Severe Hepatotoxicity of Mithramycin Therapy Caused by Altered Expression of Hepatocellular Bile Transporters


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ABSTRACT

Mithramycin demonstrates preclinical anticancer activity, but its therapeutic dose is limited by the development of hepatotoxicity that remains poorly characterized. A pharmacogenomics characterization of mithramycin-induced transaminitis revealed that hepatotoxicity is associated with germline variants in genes involved in bile disposition: ABCB4 (multidrug resistance 3) rs2302387 and ABCB11 [bile salt export pump (BSEP)] rs4668115 reduce transporter expression (P < 0.05) and were associated with grade 3 transaminitis developing 24 hours after the third infusion of mithramycin (25 mcg/kg, 6 hours/infusion, every day × 7, every 28 days; P < 0.0040). A similar relationship was observed in a pediatric cohort. We therefore undertook to characterize the mechanism of mithramycin-induced acute transaminitis. As mithramycin affects cellular response to bile acid treatment by altering the expression of multiple bile transporters (e.g., ABCB4, ABCB11, sodium/taurocholate cotransporting polypeptide, organic solute transporter α/β) in several cell lines [Huh7, HepaRG, HepaRG BSEP (–/–)] and primary human hepatocytes, we hypothesized that mithramycin inhibited bile-mediated activation of the farnesoid X receptor (FXR). FXR was downregulated in hepatocellular carcinoma cell lines and in mice expressing the farnesoid X receptor (FXR) reporter activity (P < 0.001). Mithramycin promoted glycochenodeoxycholic acid–induced cytotoxicity in ABCB11 (–/–) cells and increased the overall intracellular concentration of bile acids in primary human hepatocytes grown in sandwich culture (P < 0.01). Mithramycin is a FXR expression and FXR transactivation inhibitor that inhibits bile flow and potentiates bile-induced cellular toxicity, particularly in cells with low ABCB11 function. These results suggest that mithramycin causes hepatotoxicity through derangement of bile acid disposition; results also suggest that pharmacogenomic markers may be useful to identify patients who may tolerate higher mithramycin doses.

SIGNIFICANCE STATEMENT

The present study characterizes a novel mechanism of drug-induced hepatotoxicity in which mithramycin not only alters farnesoid X receptor (FXR) and small heterodimer partner gene expression but also inhibits bile acid binding to FXR, resulting in deregulation of cellular bile homeostasis. Two novel single-nucleotide polymorphisms in bile flow transporters are associated with mithramycin-induced liver function test elevations, and the present results are the rationale for a genotype-directed clinical trial using mithramycin in patients with thoracic malignancies.

Introduction

Mithramycin is a DNA-binding antineoplastic agent initially assessed as a cancer therapeutic in the 1960s (Kofman and Eisenstein, 1963; Sewell and Ellis, 1966; Ream et al., 1968). Previous work demonstrated that the antitumor effects of mithramycin are primarily mediated by direct and selective inhibition of specificity protein 1 binding to guanine- and...
cytosine-rich promoter elements (Blume et al., 1991; Lombó et al., 2006), resulting in a variety of anticaner effects: decreased cell growth, increased apoptosis, decreased angiogenesis, and tissue differentiation (Li and Davie, 2010; Safe et al., 2014). Mithramycin simultaneously activates p53 signaling in thoracic tumors, resulting in growth arrest, senescence, and apoptosis (Zhang et al., 2012). Additional preclinical work has implicated mithramycin as an inhibitor of the EWS-FLI1 oncogene that drives approximately 85% of Ewing’s sarcomas (Grohar et al., 2011).

Given the dramatic dose-dependent inhibitory effects of mithramycin on stem cell signaling (Zhang et al., 2012; Singh et al., 2017) with concomitant decreases in proliferation and tumorigenicity of lung and esophageal cancer, as well as malignant pleural mesothelioma (MPM) cells in vitro and in vivo (Zhang et al., 2012; Rao et al., 2016), recent efforts have focused on evaluation of mithramycin in patients with thoracic malignancies. Despite its promising preclinical activity in numerous advanced malignancies (Grohar et al., 2011; Sankpal et al., 2012; Zhang et al., 2012; Rao et al., 2016; Li et al., 2017; Liu et al., 2017; Singh et al., 2017), administration of mithramycin in cancer patients has been accompanied by systemic toxicities (Curreri and Ansfeld, 1960; Baun, 1968; Kennedy, 1970), including hepatocellular damage (Green and Donehower, 1984), severe transaminitis (Reuben et al., 2010; Grohar et al., 2017), and acute hepatic injury within days of initiating therapy (https://livertox.nih.gov/Plicamycin.htm). Prolonged cell culture concentrations between 20 and 100 nM (every 24 hours) are sufficient to induce growth arrest and apoptosis in cancer cells, and a 50–100 nM plasma concentration is required to induce tumor regression in animals; however, these concentrations have not been clinically achieved in humans (Sankpal et al., 2012; Rao et al., 2016; Grohar et al., 2017; Li et al., 2017; Liu et al., 2017; Singh et al., 2017).

The mechanism of mithramycin-induced hepatotoxicity currently remains poorly characterized and has limited its clinical use. In an ongoing clinical trial at the National Cancer Institute, significant dose-limiting hepatotoxicity that was observed in approximately 75% of patients treated with mithramycin prompted initiation of a pharmacogenetics-based investigation of potential corresponding single-nucleotide polymorphisms (SNPs). To elucidate mithramycin-induced hepatic injury and optimize mithramycin therapy, we discovered and characterized a novel mechanism by which mithramycin blocks hepatic transport of bile acids, resulting in cholestasis and subsequent hepatotoxicity. Our findings support the continued clinical evaluation of mithramycin using genotype-directed precision medicine techniques to identify patients with genetic profiles most conducive to mithramycin therapy.

Materials and Methods

Patients, Treatments, and Clinical Samples. Twelve patients (10 males; two females; median age 51 years; range 40–72 years) with refractory thoracic malignancies received 6 hours mithramycin infusions every day × 7, every 28 days. Response rates following two cycles, pharmacokinetics, and systemic toxicities (cycle 1) were evaluated. Genotype analysis of mithramycin-treated patients was conducted using the drug metabolizing elimination and transport (DMET) array (Affymetrix, Santa Clara, CA). Human hepatocytes for gene expression analysis were collected from cadavers (Bioreclamation, Baltimore, MD). Additional samples were obtained from a previously published study testing mithramycin outcomes in pediatric patients (Grohar et al., 2017). Written informed consent was obtained from all patients before enrollment on the trials (NCT01624090 and NCT01610570), and all studies were approved by the Institutional Review Board of the National Cancer Institute.

Pharmacokinetics. Blood samples were collected at predose, mid infusion (3 hours post start), end of infusion (6 hours post start), and 0.25, 0.5, 7, 2, 3, 5, 7, 9, 11, and 18 hours post end of infusion on cycle 1, day 1. An end of infusion sample was collected on cycle 1, day 2, as was a predose and end of infusion on cycle 1, day 5. For severe combined immunodeficiency mice (strain code 561; Charles River, Wilmington, MA) plasma was frozen after 5, 10, and 15 minutes, respectively, following a 1.27 µg/kg dose in normal saline. The plasma or animal liver homogenates were frozen until analysis by a validated assay, as previously published (Roth et al., 2014). Individual pharmacokinetic parameters were calculated using noncompartmental methods utilizing Phoenix WinNonlin v6.3 (Certara, Cary, NC). The National Cancer Institute is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the National Cancer Institute Animal Care and Use Committee.

Reagents. Chenodeoxycholic acid (CDCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDC), deoxycholic acid, litholic acid, cyclosporine A (CsA), as well as mithramycin were purchased from Sigma-Aldrich (St. Louis, MO). Deuteration-labeled (d4)-CDCA was purchased from Toronto Research Chemicals (Toronto, Canada).

Transporter-certified human primary hepatocytes were obtained from ThermoFisher Scientific (Hu8246; Waltham, MA). Primary human hepatocytes were cultured with proprietary cell culture media formulations developed by Qualyst Transporter Solutions (QTS), now a part of ADME-Tox Division of BioIVT. QualGro Seeding Medium and QualGro Culture Induction Medium supplemented with Matrigel (Corning, Tewksbury, MA) were from QTS (Durham, NC). The base medium (Dulbecco’s modified Eagle’s medium) used by QTS and additional supplements, including FBS, used for cell culture were from Gibco (Carlsbad, CA) and Corning.

Cell Line and Maintenance. Human hepatocellular carcinoma cells (HuH7) were purchased from National Institutes of Biomedical Innovation, Health, and Nutrition (JCRB Cell Bank, Osaka, Japan). HuH7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. HepaRG 5F control cells (MTOX1010) and bile salt export pump (BSEP) knockout HepaRG cells (MTOX1018) were obtained from Sigma-Aldrich and cultured in William’s E media containing HepaRG thaw, plate, and general purpose medium and GlutaMAX supplements (Thermo Scientific), 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were incubated at 37°C in an atmosphere containing 5% CO2 and 95% humidity.

Sandwich-Cultured Primary Human Hepatocyte Cultures. Sandwich-cultured human hepatocytes (SCHH) were established by thawing transporter-certified cryopreserved hepatocytes, according to the manufacturer’s instructions. Once thawed, the cells were suspended in QualGro seeding medium, a QTS proprietary product, at a density of 0.8 million viable cells/ml and seeded onto BioCoat 24-well (~0.4 million cells/well) cell culture plates purchased from Corning. Following the initial seeding, cells were allowed to attach for 2–4 hours, and then were rinsed and fed with 500 µl/well warm (~37°C) QualGro seeding medium. After a culture time of 18–24 hours, the seeding medium was removed. Subsequently, the cells were fed and overlaid with QualGro induction medium supplemented with 0.25 mg/ml Matrigel (Corning).

Gene Expression Assays. Cells were cultured, as described above, and treated with vehicle control (0.1% DMSO), TCA (100 µM), CDCA (100 µM), mithramycin (25 nM), or a combination...
for 72 hours. Total RNA extraction was performed using the QiAamp
red and RNaseasy mini kit (Qiagen, Valencia, CA) per the
manufacturer’s protocol. RNA concentration was determined using a
NanoDrop spectrophotometer (Molecular Devices, Sunnyvale, CA).
RNA from cells was reverse transcribed per 30 µl cDNA synthesis
reaction using the Superscript III First-Strand Synthesis System for
reverse-transcription polymerase chain reaction (PCR; Invitrogen)
per the manufacturer’s protocol. Detection of ABCB11, ABCB4, FXR,
NTCP, OSTα, and OSTβ transcript expression was conducted using
TaqMan gene expression assays [Life Technologies (Thermo),
Grand Island, NY] per the manufacturer’s instructions on a StepOnePlus
Real-Time PCR system (Applied Biosystems, Foster City, CA).

Human ABC and Drug Transporter Arrays. Huh7, HepaRG,
and BSEP knockout HepaRG cells were cultured, as described above,
and treated with vehicle control (0.1% DMSO) or a noncytotoxic
or minimally cytotoxic) concentration of mithramycin (25 nM) for
72 hours. Detection of different transporter transcripts was conducted
using TaqMan human ABC transporter and human drug transporter
arrays [Life Technologies (Thermo)] per the manufacturer’s instruc-
tions on a StepOnePlus Real-Time PCR system (Applied Biosystems).

Western Blot Analysis. Cells were cultured, as described above,
and plated in 10-cm tissue culture dishes. Cells were treated with vehicle control (0.1% DMSO) or a noncytotoxic
or minimally cytotoxic) concentration of mithramycin (25 nM) for
72 hours. Cells were then lysed with ice-cold radioimmunoprecipitation assay lysis buffer (Sigma-
Aldrich) complete with protease inhibitors (Nacalai Tesque, Kyoto,
Japan). After 30 minutes of incubation on ice, cell lysates were
centrifuged at 7500 rpm for 10 minutes. Supernatants were collected,
and total protein concentrations were determined using a bichinonic
acid assay (Thermo Scientific), according to the manufacturer’s protocol.

Cell lysates were subjected to SDS-PAGE and analyzed by Western
blotting with anti-ABCB11 polyclonal antibody (ab112494; Abcam),
anti-ABCB4 polyclonal antibody (PA5-13106; Invitrogen), anti-actin
(C-2) monoclonal antibody (sc-8432; Santa Cruz Biotechnology), anti-
farnesoid X receptor (FXR)-A polyclonal antibody (AB10304; Millipore),
anti-SLC10A1 polyclonal antibody (HPA042727; Sigma-Aldrich), anti-
organic solute transporter (OST) (C-term) polyclonal antibody
(SAB1306154; Sigma-Aldrich), anti-SLC51B polyclonal antibody
(HPA008533; Sigma-Aldrich), and anti-glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) monoclonal antibody (G8795; Sigma-Aldrich).

Primary antibodies were immunoreacted with fluorescent
or confugated goat anti-mouse and anti-rabbit IgG. Bound antibodies
were visualized, and densitometry was completed via the Odyssey
Infrared Imaging System and Odyssey software (LI-COR). Antibody
dilutions were as follows: rabbit pAB to ABCB11, 1:1500; rabbit pAB
to ABCB4, SLC10A1, and SLC51B, 1:100; rabbit pAB to FXR, 1:500;
rabbit pAB to OSTα and mouse mAB to actin, 1:1000; mouse mAB
to GAPDH, 1:2000; M800 = IRDye 800CW goat anti-mouse (incubated
with actin) 1:20,000, R800 = IRDye 800CW goat anti-rabbit (incub-
bated with ABCB11 and ABCB4) 1:15,000, R800 = IRDye 800CW
anti-rabbit (incubated with FXR, SLC10A1, OSTα, and SLC51B)
1:10,000, and M680 = IRDye 680RD goat anti-mouse (incubated with
GAPDH) 1:20,000.

TCA Uptake Assay. Huh7 cells were cultured, as described above,
and plated in 96-well plates. TCA uptake assay was performed
utilizing Gentest BSEP Vehicule Assignment Kit (Promega) per the
manufacturer’s instructions.

Luciferase Reporter Assays. Cells were cultured, as described above,
and plated into 96-well plates with Opti-MEM reduced serum
media (Invitrogen). Twenty-four hours after transfection, cells were treated with mithramycin
(25 nM) in Opti-MEM media with 0.5% FBS. Twenty-four hours after
treatment, cell Firefly luciferase activity was determined and normalized
to Renilla luciferase activity using the Dual-Luciferase Reporter Assay
System (Promega, Madison, WI). Measurements were taken on a
GloMax-Multi Detection System microplate reader (Promega).

Human FXR (NR1H4) and retinoic acid receptor (RXRα (NR1B1)
Nuclear Receptor Reporter Assay Systems as well as Live Cell
Multiplex Assays were purchased from Indigo Biosciences (State
College, PA). Functional activities of DY268 (reference antagonist;
concentration range 0–33,333 nM) and mithramycin (concentration
range 0–300 nM) in the presence of reference agonist GW4066 (300 nM),
retinoic acid (120 nM), or the FXR-ligand CDCa (50 µM) were assessed,
according to the manufacturer’s instructions. Fluorescence and lumi-
nescence measurements were taken using the SpectraMax M2 Micro-
plate Reader and the GloMax-Multi Detection System, respectively.

Bile Acid Cytotoxicity Analysis. HepaRG wild-type and BSEP
knockout cells were cultured, as described above, and treated with
vehicle control (0.1% DMSO) or mithramycin (25 nM) for 72 hours.
Cells were then treated with media containing 100 µM GCDC,
dehydoxycholic acid, or lithocholic acid for 24 hours. Cell viability was
assayed using the Dojindo Cell Counting Kit-8 (Dojindo Molecular
Technologies, Rockville, MD), according to the manufacturer’s
instructions.

Hepatobiliary Disposition of Endogenous Bile Acids in
SCHH. SCHH were established, as described above, and the hepatob-
iliary disposition of endogenous bile acids was assessed following
72 hours of treatment with d4-CDCa (100 µM), Ca (10 µM),
or mithramycin (20 µM). SCHH were treated daily beginning on day 2
of culture for 3 days with fresh dosing solutions composed of test article
and QualGro induction medium. All compound stock solutions were
prepared in 100% DMSO and diluted 1000:1 directly into QualGro
induction medium. Twenty-four hours after last dosing, assessments of
gene expression and endogenous bile acid disposition was determined.

Following the designated exposure period, SCHH were washed
once with one volume of Hank’s balanced salt solution and lysed
by addition of 0.3 ml Qiagen RNeasy lysis buffer supplemented
with β-mercaptoethanol and frozen at –80°C until processed for total
mRNA isolation and subsequent quantitative reverse-transcription
PCR analysis, as described above.

After 72 hours of exposure, SCHH were prepared for endogenous bile acid
disposition analysis. Cell culture medium was removed from SCHH
and stored at –80°C for endogenous bile acid composition analysis. After
removal of cell culture media, the tight junctions of untreated and treated
SCHH were modulated using B-CLEAR technology (QTS), as previously
described (Jackson et al., 2016; Zhang et al., 2017). Sample preparation
and bioanalysis of endogenous bile acids including tauro-CDCa (TCDCa),
glyco-CDCa (GCDCa), glycocholic acid (GCA), and TCA using liquid
chromatography with tandem mass spectrometry detection were per-
formed, as previously described (Jackson et al., 2016; Zhang et al., 2017).

Statistical Considerations. Associations of liver function test
parameters and genotypes were tested using Cochran–Armitage, Mann–Whitney,
and Jonckheere–Terpstra tests, as appropriate. Transporter expression
levels were log-transformed, standardized to controls, analyzed as
t-distributed variates for estimates of means and confidence intervals,
and then exponentiated back as foldchanges. Transporter expression
treated with CDCa or GCDC alone or in combination with mithramycin
was tested in one-way ANOVA of log-transformed levels or in Mann–
Whitney tests, depending on consistency with distributional assump-
tions. Bile acid accumulation and nuclear receptor reporter assay data
were also assessed by one-way ANOVA, with the latter corrected by the
Hochberg method for the number of receptors tested. Data analysis
was conducted with SAS/STAT (software version 9.3), and plots
were generated using GraphPad Prism (version 7). All data are presented as
the mean and S.E.M., unless otherwise indicated. P values are two-tailed
and are not corrected for multiple comparisons, except as noted above.

Results

Genetic Variants Associated with Mithramycin-
Induced Hepatotoxicity. Patients with various malignan-
cies (six MPM, two esophageal cancers, two lung cancers, two
synovial sarcomas with pulmonary metastases; n = 12) were treated with mithramycin. Twenty cycles were administered in 12 patients, all of whom were evaluable for toxicities. No objective responses were observed in seven evaluable patients. Nine patients experienced transient, asymptomatic dose-limiting transaminasitits at 25 mcg/kg per infusion. Percutaneous biopsies revealed apoptotic hepatocellular death. Three patients (two with MPM; one esophageal cancer) had no hepatotoxicity and tolerated dose escalation to 30 mcg/kg per infusion. Peak and steady state mithramycin levels were 20 and 8 nM, respectively.

However, the development of dose-limiting transaminasitis in nine of these individuals prevented the achievement of plasma concentrations sufficient for tumor regression in preclinical animal models (50–100 nM) (Rao et al., 2016), instead resulting in a C\text{max} of approximately 20 nM. Clinical LFTs revealed that mithramycin caused a >13-fold increase in geometric mean (95% confidence interval) of both alanine aminotransferase (ALT) [21 U/l (12–36) vs. 363 U/l (138–895)] and aspartate aminotransferase (AST) [20 U/l (13–29) vs. 269 U/l (116–622)], indicative of hepatocellular damage and/or injury. Of the 12 patients treated in this study, eight patients experienced clinically significant (≥grade 3) transaminase elevations approximately 24 hours following the third infusion of mithramycin (Fig. 1A). AST and ALT levels were strongly correlated; 10 patients had equal grades of ALT and AST elevation, whereas two patients with grade 1 AST had grades 0 and 2 ALT toxicity. Mithramycin pharmacokinetic parameters were not associated with transaminase elevations following mithramycin therapy (P ≥ 0.20; Fig. 1, B–D).

We therefore sought to identify interindividual genetic variations potentially underlying the observed transaminase elevations. Using the Affymetrix DMET Plus array (Arbitrio et al., 2016), genotyping analysis revealed polymorphisms in two genes (ABCB4 rs2302387 and ABCB11 rs4668115; encoding the transporters multidrug resistance 3 and BSEP, respectively) most strongly associated with AST and ALT elevations (P ≤ 0.027; Supplemental Fig. 1). Individuals carrying wild-type alleles at both loci (n = 4) had grade 0–2 AST and ALT increases, whereas all other patients with any variant in either gene (n = 8) had ≥grade 3 transaminase elevations (both P = 0.0040; Fig. 1E). These polymorphisms are in strong linkage with numerous SNPs in these genes (Supplemental Fig. 2). Other polymorphisms were either significantly associated with AST and ALT rises [rs6774801 (CYP8B1) and rs506008 (GSTM4) (P = 0.042), or trending with them [rs717620 (ABCC2, encoding MRP2) (P = 0.065); Supplemental Fig. 1]. Using a cellular uptake assay, we further determined mithramycin is not an inhibitor of ABCB11 (BSEP) transport (Supplemental Fig. 3).

We acquired human hepatocytes collected from cadavers to confirm that patients who were wild-type at ABCB11 rs4668115 had greater expression of ABCB11, whereas those who were wild-type at both sites had greater expression of both ABCB4 and ABCB11 (Fig. 1F). We additionally validated our findings using clinical samples from a study evaluating mithramycin outcomes in pediatric patients (Grohar et al., 2017), and again observed a statistically significant association between the ABCB11 SNP and mithramycin-induced LFT elevations (P = 0.11 for both ALT and AST elevations) (Fig. 1G). Due to unsuccessful PCR, we were unable to ascertain genotype at ABCB4 in these samples. Because variation in the genes identified by DMET analysis to be strongly associated with mithramycin-mediated AST and ALT elevations could alter bile synthesis and transport (Bohan and Boyer, 2002; Arbitrio et al., 2016), we hypothesized that mithramycin induces cholestasis by altering the expression of hepatic transporters regulating bile flow, primarily ABCB4 and ABCB11.

Mithramycin Alters Expression of Hepatic Bile Acid Transporters. Bile acid transport in the liver is mediated by numerous transporters functioning on both the canalicular and basolateral membranes of hepatocytes. These hepatic transporters are involved in bile acid emulsification and in regulating the intracellular and biliary concentrations of bile acids (Fig. 2A) (Bohan and Boyer, 2002; Dawson et al., 2009). Given that both genetic defects and alterations in hepatobiliary bile transporters have been implicated in the pathogenesis and pathophysiology of cholestatic liver injury (Hali, 2013), we evaluated the effects of mithramycin treatment (at the C\text{max} concentration of approximately 25 nM, as observed in Fig. 1B) on transporter expression in human liver cell lines. We first confirmed that mithramycin (1.27 μg/kg) plasma concentration in mice matched hepatic concentrations 10 minutes after dosing [mean (95% confidence interval) = 0.54 (0.40–0.69) and 0.44 nM (0.00–1.04), for plasma and liver, respectively, n = 6 per treatment]. In Huh7 cells, mithramycin (25 nM, 72 hours) was minimally cytotoxic (Fig. 2B) and lowered the expression of SLC5A1 (encoding OATpA), ABCB11, and SLC1B3 while simultaneously increasing the expression of SLC5B1 (encoding OATpB), ABCB3 (encoding MRP3), ABCB4, and ABCB1 (encoding P-glycoprotein; P = 0.023; Fig. 2C). Mithramycin also altered the expression of many other uptake and efflux transporters (Supplemental Fig. 4).

As ABCB4 and ABCB11 are both regulated by similar factors that are affected by bile acid treatment (Plass et al., 2002; Huang et al., 2003), we next assessed whether mithramycin treatment affected the biliary-mediated induction of these transporters. CDCA caused a 1.55-fold upregulation of ABCB4 that was not abrogated by mithramycin. CDCA also upregulated ABCB11 (57-fold), and mithramycin strongly interfered with biliary-mediated ABCB11 inducibility (P = 0.0022; Fig. 2D). In HepaRG and HepaRG BSEP (−/−) cells, mithramycin lowered the expression of most transporters while raising the expression of some (Fig. 2E). Upon administration of bile acids, GCDC lowered the expression of both ABCB4 and ABCB11 in HepaRG cells by 34% and 28%, respectively, whereas CDCA lowered the expression of ABCB4 by 20% and raised the expression of ABCB11 (2.5-fold) (Fig. 2F). Other transporters involved in bile acid uptake and efflux [sodium/taurocholate cotransporting polypeptide (NTCP), OSTa, and OSTb] (Bohan and Boyer, 2002; Dawson et al., 2009) were also differentially regulated by administration of mithramycin and bile acids (Fig. 2G). We therefore reasoned that mithramycin interferes with the expression of several transporters contributing to normal bile acid homeostasis in the liver.

Mithramycin-Mediated Downregulation of FXR Is Associated with Increased Bile Acid Toxicity. Bile acid synthesis, metabolism, and circulation are under tight regulation by FXR, for which bile acids are physiologic ligands (Makishima et al., 1999). FXR mediates hepatic bile acid efflux by inducing the expression of ABCB4 and ABCB11 to transport phosphatidylcholine and bile, respectively, to the canalicular lumen (Plass et al., 2002; Huang et al., 2003). FXR
Twelve adult patients with thoracic malignancies were administered mithramycin in a Phase II clinical trial. (A) LFT values were conducted in 20 cycles and analyzed for highest grade toxicity. Pharmacokinetic analysis was conducted, and ALT and AST toxicity grade were plotted vs. (B) C_{max}, (C) area under the curve, and (D) clearance. Means are shown by bars. (E) The highest ALT or AST test value for each patient was associated with a combination of two SNPs in ABCB4 (rs2302387) and ABCB11 (rs4668115). (F) Total hepatocyte ABCB4 and ABCB11 gene expression was measured using quantitative PCR and plotted according to combined rs2302387/rs4668115 genotype. (G) Genotype of ABCB11 (rs4668115) was confirmed to be associated with LFT elevations in pediatric patients treated with mithramycin. *P < 0.05 and **P < 0.01.
inhibition would also be expected to directly increase the expression of OSTβ and indirectly increase NTCP expression by blocking FXR-regulated SHP transcription. Although NTCP expression decreased, the effects of direct FXR inhibition were observed in the HepaRG cell line. We therefore assessed the effects of mithramycin on FXR and SHP expression and function. Mithramycin lowers FXR relative mRNA and protein expression in Huh7, HepaRG, Bsep (-/-) knock-out cells, and in primary human hepatocytes (67%, 57%, 55%, 34%, respectively; P < 0.0001; Fig. 3, A and B).

Mithramycin also inhibited the ligand-dependent (CDCA and GW4064) signaling of a fusion receptor of the FXR ligand binding domain and a galactose-induced gene 4 (Gal4) DNA binding domain from yeast (Fig. 3C) (Merk et al., 2014) and exhibited stronger inhibition of CDCA-FXR signaling than DY268 by 25% (P = 0.0006). Mithramycin did not inhibit a RXR ligand binding domain linked to the same Gal4 DNA binding domain (Supplemental Fig. 5), despite its ability to bind guanine- and cytosine-rich DNA promoter elements. In Huh7, HepaRG, and HepaRG BSEP (-/-) cells, mithramycin did not consistently inhibit other nuclear receptors in reporter assays (androgen receptor, glucocorticoid receptor, hepatocyte nuclear factor 4, liver x receptor alpha, peroxisome proliferator-activated receptor alpha, progesterone receptor, retinoic acid receptor, retinoid x receptor, vitamin D receptor), although it unexpectedly decreased basal estrogen receptor activity (P = 0.015 for Huh7, P = 0.0026 for HepaRG, and P = 0.0029 for BSEP knock-out cells; Supplemental Fig. 6).

In spite of FXR inhibition, SHP expression increased after mithramycin treatment (Fig. 3D), which is consistent with the unexpected decrease in HepaRG NTCP expression despite FXR inhibition (Fig. 2G).

We next hypothesized that, by deregulating bile acid-mediated FXR regulation, mithramycin treatment would increase the cytotoxicity of GCDC treatment in BSEP (-/-) cells lacking BSEP expression. Mithramycin did not increase
GCDC (100 μM) toxicity in wild-type HepaRG cells, but did result in additional cytotoxicity in BSEP (−/−) cells (Fig. 3E). The data suggest mithramycin blocks both FXR expression and the ligand-dependent activity of FXR, resulting in bile acid–induced toxicity in cells that have a lower capacity to efflux bile.

Disruption of Bile Acid Transporter Expression and Disposition Facilitates Intrahepatic Bile Acid Accumulation. As standard monolayer cell cultures do not fully retain hepatic morphology and liver-specific metabolic functions (Marion et al., 2007), we evaluated mithramycin-induced alterations of transporter expression and bile acid disposition in SCHH. We first genotyped several lots of hepatocytes and identified one lot heterozygous at both ABCB4 rs2302387 and ABCB11 rs4668115 (C/T and G/A). A noncytotoxic concentration of mithramycin (20 nM, 72 hours) lowered the expression of SLC51A, SLC51B, SLC10A1, SLCO1B3, ABCB3, ABCC4, ABCB4, ABCB11, and ABCB1, while increasing SLCO1B1 expression (Fig. 4A) and altering the expression of many other uptake and efflux transporters (Supplemental Fig. 7). These data suggest that, contrary to observations in Huh7 and HepaRG cells grown in a monolayer, mithramycin reduces the expression of most bile transporters in polarized primary human hepatocytes grown in sandwich culture.

After 72-hour treatment with either DMSO, d4-CDCA, CsA, or mithramycin, accumulations of TCDDA, GCDDCA, GCA, and TCA were measured in the cell culture media, within the cell, and within the bile canaliculus. When compared with vehicle control, mithramycin treatment displayed a trend toward increased intracellular GCA accumulation (P < 0.068; Fig. 4C). Mithramycin significantly decreased the accumulation of TCDDA in cell culture media (P = 0.018), while increasing accumulation within the cell (P ≥ 0.09; Fig. 4D). The same effect was observed with the accumulation of highly toxic GCDDCA in both the cell culture media (P = 0.041) and within the cell (P = 0.0048; Fig. 4E). Whereas treatment with BSEP regulators d4-CDCA and CsA affected bile acid production, mithramycin did not alter total bile acid content in the sandwich culture system (Fig. 4F). Consistent with the hypothesis that mithramycin treatment results in bile acid accumulation within cells, mithramycin treatment significantly increased accumulation of all bile acids when compared with DMSO treatment by 35% (P < 0.0001), in direct contrast to d4-CDCA and CsA (Fig. 4G).

Discussion

In the present study, we sought to examine potential mechanisms driving mithramycin-induced severe hepatotoxicity and optimize mithramycin therapy to achieve clinically useful plasma concentrations in a subset of patients with favorable genotypes. In an ongoing clinical trial, significant LFT changes and severe transaminitis, indicative of hepatic injury, resulting from 3-day mithramycin therapy prevented the achievement of plasma concentrations above 20 nM in patients with various thoracic malignancies. Using a pharmacogenomic drug metabolism multigene panel, we found mithramycin-induced AST and ALT elevations to be most strongly correlated with genetic
variants in ABCB11 and ABCB4, which are both involved in the majority of canalicular bile flow in the liver (Chan and Vandeberg, 2012).

Previous studies have shown that allelic variants and mutations leading to severe deficiencies of ABCB11 and ABCB4 function are associated with phenotypically-related
hereditary liver disorders: benign recurrent intrahepatic cholestasis types 2 and 3 and progressive familial intrahepatic cholestasis types 2 and 3 (Curreri and Ansfeld, 1960; Sewell and Ellis, 1966; Chan and Vandenberg, 2012; Srivastava, 2014). Toxic accumulation of bile acids in the liver is a known cause of hepatocyte injury (Chiang and Ferrrell, 2018), and each of these diseases results from impairment of bile homeostasis, resulting in cholestasis, derangement of liver function, and transaminis. We found that mithramycin toxicity was also associated with other genes involved in bile disposition, including \textit{ABCC2} and \textit{CYP8B1}. These findings led to the hypothesis that mithramycin-induced hepatotoxicity occurs via cellular deregulation of bile homeostasis, particularly in patients expressing less hepatic ABCB11 and ABCB4.

The present data are the first to suggest that the \textit{ABCB4} rs2302387 (L59L) and \textit{ABCB11} rs4148115 polymorphisms are associated with expression of both transporters in patient-derived human hepatocytes, and that these variants have clinical consequences. The synonymous \textit{ABCB4} rs2302387 polymorphism (L59L) is in strong linkage disequilibrium with a polymorphism (rs4148805, −1584C>T) that is known to disrupt a critical "C" base in an enhancer element of \textit{ABCB4} (CCAAT vs. TCAAT) and thereby reduces \textit{ABCB4} expression in hepatocytes (Jang et al., 2013). The rs4148115 polymorphism is in a large disequilibrium block as well; however, it is not in strong linkage with polymorphisms that have previously been associated with BSEP function (Lang et al., 2007; Nieuweboer et al., 2015). Mapping studies are required to fully clarify which variant(s) drives differences in hepatic expression, but rs2302387 and rs4148115 are nevertheless associated with mithramycin-induced acute transaminis in two independent studies taking place in adult patients with thoracic malignancies and in pediatric patients with Ewing sarcoma. Mithramycin toxicity is therefore predicted to be most severe in patients harboring variant genotypes conferring low expression of \textit{ABCB4} and \textit{ABCB11}.

Consistent with its role as a transcription factor inhibitor, mithramycin blocks approximately 75% of transcription in animal liver (Yarbro et al., 1966). Our data further indicate that mithramycin alters the expression of most bile transporters in various hepatocyte cell lines. This effect was most pronounced in HepaRG cells lacking BSEP, in which most transporters were downregulated. In HepaRG wild-type cells, coadministration of bile acids with mithramycin significantly decreased the expression of the canalicular bile transporters, \textit{ABCB11} and \textit{ABCB4}. HepaRG cells appear to respond to \textit{ABCB11} and \textit{ABCB4} inhibition by reducing the expression of bile uptake (\textit{SLCO1A1}, NTCP), while increasing expression of the basolateral efflux transporters (\textit{SLC5A1/B, OSTA/β}). In primary human hepatocytes, mithramycin reduced the expression of all bile transporters (except \textit{SLCO1B1}), resulting in greater intracellular concentrations of bile species. Bile acids act as signaling molecules to activate certain hepatic nuclear receptors, including the major bile acid sensor FXR. Once activated, FXR alters the expression of numerous hepatic transporters to decrease bile acid uptake while upregulating bile acid efflux (Cui et al., 2012; Jackson et al., 2018). Consistent with these findings, we found that mithramycin inhibits both the transcription and expression of FXR, as well as its association with FXR ligand-activating bile acids (i.e., CDCA), leading to subsequent deregulation of bile acid homeostasis in hepatocytes. Such inhibition of FXR with concurrent deregulation of bile transport is in contrast with liver injury caused by direct ABCB11 inhibitors. For instance, CsA blocks ABCB11 and activates FXR-mediated compensatory mechanisms as intracellular bile accumulates (Jackson et al., 2018), whereas mithramycin downregulates ABCB11 while simultaneously blocking FXR-mediated cellular responses.

Consistent with this view and unlike CDCA and CsA, mithramycin did not decrease bile synthesis. To our surprise, we also did not find mithramycin to be an inhibitor of BSEP-regulated bile acid transport, as are a variety of other hepatotoxic therapeutics (Qiu et al., 2016). Mithramycin instead potentiated GCDC-induced toxicity in BSEP (−/−) cells. We did not find that mithramycin inhibited a variety of other nuclear receptors; albeit, we did not test whether mithramycin inhibited these in the presence of ligand.

Mithramycin appears to function as both an inhibitor of FXR gene expression and a strong antagonist of ligand-induced activation of FXR. Mithramycin therefore deregulates bile flow through several hepatic transporters and results in intracellular accumulation of bile acids. In HepaRG BSEP (−/−) cells, such accumulation of bile results in cytotoxicity. Taken together, these data provide evidence that mithramycin induces cholestasis in individuals harboring genetic profiles susceptible to bile acid accumulation (i.e., \textit{ABCB4} and/or \textit{ABCB11} gene variants). A dose–escalation clinical trial is currently underway to test mithramycin therapy in patients identified as carrying only wild-type alleles at both rs2302387 and rs4668115 in germline DNA (NCT01624090). Following accrual of three additional patients to this trial, we found that CYP8B1 became even more strongly associated with mithramycin-induced hepatotoxicity and that a hepatic transporter, RALBP, was also associated with mithramycin outcome (data not shown). We are currently amending the clinical trial to account for these SNPs in addition to rs2302387 and rs4668115, and we are characterizing the mechanism by which CYP8B1 and RALBP SNPs affect mithramycin-induced LFT elevations. Identifying patients who are at risk for mithramycin-induced hepatic injury could inform whether patients with optimal genetic profiles can tolerate this therapy at higher concentrations, potentially recapitulating drug exposure levels achieved in preclinical studies and improving clinical responses.

Authorship Contributions

\textbf{Participated in research design:} Sissung, Peer, Jackson, Brouwer, Grohar, Glod, Widemann, Heller, Schrump, Figg.

\textbf{Conducted experiments:} Sissung, Huang, Hauke, McCrea, Peer, Barbier, Strope, Ley, Zhang, Hong, Jackson, Grohar, Glod, Widemann, Heller, Schrump, Figg.

\textbf{Contributed new reagents or analytic tools:} Widemann, Schrump, Figg.

\textbf{Performed data analysis:} Sissung, Peer, Venzon, Jackson.

\textbf{Wrote or contributed to the writing of the manuscript:} Sissung, Huang, Venzon, Jackson, Brouwer, Schrump, Figg.

References


