb1a/1b and Abcc2 (Abcb1a/1b/Abcc2−/− mice) resulted in drastically increased hepatotoxicity, whereas the single knockouts displayed only little (Abcb1a/1b−/−) or modest (Abcc2−/−) hepatotoxicity. Strikingly, the additional deletion of Cyp3a in Cyp3a/Abcb1a/1b/Abcc2−/− mice resulted in nearly complete loss of the hepatotoxicity, although the liver levels of parent trabectedin were increased. Trabectedin metabolite levels, however, were markedly decreased in this strain. These data indicate that CYP3A-generated trabectedin metabolites, rather than the parent trabectedin, are primarily causing the hepatotoxicity. Moreover, Abcb1a/1b and Abcc2 together seem to remove the most hepatotoxic CYP3A-generated trabectedin metabolites from the liver. The highly increased hepatotoxicity and trabectedin metabolite levels (but not parent trabectedin levels) in liver of Abcc2/Abcc3−/− mice show that Abcc3 also plays an important role in clearing hepatotoxic trabectedin metabolites from the liver. Together, our data indicate that Abcb1a/1b, Abcc2, and Abcc3 have a profound and partially redundant function in protection from trabectedin-mediated hepatotoxicity.

Despite the potentially important clinical implications, there is only very limited information available about the effect of drug transporters on trabectedin-mediated hepatotoxicity. We showed in vitro that trabectedin is transported by ABCC2/Abcc2 and, albeit only modestly, by ABCC3. Previous in vitro studies have already shown that trabectedin is a substrate for...
ABCB1/Abcb1a (17). Because trabectedin is heavily metabolized, in vitro transport studies with the parent compound have only limited value as they do not provide information about the transport of the various trabectedin metabolites. In vivo studies with knockout mice for these drug transporters are therefore more appropriate to assess the actual impact of these drug transporters. We found a disproportionate increase in hepatotoxicity in mice that lack, in addition to Abcc2, either Abcb1a/1b or Abcc3, in comparison with the single knockout mice for these drug transporters. Hence, these drug transporters can, for a large part, take over the function of each other in the protection against trabectedin-mediated hepatotoxicity. Nevertheless, we observed significant hepatotoxicity in Abcc2−/− mice, indicating that Abcb1a/1b and Abcc3 could not completely take over the hepatoprotective function of Abcc2.

Several studies have indicated a primary role for CYP3A in the metabolism of trabectedin. In agreement, we found higher levels of trabectedin in the plasma and livers of Cyp3a−/− mice. However, although we had expected to find (severe) hepatotoxicity in Cyp3a−/− mice, this seemed to be only very modest, especially in comparison with the Abcc2−/− mice. Despite the prominent role for CYP3A in trabectedin metabolism, our data indicate that trabectedin can still be metabolized by other enzymes, in line with previous in vitro studies that have shown that other (human) CYPs can also metabolize trabectedin (8, 9). It is, however, important to realize that this mixture of metabolites formed by enzymes other than CYP3A apparently does not cause such severe hepatotoxicity. Based on the profoundly reduced trabectedin-mediated hepatotoxicity in Cyp3a/Abcb1a/1b/Abcc2−/− mice, we could substantiate that the metabolites formed by CYP3A are primarily responsible for the severe hepatotoxicity in Abcb1a/1b/Abcc2−/− mice. Accordingly, the role of CYP3A in trabectedin-mediated hepatotoxicity is rather complex, and can be seen as either toxifying or detoxifying depending on the presence or absence of the drug transporters. As long as the toxic metabolites formed by CYP3A can be transported out of the liver by Abcc2 and Abcb1a/1b or Abcc3, metabolism by CYP3A can be seen as a detoxification pathway. However, when the transport by Abcc2 and Abcb1a/1b or Abcc3 is absent or inhibited, CYP3A-dependent metabolism could rather be seen as a strong toxification mechanism.

Few toxicologic data are available about the metabolites of trabectedin. Although of great interest, attempts to structurally identify the trabectedin metabolites have been unsuccessful (6, 7). It is known, however, that trabectedin is metabolized to a plethora of metabolites, each likely to have a different toxicity profile and different affinities toward drug transporters. It is important to realize that (toxic) metabolites transported by Abcc2 could be different from the ones transported by Abcb1a/1b and/or Abcc3. Furthermore, trabectedin metabolites could be more hepatotoxic than trabectedin itself. Even when similarly toxic as the parent compound, a possibly longer residence time of certain metabolites in the liver could result in more severe hepatotoxicity.

These findings have important implications for the clinical use of trabectedin. It has been shown that pretreatment with dexamethasone reduces the hepatotoxic effects of trabectedin (12, 13). Although not completely understood, it was suggested that this protective effect could be due to increased metabolism by CYP3A enzymes (14, 15). Although dexamethasone is indeed a well-known inducer of CYP3A, it is also a potent inducer of ABCC2 (30). As a result, taking our data into account, the hepatoprotective effects of dexamethasone could well be the result of ABCC2 induction rather than CYP3A induction. In this respect, it is important to realize that when dexamethasone is given to patients that have no or dysfunctional ABCC2 (e.g., Dubin-Johnson patients), only CYP3A will be induced. As our study shows, this would likely exacerbate the hepatotoxic effects of trabectedin rather than reduce them. Thus, in general, patients with reduced or absent ABCC2 activity due to polymorphisms could be more susceptible to trabectedin hepatotoxicity. Therefore, screening of patients for MRP2 polymorphisms before the start of the trabectedin treatment might be considered, especially in case dexamethasone is coadministered.

Trabectedin is currently tested in several clinical trials against various types of tumors (see Introduction) and thus has the potential to become more widely used in the future. Combination treatments of trabectedin with other anticancer drugs, such as
doxorubicin, paclitaxel, and docetaxel, are also currently investigated in clinical trials (31–33). Importantly, doxorubicin, paclitaxel, and docetaxel are all substrates for ABCB1 and/or ABCC2 (22, 34, 35), and possible drug-drug interactions leading to severe hepatotoxicity could thus arise. Like most anticancer drugs, trabectedin has a narrow therapeutic window. Improved insight on and awareness of those systems that affect the disposition and toxicity of trabectedin, such as CYP3A and drug transporters, will be crucial to allow optimally safe use of this drug.

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References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
ABCC2, ABCC3, and ABCB1, but not CYP3A, Protect against Trabectedin-Mediated Hepatotoxicity


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