DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 77420

Title Page

Progressive and preferential cellular accumulation of hydrophobic bile acids induced by cholestatic drugs is associated with inhibition of their amidation and sulfation

Ahmad Sharanek, Audrey Burban, Lydie Humbert, Christiane Guguen-Guillouzo, Dominique Rainteau and André Guillouzo

Inserm UMR991, Liver Metabolism and Cancer, Rennes, France (A.S., A.B., C.G-G., A.G.); Université de Rennes 1, Rennes, France (A.S., A.B., C.G-G., A.G.); ERL Inserm U1157/UMR7203, Faculté de Médecine Pierre et Marie Curie, Site Saint Antoine, Paris, France (L.H., D.R.).

Running Title Page

Running title: *Cholestatic drugs inhibit bile acid amidation*

Address correspondence to: André Guillouzo, Inserm UMR 991, Faculté des Sciences Pharmaceutiques et Biologiques, 35043 Rennes Cedex, France. Fax: +33 2 99 54 01 37. E-mail: andre.guillouzo@univ-rennes1.fr

Text pages: 21

Number of tables: 2

Figures: 7

References: 50

Abstract: 250

Introduction: 722

Discussion: 1494

Abbreviations:

BAAT, bile acid-CoA: amino acid N-acetyltransferase; BACs, bile acid-CoA synthetase; BA, bile acid; BC, bile canaliculi; BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; CDCA-3S, chenodeoxycholic acid 3 sulfate; CDF, 5(6)-carboxy-2′,7′-dichlorofluorescein; CDFDA, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate; CoA, coenzyme A; CPZ, chlorpromazine; CsA, cyclosporine A; CYP27A1, cytochrome P450 27A1; CYP7A1, cytochrome P450 7A1; CYP8B1, cytochrome P450 8B; DCA, deoxycholic acid; DCA-3S, deoxycholic acid 3 sulfate; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HO1, heme oxygenase 1; HPLC-MS/MS, high-pressure liquid chromatography with tandem mass spectrometry; LCA, lithocholic acid; LCA-3S, lithocholic acid 3 sulfate; MDR, multidrug resistance protein; MTT, methylthiazoletetrazolium; MRP, multidrug resistance-associated

protein; NTCP, sodium taurocholate co-transporting polypeptide; OATP, organic anion transporting protein; PBS, phosphate-buffered saline; ROCK/MLCK, Rho-kinase/myosin light chain kinase; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; SULT2A1, sulfotransferase 2A1; Tac, tacrolimus; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA; taurodeoxycholic acid; TLCA; taurolithocholic acid; TLCA-3S, taurolithocholic acid 3 sulfate; Tol, tolcapone; Tro, troglitazone; TVX, trovafloxacin.

Abstract

Drug-induced intrahepatic cholestasis is characterized by cellular accumulation of bile acids (BA) whose mechanisms remain poorly understood. The present study aimed to analyze early and progressive alterations of BA profiles induced by cyclosporine A, chlorpromazine, troglitazone, tolcapone, trovafloxacin and tacrolimus after 4h, 24h and 6 daily treatments of differentiated HepaRG cells. In BA-free medium the potent cholestatic drugs, cyclosporine A, chlorpromazine and troglitazone, reduced endogenous BA synthesis after 24h, while the rarely cholestatic drugs, tolcapone, trovafloxacin and tacrolimus, reduced BA synthesis only after 6 days. In the presence of physiological serum BA concentrations, cyclosporine A, chlorpromazine and troglitazone induced early and preferential cellular accumulation of unconjugated lithocholic, deoxycholic and chenodeoxycholic acids that increased 8-12- and 47-50-fold after 24h and 6 days respectively. Accumulation of these hydrophobic BAs resulted from strong inhibition of amidation and in addition, for lithocholic acid reduction of its sulfo-conjugation, and was associated with variable alterations of uptake and efflux transporters. Trovafloxacin also caused BA accumulation, especially after 6 days, while tolcapone and tacrolimus were still without effect. However, when exogenous BAs were added to the medium at cholestatic serum concentrations, a 6-day treatment with all drugs resulted in cellular BA accumulation with higher folds of chenodeoxycholic and lithocholic acids. At the tested concentration, tolcapone had the lowest effect. These results bring the first demonstration that major cholestatic drugs can cause preferential and progressive *in vitro* cellular accumulation of unconjugated toxic hydrophobic BAs, and bring new insights in mechanisms involved in drug-induced cellular accumulation of toxic BAs.

DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Bile acids (BAs) are the major components of bile, representing 67%. BAs play an important role in digestion through emulsification of lipids and they are critical for regulation of glucose and cholesterol homeostasis (Lefebvre et al., 2009; Crocenzi et al., 2012). The two primary BAs, chenodeoxycholic (CDCA) and cholic (CA) acids, are synthesized in the hepatocytes from cholesterol oxidation then conjugated (amidated) with either glycine or taurine by bile acid-CoA synthetase (BACs) and bile acid-CoA: amino acid N-acetyltransferase (BAAT) in hepatocytes giving rise to taurocholic acid (TCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and taurochenodeoxycholic acid (TCDCA) (Chiang, 2009; Monte et al., 2009; Anwer, 2014). BAs may also be metabolized by different liver enzymes such as cytochrome P450s, and glucuronosyl- and sulfo-transferases (Chiang, 2009). After their formation BAs are secreted into bile and stored in the gallbladder. When secreted into the small intestine, the gut microbiota converts CDCA and CA by 7α-dehydroxylation to the secondary BAs lithocholic (LCA) and deoxycholic (DCA) acids (Monte et al., 2009). The enterohepatic circulation allows for re-uptake of about 95% of both primary and secondary BAs from the intestinal lumen into the blood (Chiang, 2009; Anwer, 2014). Hepatic elimination and enterohepatic circulation of BAs are accomplished by a wide range of uptake and efflux transporters (Anwer, 2012). Circulating BAs are taken up into hepatocytes by the sodium taurocholate co-transporting polypeptide (NTCP) and organic anion transporting proteins (OATPs) located on the sinusoidal membrane of hepatocytes. Glycine- and taurine-conjugated BAs are the main physiological substrates of NTCP (Hata et al., 2003; Dawson et al., 2009), while unconjugated and sulfated BAs are weak substrates for NTCP and efficiently imported by members of the OATP family (Hata et al., 2003; Dawson et al., 2009).

In human liver, the bile salt export pump (BSEP) located on the canalicular membrane of hepatocytes is responsible for secretion of major BA species into bile including unconjugated, taurine- and glycine- amidated monovalent BA species (Stieger, 2011). In human, but not in rodents, BSEP transports some sulfated BAs (Hayashi et al., 2005); however, secretion of

most sulfated BAs is mediated by the multidrug resistance protein-2 (MRP2) and multidrug resistance protein-1/3 (MDR1/3) (Akita et al., 2001; Lam et al., 2005).

Intrahepatic accumulation of BAs or their conjugated salts can be caused by a number of drugs (Amieva et al., 1994); it defines a pathological condition, cholestasis, in which bile secretion and flow are impaired and toxic BAs accumulate in the liver. The hydrophobic DCA, LCA and mainly CDCA, are the most toxic BAs (Kojima et al., 2008). Indeed, in large population-based studies bland and mixed cholestatic patterns were found to represent up to 60% of drug-induced liver injury cases (Bjornsson and Jonasson, 2013). Several mechanisms underlying drug-induced cholestasis have been reported. A cholestatic agent can interfere in the activity of BA uptake and efflux transporters and with bile canaliculi (BC) motility, BA intracellular trafficking and BA metabolism and conjugation (Rodrigues et al., 2014). A disturbance of any one of these important processes can alter the balance of the BA pool and increase intracellular concentrations of toxic BAs. All these alterations converge in perturbation of the BA homeostasis pool leading to cholestasis.

Many drugs can cause cholestasis in humans without any significant liver injury in preclinical animal models (Fattinger et al., 2001; Kojima et al., 2003; Kostrubsky et al., 2003; Pauli-Magnus and Meier, 2006; Morgan et al., 2010). This could be attributed, at least in part, to the species differences in BA composition and regulation between humans and animals (Chiang, 2009). Presently, there is little information regarding the early changes in BA profiles and disposition in human liver exposed to cholestatic drugs. Recently, we demonstrated a dose-dependent accumulation of LCA in cell layers of HepaRG cells treated with cyclosporine A (CsA) (Sharanek et al., 2015). Importantly, these cells produce conjugated primary BAs at similar levels as primary human hepatocytes and as in clinic, they can exhibit typical cholestatic features after treatment with cholestatic drugs, including BC deformations, impairment of the Rho-kinase/myosin light chain kinase (ROCK/MLCK) pathway, inhibition of BA transporters, accumulation of endogenous BAs and initiation of compensatory mechanisms (Antherieu et al., 2013; Sharanek et al., 2015; Sharanek et al., 2016; Burban et al., 2017). These data led us to use metabolically competent and highly polarized HepaRG cells (Aninat et al., 2006;

Bachour-El Azzi et al., 2015) to investigate changes in BA profiles and disposition induced by cholestatic drugs as a function of time of treatment and in the absence or presence of exogenous BAs. Based on their clinical side effects, two categories of cholestatic drugs were selected: the potent cholestatic CsA, chlorpromazine (CPZ), troglitazone (Tro), and the rarely cholestatic trovafloxacin (TVX), tolcapone (Tol) and tacrolimus (Tac). Our results show early and progressive changes in BA intracellular content and profiles after single and repeated daily additions of the 6 cholestatic drugs and that these changes were dependent on the tested drug and the amount of added exogenous BAs to the culture medium.

Materials and Methods

Reagents

CsA, CPZ, TVX, Tol, Tro, methylthiazoletetrazolium (MTT), 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) and all exogenous BAs were purchased from Sigma (St. Quentin Fallavier, France). Tac was provided by Tocris Bioscience (Bristol, UK). ^{3}H-Taurocholic acid (^{3}H-TCA) was from Perkin Elmer (Boston, MA). Hoechst dye was obtained from Promega (Madison, Wisconsin). Other chemicals were of reagent grade.

Cell cultures

HepaRG cells were seeded at a density of 2.6×10^4 cells/cm^2 in Williams' E medium supplemented with 10% Hyclone® fetal bovine serum (Thermo-Fisher scientific, Waltham, MA, USA), 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, and 50 µM hydrocortisone hemisuccinate. After 2 weeks, HepaRG cells were shifted to the same medium supplemented with 1.7 % dimethyl sulfoxide (DMSO) for 2 more weeks in order to obtain differentiated cultures with maximum functional activities. At this time, the cultures contained around the same number of HepaRG hepatocytes and progenitors/primitive biliary cells and were ready for use (Cerec et al., 2007).

Cell treatment

Before starting the treatments HepaRG cells were incubated for 24h in a medium supplemented with 2% fetal bovine charcoal-stripped serum (BA-free serum) (Sigma,

reference: F6765) to eliminate bovine BAs accumulated into the cells during the previous culture period in a serum-supplemented medium (Sharanek et al., 2015). No individual BA was detected in charcoal-stripped serum. Then, the cells were treated with the tested compounds dissolved in BA-free medium supplemented with 2% charcoal-stripped serum and 1% DMSO for different time points, in the absence or presence of BA mixtures composed of 8 individual BAs at concentrations found in the serum of either normal individuals (1X) or cholestatic patients (60X) (Table 1). Because cholestatic drugs can alter BA uptake, the two BA mixtures were added 24h before their simultaneous addition with tested compounds.

Cell viability

Cytotoxicity of BA mixtures and tested drugs was evaluated using the MTT colorimetric assay. Briefly, the cells were seeded in 96-well plates and treated with various concentrations of tested compounds either alone or combined with BA mixtures for 24h or 6 days. After medium removal, 100µl of serum-free medium containing MTT (0.5mg/ml) was added to each well and incubated for 2h at 37°C. The water-insoluble formazan was dissolved in 100µl DMSO, and absorbance was measured at 550 nm.

Phase-contrast cell imaging

Phase-contrast images of HepaRG cells were captured with an AxioCam MRm camera using phase-contrast microscopy. An inverted microscope (Zeiss Axiovert 200M) equipped with a thermostatic chamber (37°C and 5% CO_2) to maintain the cells under normal culture conditions was used.

Measurement of bile acids

Standard stock solutions were prepared in methanol at a concentration of 1mg.mL^{-1} and stored in a sealed container at -20°C. The stock solutions were pooled and diluted to obtain mixed calibration BA solutions with concentrations ranging from 31.3ng.mL^{-1} to 31.3µg.mL^{-1}. Standard solutions were available for 28 BAs, i.e. CDCA; TCDCA; GCDCA; chenodeoxycholic acid 3 sulfate (CDCA-3S); CA; TCA; GCA; cholic acid 3 sulfate; DCA; taurodeoxycholic acid (TDCA); glycodeoxycholic acid (GDCA); deoxycholic acid 3 sulfate (DCA-3S); LCA; taurolithocholic acid (TLCA); glycolithocholic acid; lithocholic acid 3 sulfate (LCA-3S);

taurolithocholic acid 3 sulfate (TLCA-3S); glycolithocholic acid 3 sulfate; ursodeoxycholic acid; tauro-ursodeoxycholic acid; glycol-ursodeoxycholic; ursodeoxycholic acid 3 sulfate; tauro-ursodeoxycholic acid 3 sulfate; glyco-ursodeoxycholic acid 3 sulfate ; taurohyodeoxycholic acid; hyodeoxycholic acid; hyocholic acid; and α-muricholic acid. Depending on culture conditions, 15 out of 28 BAs were detected, i.e. CDCA; TCDCA; GCDCA; CDCA-3S; CA; TCA; GCA; DCA; TDCA; GDCA; DCA-3S; LCA; TLCA; LCA-3S; TLCA-3S.

Samples preparation and analysis

Both cells and media were collected from HepaRG cell cultures treated with the 6 tested compounds combined or not with BA mixtures. Before analysis, the samples were lyophilized, and then 1ml of water was added to the dried samples, homogenized using a PolyTron® homogenizer for 30sec and clarified by centrifugation at 20 000 × g for 20min. The supernatant was collected and extracted using a SPE cartridge. BA content was measured using high pressure liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS). The chromatographic separation of BAs was carried out on a Zorbax eclipse XDB-C18 (Agilent Technology, Garches, France) fitted on an Agilent 1100 HPLC system (Massy, France) as previously described for human samples (Humbert et al., 2012). The column temperature setting was 35°C. The mobile phases consisted of (A) ammonium acetate 15mmol/l, pH 5.3 and (B) methanol at 65:35 (v/v). BAs were eluted by increasing B in A from 65 to 95 (v/v) for 30min. Separation was achieved at a flow rate varying between 0.3 and 0.5ml/min for 30min. Mass spectra were obtained using an API® 2000 Q-Trap (AB-Sciex, Concord, Canada) equipped with a TurboIon electrospray (ESI) interface set in the negative mode (needle voltage – 4500V) with nitrogen as the nebulizer set at 40 (arbitrary pressure unit given by the equipment provider). Curtain and heater pressures were set at 20 and 40 (arbitrary units), respectively and the ion source temperature was set at 400°C. Declustering and entrance potentials were set at −60V and −10V, respectively. The MS/MS detection was operated at unit/unit resolution. The acquisition dwell time for each transition monitored was 70msec. Data were acquired by the Analyst® software (version 1.4.2, AB-Sciex) in the Multiple Reaction Monitoring mode.

Na^+-dependent taurocholic cotransporting polypeptide activity

Activity of the NTCP transporter was estimated through determination of sodium-dependent intracellular accumulation of the radio-labeled taurocholic acid (^{3}H-TCA) substrate. After treatment with tested drugs, cells were incubated with 43.3nM of radio-labeled TA for 30 min. Cells were then washed twice with PBS and lysed with 0.1 N NaOH. Accumulation of radiolabeled substrate was determined through scintillation counting.

Carboxy dichlorofluorescein (CDF) clearance

After treatment, the cells were washed with warm Williams' E medium without phenol red and incubated in 3 µM CDFDA for 30 min at 37 °C in the medium used for passive intracellular accumulation. Upon hydrolysis by intracellular esterases, CDFDA was converted to fluorescent CDF (excitation/emission: 488/509 nm) and directed towards the biliary pole by membrane transporters, particularly MRP2. After washing, imaging was performed using a Cellomics. ArrayScan VTI HCS Reader (Thermo Scientific). The number of CDF accumulating BC was quantified using ImageJ 1.48 software.

Taurocholate acid clearance

Cells were first exposed to 43.3 nM ^{3}H-TCA for 30 min to induce its intracellular accumulation, washed with standard buffer and then treated with the tested drugs for 4h in a standard buffer containing Ca^{2+} and Mg^{2+}. After incubation, the cells were washed and scraped in 0.1 N NaOH, and the remaining radiolabelled substrate was measured through scintillation. ^{3}H-TCA clearance was determined based on its accumulation in cell layers (cells + BC) and calculated relative to the control.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was extracted from 10^6 HepaRG cells with the NucleoSpin RNA isolation Kit (Macherey-Nagel, Düren, Germany). RNAs were reverse-transcribed into cDNA and RT-qPCR was performed using a SYBR Green mix. Primer sequences are listed in Supplemental Table 1.

Data analysis

The amounts of total and individual BAs in both cell layers and supernatants samples were measured. To keep the same unit, BAs in both cell layers and supernatants were expressed in µg/10^6 hepatocytes. The basal primitive biliary cells did not express BA metabolizing enzymes and transporters (Sharanek et al., 2014; Sharanek et al., 2015). Therefore, these cells were neglected for calculation of BA content. Only HepaRG hepatocytes were considered and estimated to represent 50% of total cells in the cultures (Cerec et al., 2007).

Statistical analysis

One-way ANOVA with Bonferroni's multiple comparison test (GraphPad Prism 5.00) was performed to compare data. Values of 4h samples corresponded to 4h treatments, those of 24h and 6-day samples to 24h treatments (the last 24h for 6 days). Each value corresponded to the mean ± standard error of mean (SEM) of three independent experiments. Data were considered significantly different when $p<0.05$.

Results

Effects of tested drugs on bile canaliculi morphology and bile acid transporters

We recently showed that BC deformation is an important feature of drug-induced cholestasis (Sharanek et al., 2016). In the current work, six cholestatic compounds were selected: CsA, CPZ, Tro, Tol, TVX and Tac. The lowest drug concentrations that caused obvious BC deformations without inducing cytotoxicity to HepaRG cells after 6 daily repeated additions, were selected (Figure 1A and B). After 4h, CsA (5µM), CPZ (15µM), Tol (10µM), TVX (10µM) and Tac (5µM) induced dilatation of BC which persisted without obvious aggravation after 6 daily repeated treatments. Noticeably, in the conditions used in this study CPZ caused a slight dilatation of BC. Tro previously found to cause constriction of BC at concentrations higher than 50µM, was cytotoxic after 6 daily repeated treatments at such concentrations. Consequently, it was used at 10µM, a concentration that did not obviously alter BC morphology but impaired BA transporters.

DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

The effect of tested drugs on Na^+-dependent BA uptake activity was estimated through measurement of intracellular accumulation of ^{3}H-TCA after 4 and 24h treatment of HepaRG cells. Both CsA and Tro reduced ^{3}H-TCA uptake by 70% after 4h compared to untreated cells. No significant inhibition was observed with other tested drugs even after 24h treatment (Figure 1C). To estimate effects of tested drugs on BSEP activity, HepaRG cells were incubated with ^{3}H-TCA for 30 min and then treated for 4h with the tested drugs in standard buffer containing Ca^{2+}/Mg^{2+}. At the tested concentrations CsA and CPZ reduced ^{3}H-TCA canalicular efflux to 42 and 60% respectively. TVX and Tol also slightly reduced ^{3}H-TCA efflux to 70% compared to untreated cells. No significant inhibition was observed with Tro and Tac (Figure 1D). To estimate MRP2 activity, HepaRG cells were exposed to the tested drugs for 4 or 24h followed by incubation with CDF, an MRP2 substrate. CDF fluorescent substrate was visualized in BC lumen of untreated cells. After 4h treatment, CsA, Tro and Tac inhibited canalicular excretion of CDF by 75, 77 and 53% respectively compared to untreated cells; no effect was observed with the 3 other drugs even after 24h treatment (Figure 1E and F).

Effects of tested cholestatic drugs on endogenous BA profiles and disposition

BA profiles and disposition were analyzed in both cell layers and supernatants of HepaRG cell cultures incubated in a medium supplemented with 2% BA-free charcoal-stripped serum. After 4h, total BA content reached 0.12±0.01µg (0.09±0.01 and 0.04±0.01µg in supernatants and cell layers, respectively). After 24h, this total amount increased to 0.28±0.02µg and after 6 days, i.e. 24h following the last medium renewal, peaked at 0.42±0.03µg, representing around 1.5-fold the values measured at 24h. This increase in total BAs was mainly due to tauro-conjugates. Glyco-conjugates (GCDCA) remained nearly unchanged (Figure 2A-C). Unconjugated BAs were not detected. Altogether, these data reflected the capacity of HepaRG cells to synthesize, conjugate and secrete BAs whose levels were estimated to reach 0.28µg BAs/10^6 hepatocytes/day versus 0.13µg BAs/10^6 hepatocytes/day in serum-free medium (Sharanek et al., 2015).

BA profiles were then analyzed following treatment of HepaRG cells with the 6 cholestatic drugs in a medium containing 2% BA-free serum for 4h, 24h and 6 days. After 4h, whatever

the tested drug, total BA content (cells+supernatant) remained unchanged compared to untreated cells. However, changes in the supernatant/cell layer ratio were observed. Interestingly, an accumulation of total BAs in cell layers peaking at 0.01±0.01µg (1.8-fold) and 0.06±0.01µg (1.6-fold) was observed with CsA and CPZ respectively, compared to 0.04±0.01µg in their untreated counterparts. Cellular BAs corresponded to an accumulation of conjugates of primary BAs (TCDCA, GCDCA and TCA). Importantly, BA total amounts and profiles were not changed with the 4 other drugs in BA-free medium after 4h (Figure 2D).

By contrast, intracellular BA accumulation was no longer observed after 24h treatment with CsA or CPZ and was associated with a reduced total BA content (cells+supernatants) to 0.24µg (88%) and 0.23µg (84%) respectively, compared to 0.28µg in untreated cells (Figure 2B), especially tauro-conjugates (TCA and TCDCA). A reduced total BA content to 0.20µg (71%) was also observed with Tro. These decreased values likely reflected an inhibition of BA synthesis by these 3 drugs while Tol, TVX and Tac were ineffective (Figure 2E).

The influence of cholestatic compounds on endogenous BA content and profiles was also analyzed after 6 daily repeated additions. Compared to the total BA content of 0.42µg measured in untreated cultures those measured in drug-treated cultures were decreased whatever the tested drug, Tol showing the lowest effect at the tested concentration. They corresponded to 0.13±0.02µg (32%), 0.13±0.01µg (31%), 0.21±0.02µg (51%), 0.31±0.08µg (75%), 0.20±0.03µg (49%) and 0.17±0.01µg (41%), with CsA, CPZ, Tro, Tol, TVX and Tac respectively (Figure 2F). Synthesis of all primary BAs was reduced.

Effects of cholestatic drugs in presence of physiological BA concentrations

To mimic physiological BA content in human, a mixture of 8 BAs at concentrations found in the serum of normal individuals (1X-BAs) (3.56±0.22µM corresponding to 4.48±0.29µg) (Scherer et al., 2009), was added to the culture medium for 4h, 24h or 6 days (Table 1). After exposure of HepaRG cells to 1X-BAs, total BA content reached 4.89±0.24µg. After 24h, unconjugated BAs dropped to 0.05±0.02µg compared to 1.14±0.07µg in non-exposed medium (Figure 3A), paralleling a corresponding increase in conjugated BAs and resulting from tauro- and glyco-

conjugation of exogenous unconjugated primary BAs (Figure 3B) and sulfation of LCA (Figure 3C).

Effects of the tested drugs on BA profiles were analyzed in HepaRG cells concomitantly incubated with the 1X-BA mixture after 4h, 24h and 6 days. In the presence of 1X-BAs, a 4h-treatment with either CsA or CPZ caused accumulation of total BAs in cell layers peaking respectively at 1.38±0.25µg (2.3-fold) and 1.53±0.26µg (2.6-fold) compared to 0.59±0.22µg in untreated cells, and this accumulation was associated with a parallel decrease in supernatants (Figure 3D). Both conjugated (TCDCA, TCA, TDCA, TLCA, GCDCA, GCA, GDCA) and unconjugated BAs (CDCA, CA, DCA, LCA) were found to accumulate in cell layers. Interestingly, unconjugated BAs preferentially accumulated in cell layers after CsA and CPZ treatment reaching 8- and 10-fold respectively, while conjugated BAs accumulated only up to 2-fold, compared to untreated cells after 4h (Figure 3E).

After 24h, no further cellular accumulation of conjugated BAs was observed (Figure 4A). By contrast, unconjugated BAs continued to preferentially accumulate reaching 26-, 19-, and 16-fold in CsA-, CPZ- and Tro-treated cells respectively compared to untreated cells (Figure 4B and C). Indeed, unconjugated CDCA DCA and LCA significantly accumulated in both cell layers and supernatants (Figure 4D-F). Sulfated LCA was found to be accumulated in cell layers and decreased in supernatants (Figure 4G). Even though unconjugated CA significantly accumulated in the supernatants of CsA-, CPZ- and Tro-treated cultures, no significant accumulation was observed in the cell layers (Figure 4H).

In parallel, tauro- and glyco-amidation of exogenous BAs was reduced to 70-75% by CsA, CPZ and Tro. Sulfation of LCA was also decreased to 68, 30 and 60% after 24h treatment with CsA, CPZ and Tro respectively, compared to untreated cells, whereas the 3 other drugs were ineffective (Figure 5A and B). These data reflected a marked inhibition of *de novo* amidation and sulfation of exogenous BAs by the 3 potent cholestatic drugs.

After 6 repeated daily additions of the 1X-BA mixture with medium renewal an accumulation of total BAs in cell layers reaching 1.95±0.04µg, 2.08±0.10µg, 1.90±0.12µg and 1.23±0.09µg was obtained with CsA, CPZ, Tro and TVX respectively, compared to 0.91±0.07 µg in

corresponding untreated cells (Figure 5C), reflecting an accumulation of almost all individual BAs. Conjugated BA species slightly accumulated in CsA-, CPZ-, Tro- and TVX-treated cells after 6 repeated additions; i.e. 1.9-, 2.1-, 1.9- and 1.3-fold respectively, compared to untreated cells (Figure 5D and F). Indeed, as observed after 24h, a 6-day treatment with CsA, CPZ, Tro and TVX, caused a marked preferential accumulation of unconjugated forms of primary and secondary BAs to reach 50-, 45-, 50- and 15-fold respectively compared to untreated cells (Figure 5E and F). Compared with a 24h exposure to the 1X-BAs mixture, more unconjugated BAs were found in cell layers of CsA-, CPZ-, Tro- and TVX-treated cultures after 6 days (Figure 5G). No significant change in BA profiles and disposition was observed with Tol and Tac.

Effects of tested drugs on BA profiles in cholestatic condition

To mimic BA content in the cholestatic condition, the mixture of 8 BAs at concentrations found in the serum of patients suffering from cholestasis (60X-BAs) (179±3µM corresponding to 225±5µg) (Scherer et al., 2009), was added to the culture medium for 4h, 24h or 6 days (Table 1).

The 60X-BAs mixture did not induce any significant cytotoxicity even after 6 days. Accordingly, no major morphological changes were evidenced, including absence of any alteration of BC structures. Only few lipid droplets were noticed in HepaRG hepatocytes exposed to the 60X-BA mixture for 6 days. When HepaRG cells were co-exposed to this BA mixture and the six cholestatic compounds no further cytotoxicity and change in BC structures were evidenced, even after 6 days (Figure 6A). In support, gene expression of albumin was not significantly modulated (Table 2).

In the presence of 60X-BAs, cell layers of untreated cells accumulated 23-fold more BAs after 4h compared to 1X-BAs and the amount remained relatively unchanged thereafter (Figure 6B). While in cell layers conjugated and unconjugated BAs represented 96 and 4% of total cellular BAs respectively in the 1X-BA condition, cellular unconjugated BA content increased to 47% in parallel to a decrease in cellular conjugated BAs to 53% in the presence of 60X-BAs (Figure 6C). These changes induced by the 60X-BAconcentration also influenced the effect of tested drugs. Indeed, 4h-treatment with CsA and CPZ in the presence of 60X-BA mixture induced

accumulation of both conjugated and unconjugated BAs in comparable proportions. Unlike in the 1X-BA condition, Tro and TVX induced cellular accumulation of total BAs as early as 4h. The effects of cholestatic drugs after 24h were comparable to their effects after 4h (Figure 6D). Interestingly, after 6 days all the tested compounds caused accumulation of total BAs, i.e. both primary and secondary BAs, peaking at 43.2±1.0, 50.4±14.5, 47.5±17.3, 44.2±16.7 and 39.1±14.2µg after treatment with CsA, CPZ, Tro, TVX and Tac respectively, compared to 14.2±2.8µg in untreated cells, corresponding to 3.0-, 3.6- 3.3-, 3.1- and 2.7-fold increase respectively. At the tested concentration, Tol showed a low potency to cause accumulation of BAs with a total amount of 27.9±4.9µg (1.9-fold) (Figure 6D). Compared to 24h, after 6-day exposure to the 60X-BA mixture, the tested drugs caused 2-3-fold more total LCA and CDCA accumulation in cell layers while total CA remained nearly unchanged (Figure 6E-G).

Cholestatic drugs and exogenous BAs deregulate expression of various target genes

Many genes involved in BA transport, synthesis, and metabolism can be deregulated by cholestatic drugs and intracellular accumulation of BAs (Antherieu et al., 2013; Liu et al., 2014; Sharanek et al., 2014). A set of target genes was analyzed by RT-qPCR after 24h and 6 daily treatments with various cholestatic drugs in absence or presence of exogenous BAs. Genes related to BA metabolism and transport included the nuclear receptor FXR, efflux transporters (BSEP, MDR1, MDR3, MRP2, MRP3 and MRP4), the uptake transporter NTCP, and BA synthesis and conjugation enzymes (CYP7A1, CYP8B1, CYP27A1, BAAT and SULT2A1). No significant effects were evidenced after 24h whatever the experimental condition. By contrast, various genes were found to be modulated after 6 days. 1X- and 60X-BA mixtures alone induced BSEP expression by 1.6- and 4.3-fold respectively; this fold-induction with 60X-BAs was not significantly modulated by cholestatic drugs. MDR1, MDR3, BAAT and SULT2A1 were also up-regulated while CYP7A1, CYP27A1 and NTCP were down-regulated to a variable extent by the 60X-BA mixture (Table 2).

The 6 cholestatic drugs differently modulated expression of the tested genes; all but Tol, decreased BSEP expression. NTCP expression was inhibited by CPZ and TVX, and induced by CsA. All drugs but Tol reduced CYP7A1, CYP27A1 and CYP8B1 gene expression without

any further aggravation in the presence of exogenous BAs. CsA, CPZ and Tro reduced expression of BAAT and SULT2A1, whereas TVX and Tac showed similar effects only in the presence of exogenous BAs (Table 2). Genes related to oxidative and ER stress were also differently deregulated by these tested drugs. While CPZ and Tro caused overexpression of HO1, CsA, TVX and to a lower extent CPZ induced that of ER stress-related genes (ATF4 and CHOP). Tol and Tac had no significant effects. Co-addition of exogenous BAs aggravated oxidative and ER stress response, especially the 60X-BA mixture (Table 2).

Discussion

In the present work, we analyzed the first steps leading to drug-induced accumulation of BAs in human liver using HepaRG hepatocytes. Our data showed that BA accumulation profile and disposition were dependent on the tested cholestatic drug, co-exposure to exogenous BAs and treatment duration and that treatment with the major cholestatic drugs resulted in early, preferential and progressive cellular accumulation of unconjugated forms of toxic hydrophobic BAs.

At the tested concentrations that were not cytotoxic even after 6 daily exposures in presence of 1X- or 60X-BA mixtures, CsA, CPZ, Tol, TVX and Tac caused dilatation of BC as previously reported (Burbank et al., 2016). Only Tro appeared to be ineffective at the low tested concentration; like CPZ and CsA, Tro has been found to induce BC constriction at high concentrations (Antherieu et al., 2013; Sharanek et al., 2014; Burbank et al., 2016).

Marked differences in their potential to accumulate total and individual BAs in HepaRG cells were observed between the tested cholestatic drugs. On the basis of the different patterns of BA profiles after treatment in the absence or presence of exogenous BA mixtures, it was possible to classify the 6 drugs into 2 groups: group 1 that corresponded to the three potent cholestatic compounds (CsA, CPZ, Tro) and group 2 that included the 3 other drugs (TVX, Tol, Tac); noteworthy, these two groups correspond to potent and rarely cholestatic drugs in clinic respectively (Burbank et al., 2016). Cellular accumulation of BAs was evidenced in cultures treated with CPZ and CsA for 4h, likely due to inhibition of BSEP transport. However, after 24h

a decrease in total BA content (cell layers+supernatants) was observed with group 1 drugs while similar contents were still observed in untreated and group 2 drug-treated cultures. After 6 days, the decrease in intracellular BA content was strongly amplified with CsA, CPZ, Tro and Tac but not with Tol and only slightly with TVX. Total BA contents were decreased with all the drugs, especially CsA and CPZ; with these two last drugs they were reduced to one third of the content measured in corresponding control cultures. These data could be explained by inhibition of not only BA synthesis but also neo-synthesized BA re-uptake by NTCP and activation of compensatory secretion via the sinusoidal transporters MRP3/4 (Zollner et al., 2003; Sharanek et al., 2015). In support, BA metabolizing enzymes were down-regulated except with Tol after 6-day treatment.

To mimic the physiological *in vivo* situation, a cocktail of 8 human primary and secondary BAs in their unconjugated and conjugated forms were added to the culture medium at corresponding serum concentrations (1X-BA mixture). Group 1 molecules caused the earliest and highest accumulation of total BAs. Four major differences with group 2 were evidenced. First, BA accumulation was already observed with CsA and CPZ after 4h and with Tro after 24h while the 3 other drugs remained ineffective. Second, group 1 drugs caused cellular accumulation of greater amounts of BAs compared to group 2 molecules in the different experimental conditions tested. Third, unconjugated hydrophobic BAs (CDCA, DCA and LCA), preferentially accumulated in cell layers from cultures treated with group 1 drugs during the first 24h. Fourth, preferential accumulation of these hydrophobic BAs in group 1 treated cells increased as a function of treatment duration, from 8-12-fold after 4h to 45-50-fold after 6 daily repeated additions. Such progressive accumulation of toxic BAs is relevant with *in vivo* chronic development of drug-induced cholestasis. Indeed, while in most instances, acute drug-induced cholestasis is reversible after drug ceasing, continuous therapy with cholestatic drugs can lead to chronic liver disease (Padda et al., 2011). Noticeably, TVX effects on BA accumulation were close to those of group 1 drugs after 6 days in the presence of 1X-BAs. In addition to acute liver failure (Stahlmann, 2002) rare cases of cholestasis have been reported with this antibiotic

(Lucena et al., 2000). It cannot be excluded that the number of cholestasis cases would have been higher if hepatic failure had appeared later after the start of treatment.

The increase in total unconjugated BA content in both cell layers and supernatants of cultures treated with CsA, CPZ and Tro resulted from inhibition of amidation and sulfation activities. Indeed, amidation of BAs requires sequential enzymatic reactions that mainly involved two enzymes, namely BACs and BAAT. Unconjugated BAs are first activated with coenzyme A (CoA) by the fatty acid transport protein 5, which is located on the basolateral membrane of hepatocytes and is responsible for major BACs activity (Doege et al., 2006). Next, CoA-activated BAs are substrates for BAAT that is predominantly present in peroxisomes (Pellicoro et al., 2007). This requires CoA-activated unconjugated BAs to be transported into peroxisomes, followed by glycine and taurine conjugation. Therefore, inhibition of BA amidation could result from interference of drugs with one or more of these essential steps, (i) reduced expression and/or activity of BACs preventing CoA-BA formation, (ii) blockage of CoA-BA translocation to the peroxisomes and (iii) interference with expression and/or activity of BAAT preventing amidation of CoA-BA with taurine and glycine in the peroxisomes (Figure 7). In support, BACs was found to be inhibited with 10 µM CsA, without any significant inhibition of BAAT activity (Vessey and Kelley, 1995).

The secondary and most lipophilic BA, LCA, was entirely sulfo-conjugated and secreted within the first 24h in untreated and group 2 drug-treated cultures. By contrast, CsA, CPZ and Tro caused inhibition of LCA sulfation associated with its accumulation in cell layers. Inhibition of LCA sulfation could be at least partly related to an inhibition of SULT2A1. Noteworthy, high LCA levels were measured in the serum of patients treated with CsA and of patients with intrahepatic cholestasis of pregnancy (Myara et al., 1996; Lucangioli et al., 2009), supporting the clinical relevance of our *in vitro* findings.

Noteworthy, contrary to their similar effects on cellular accumulation of both conjugated and unconjugated BAs, CPZ, CsA and Tro differently affected activity of uptake and efflux transporters. CsA has been found to be a strong competitive inhibitor for BSEP (Watanabe et al., 2007) and to alter membrane fluidity (Yasumiba et al., 2001). CPZ can induce ROS

generation leading to alteration of BA transporters in HepaRG cells (Antherieu et al., 2013). Tro-sulfate competitively inhibits BSEP (Ivetic and Ridley, 2004). In support, after 4h treatment at the tested concentrations CPZ and CsA inhibited efflux of radiolabeled ^{3}H-TCA, the major substrate of BSEP while Tro, like CsA, inhibited ^{3}H-TCA uptake by NTCP and CDF efflux by MRP2 (Figure 7). Therefore, even if preferential cellular accumulation of unconjugated hydrophobic BAs could be explained by the reduced capacity of BA efflux transporters it appeared that inhibition of amidation represented a major step in intracellular accumulation of BAs.

Addition of exogenous BAs at cholestatic concentrations (60X-BA mixture), also considerably affected BA contents and profiles in both cell layers and supernatants. The disposition of unconjugated and conjugated BAs was different in cell layers of cultures exposed to 60X-BA versus 1X-BAs. Indeed, 23-fold more BAs were accumulated in cell layers and unconjugated BAs represented 47% versus 4% in the presence of 1X-BA concentrations after 24h. These differences could be explained by strong inhibition of amidation activity and efflux transporters in the presence of BA overload. Noticeably, group 2 molecules, also accumulated BAs after 6 daily repeated exposure to 60X-BAs. Tol slightly accumulated BAs, suggesting that it had the lower cholestatic potential. In agreement, in clinic even though Tol has been associated with liver toxicity and mortality (Watkins, 2000), only one single case of cholestasis has been reported in the LiverTox website (www.livertox.nih.gov).

Although compared to 1X-BAs, more BAs were accumulated in cell layers after 6 daily repeated exposure to 60X-BAs, no cytotoxicity was observed. In agreement, serum and intrahepatic BAs at concentrations that are observed during cholestasis *in vivo* are largely non-hepatotoxic to isolated hepatocytes and liver cell lines unless the BA composition is toxified by addition of the hydrophobic BAs CDCA, LCA or DCA (Suda et al., 2011). Necrosis was evidenced in the human hepatocytes exposed to GCDCA only at concentrations of 2mM or higher for 24h (Wakabayashi et al., 2006). LCA and DCA were not cytotoxic to HepaRG cells at concentrations lower than 200µM after a 24h exposure (Sharanek et al., 2015), supporting

the prolonged survival of hepatocytes in cholestatic livers despite high intrahepatic and serum BA levels.

In conclusion, we report the first *in vitro* in-depth analysis of intracellular accumulation of total and individual BAs caused by various cholestatic drugs in the presence of exogenous BAs and as a function of culture time. Our results demonstrate that the most potent cholestatic drugs can cause *in vitro* intracellular accumulation of unconjugated forms of the three hydrophobic BAs, CDCA, LCA and DCA, that could represent suitable markers to distinguish potent cholestatic drugs from those referred as rarely cholestastic in clinic. Moreover, they suggest that inhibition of BA amidation and sulfation represents major mechanisms of drug-induced cholestasis. However, a large number of cholestatic and non-cholestatic drugs has to be investigated to determine the specificity and robustness of this new way to predict the cholestatic potential of new drugs. Finally, since currently ursodeoxycholic acid is the only FDA-approved drug for the treatment of cholestasis and its efficacy is limited (Erlinger, 1997; Mullenbach et al., 2005) there is an urgent need for new treatments of cholestatic liver diseases. HepaRG cells could represent a consistent and easy to use *in vitro* cell model to study anti-cholestatic therapeutics.

Authorship Contributions

Participated in research design: Sharanek, Burban, Guillouzo, and Rainteau

Conducted experiments: Sharanek, Burban, and Humbert

Performed data analysis: Sharanek, Burban, Guillouzo and Rainteau

Wrote or contributed to the writing of the manuscript: Sharanek, Burban, Guguen-Guillouzo, Guillouzo and Rainteau

References

Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, and Sugiyama Y (2001) Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochim Biophys Acta* **1511:**7-16.

Amieva MR, Wilgenbus KK, and Furthmayr H (1994) Radixin is a component of hepatocyte microvilli in situ. *Exp Cell Res* **210:**140-144.

Aninat C, Piton A, Glaise D, Le Charpentier T, Langouet S, Morel F, Guguen-Guillouzo C, and Guillouzo A (2006) Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos* **34:**75-83.

Antherieu S, Bachour-El Azzi P, Dumont J, Abdel-Razzak Z, Guguen-Guillouzo C, Fromenty B, Robin MA, and Guillouzo A (2013) Oxidative stress plays a major role in chlorpromazine-induced cholestasis in human HepaRG cells. *Hepatology* **57:**1518-1529.

Anwer MS (2012) Intracellular Signaling by Bile Acids. *J Biosci (Rajshari)* **20:**1-23.

Anwer MS (2014) Role of protein kinase C isoforms in bile formation and cholestasis. *Hepatology* **60:**1090-1097.

Bachour-El Azzi P, Sharanek A, Burban A, Li R, Guevel RL, Abdel-Razzak Z, Stieger B, Guguen-Guillouzo C, and Guillouzo A (2015) Comparative Localization and Functional Activity of the Main Hepatobiliary Transporters in HepaRG Cells and Primary Human Hepatocytes. *Toxicol Sci* **145:**157-168.

Bjornsson ES and Jonasson JG (2013) Drug-induced cholestasis. *Clin Liver Dis* **17:**191-209.

Burban A, Sharanek A, Hue R, Gay M, Routier S, Guillouzo A, and Guguen-Guillouzo C (2017) Penicillinase-resistant antibiotics induce non-immune-mediated cholestasis through HSP27 activation associated with PKC/P38 and PI3K/AKT signaling pathways. *Sci Rep* **7:**1815.

Burbank MG, Burban A, Sharanek A, Weaver RJ, Guguen-Guillouzo C, and Guillouzo A (2016) Early Alterations of Bile Canaliculi Dynamics and the Rho Kinase/Myosin Light Chain Kinase Pathway

Are Characteristics of Drug-Induced Intrahepatic Cholestasis. *Drug Metab Dispos* **44:**1780-1793.

Cerec V, Glaise D, Garnier D, Morosan S, Turlin B, Drenou B, Gripon P, Kremsdorf D, Guguen-Guillouzo C, and Corlu A (2007) Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology* **45:**957-967.

Chiang JY (2009) Bile acids: regulation of synthesis. *J Lipid Res* **50:**1955-1966.

Crocenzi FA, Zucchetti AE, Boaglio AC, Barosso IR, Sanchez Pozzi EJ, Mottino AD, and Roma MG (2012) Localization status of hepatocellular transporters in cholestasis. *Front Biosci (Landmark Ed)* **17:**1201-1218.

Dawson PA, Lan T, and Rao A (2009) Bile acid transporters. *J Lipid Res* **50:**2340-2357.

Doege H, Baillie RA, Ortegon AM, Tsang B, Wu Q, Punreddy S, Hirsch D, Watson N, Gimeno RE, and Stahl A (2006) Targeted deletion of FATP5 reveals multiple functions in liver metabolism: alterations in hepatic lipid homeostasis. *Gastroenterology* **130:**1245-1258.

Erlinger S (1997) Drug-induced cholestasis. *J Hepatol* **26 Suppl 1:**1-4.

Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B, and Meier PJ (2001) The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther* **69:**223-231.

Hata S, Wang P, Eftychiou N, Ananthanarayanan M, Batta A, Salen G, Pang KS, and Wolkoff AW (2003) Substrate specificities of rat oatp1 and ntcp: implications for hepatic organic anion uptake. *Am J Physiol Gastrointest Liver Physiol* **285:**G829-839.

Hayashi H, Takada T, Suzuki H, Onuki R, Hofmann AF, and Sugiyama Y (2005) Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: a comparison of human BSEP with rat Bsep. *Biochim Biophys Acta* **1738:**54-62.

Humbert L, Maubert MA, Wolf C, Duboc H, Mahe M, Farabos D, Seksik P, Mallet JM, Trugnan G, Masliah J, and Rainteau D (2012) Bile acid profiling in human biological samples: comparison of

extraction procedures and application to normal and cholestatic patients. *J Chromatogr B Analyt Technol Biomed Life Sci* **899:**135-145.

Ivetic A and Ridley AJ (2004) Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology* **112:**165-176.

Kojima H, Nies AT, Konig J, Hagmann W, Spring H, Uemura M, Fukui H, and Keppler D (2003) Changes in the expression and localization of hepatocellular transporters and radixin in primary biliary cirrhosis. *J Hepatol* **39:**693-702.

Kojima H, Sakurai S, Yoshiji H, Uemura M, Yoshikawa M, and Fukui H (2008) The role of radixin in altered localization of canalicular conjugate export pump Mrp2 in cholestatic rat liver. *Hepatol Res* **38:**202-210.

Kostrubsky VE, Strom SC, Hanson J, Urda E, Rose K, Burliegh J, Zocharski P, Cai H, Sinclair JF, and Sahi J (2003) Evaluation of hepatotoxic potential of drugs by inhibition of bile-acid transport in cultured primary human hepatocytes and intact rats. *Toxicol Sci* **76:**220-228.

Lam P, Wang R, and Ling V (2005) Bile acid transport in sister of P-glycoprotein (ABCB11) knockout mice. *Biochemistry* **44:**12598-12605.

Lefebvre P, Cariou B, Lien F, Kuipers F, and Staels B (2009) Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* **89:**147-191.

Liu J, Lu H, Lu YF, Lei X, Cui JY, Ellis E, Strom SC, and Klaassen CD (2014) Potency of individual bile acids to regulate bile acid synthesis and transport genes in primary human hepatocyte cultures. *Toxicol Sci* **141:**538-546.

Lucangioli SE, Castano G, Contin MD, and Tripodi VP (2009) Lithocholic acid as a biomarker of intrahepatic cholestasis of pregnancy during ursodeoxycholic acid treatment. *Ann Clin Biochem* **46:**44-49.

Lucena MI, Andrade RJ, Rodrigo L, Salmeron J, Alvarez A, Lopez-Garrido MJ, Camargo R, and Alcantara R (2000) Trovafloxacin-induced acute hepatitis. *Clin Infect Dis* **30:**400-401.

Monte MJ, Marin JJ, Antelo A, and Vazquez-Tato J (2009) Bile acids: chemistry, physiology, and pathophysiology. *World J Gastroenterol* **15:**804-816.

Morgan RE, Trauner M, van Staden CJ, Lee PH, Ramachandran B, Eschenberg M, Afshari CA, Qualls CW, Jr., Lightfoot-Dunn R, and Hamadeh HK (2010) Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicol Sci* **118:**485-500.

Mullenbach R, Bennett A, Tetlow N, Patel N, Hamilton G, Cheng F, Chambers J, Howard R, Taylor-Robinson SD, and Williamson C (2005) ATP8B1 mutations in British cases with intrahepatic cholestasis of pregnancy. *Gut* **54:**829-834.

Myara A, Cadranel JF, Dorent R, Lunel F, Bouvier E, Gerhardt M, Bernard B, Ghoussoub JJ, Cabrol A, Gandjbakhch I, Opolon P, and Trivin F (1996) Cyclosporin A-mediated cholestasis in patients with chronic hepatitis after heart transplantation. *Eur J Gastroenterol Hepatol* **8:**267-271.

Padda MS, Sanchez M, Akhtar AJ, and Boyer JL (2011) Drug-induced cholestasis. *Hepatology* **53:**1377-1387.

Pauli-Magnus C and Meier PJ (2006) Hepatobiliary transporters and drug-induced cholestasis. *Hepatology* **44:**778-787.

Pellicoro A, van den Heuvel FA, Geuken M, Moshage H, Jansen PL, and Faber KN (2007) Human and rat bile acid-CoA:amino acid N-acyltransferase are liver-specific peroxisomal enzymes: implications for intracellular bile salt transport. *Hepatology* **45:**340-348.

Rodrigues AD, Lai Y, Cvijic ME, Elkin LL, Zvyaga T, and Soars MG (2014) Drug-induced perturbations of the bile acid pool, cholestasis, and hepatotoxicity: mechanistic considerations beyond the direct inhibition of the bile salt export pump. *Drug Metab Dispos* **42:**566-574.

Scherer M, Gnewuch C, Schmitz G, and Liebisch G (2009) Rapid quantification of bile acids and their conjugates in serum by liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **877:**3920-3925.

Sharanek A, Azzi PB, Al-Attrache H, Savary CC, Humbert L, Rainteau D, Guguen-Guillouzo C, and Guillouzo A (2014) Different dose-dependent mechanisms are involved in early cyclosporine a-induced cholestatic effects in hepaRG cells. *Toxicol Sci* **141:**244-253.

Sharanek A, Burban A, Burbank M, Le Guevel R, Li R, Guillouzo A, and Guguen-Guillouzo C (2016) Rho-kinase/myosin light chain kinase pathway plays a key role in the impairment of bile canaliculi dynamics induced by cholestatic drugs. *Sci Rep* **6:**24709.

Sharanek A, Burban A, Humbert L, Bachour-El Azzi P, Felix-Gomes N, Rainteau D, and Guillouzo A (2015) Cellular Accumulation and Toxic Effects of Bile Acids in Cyclosporine A-Treated HepaRG Hepatocytes. *Toxicol Sci* **147:**573-587.

Stahlmann R (2002) Clinical toxicological aspects of fluoroquinolones. *Toxicol Lett* **127:**269-277.

Stieger B (2011) The role of the sodium-taurocholate cotransporting polypeptide (NTCP) and of the bile salt export pump (BSEP) in physiology and pathophysiology of bile formation. *Handb Exp Pharmacol***:**205-259.

Suda J, Zhu L, and Karvar S (2011) Phosphorylation of radixin regulates cell polarity and Mrp-2 distribution in hepatocytes. *Am J Physiol Cell Physiol* **300:**C416-424.

Vessey DA and Kelley M (1995) Inhibition of bile acid conjugation by cyclosporin A. *Biochim Biophys Acta* **1272:**49-52.

Wakabayashi Y, Kipp H, and Arias IM (2006) Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. *J Biol Chem* **281:**27669-27673.

Watanabe N, Takashimizu S, Kojima S, Kagawa T, Nishizaki Y, and Mine T (2007) Altered cell motility behaviour and reorganization of contractile proteins after primary monolayer cultures of isolated hepatocytes. *Tokai J Exp Clin Med* **32:**34-41.

Watkins P (2000) COMT inhibitors and liver toxicity. *Neurology* **55:**S51-52; discussion S53-56.

Yasumiba S, Tazuma S, Ochi H, Chayama K, and Kajiyama G (2001) Cyclosporin A reduces canalicular membrane fluidity and regulates transporter function in rats. *Biochem J* **354:**591-596.

Zollner G, Fickert P, Silbert D, Fuchsbichler A, Marschall HU, Zatloukal K, Denk H, and Trauner M (2003) Adaptive changes in hepatobiliary transporter expression in primary biliary cirrhosis. *J Hepatol* **38:**717-727.

Footnotes

This work was partly supported by the European Community through the Innovative Medicines Initiative Joint Undertaking MIP-DILI project [grant agreement number 115336], resources of which are composed of financial contribution from the European Union's Seventh Framework Programme [FP7/20072013] and EFPIA companies' in kind contribution. Ahmad Sharanek and Audrey Burban were financially supported by the MIP-DILI project.

A.S., A.B. and L.H. contributed equally to this work

A.G. and D.R. are senior co-authors

Legends for Figures

Figure 1. Cytotoxicity and effects on bile canaliculi morphology and bile acid transporters of tested drugs in human HepaRG cells. (**A)** Representative phase-contrast images of HepaRG cells treated with 5µM CsA, 15µM CPZ, 10µM Tro, 10µM Tol, 10µM TVX and 5µM Tac for 4h. Images were captured using time lapse microscopy. Orange arrows indicate BC dilatation (bar = 50 µm). **(B)** Cytotoxicity was measured using the MTT colorimetric assay. HepaRG cells were treated daily for 6 days with tested drugs. **(C)** Uptake and **(D)** efflux of ^{3}H-TCA in HepaRG cells after 4h of treatment. (E) Quantification of CDF accumulation in BC of HepaRG hepatocytes using ImageJ 1.48 software (**F**) Representative fluorescent images showing effects of tested drugs on CDF efflux after 4h of treatment. Nuclei stained in blue (Hoechst dye). The fluorescent images were obtained with a Cellomics ArrayScan VTI HCS Reader. Data were expressed relative to those of untreated cells arbitrarily set at 100%. They represent the means ± SEM of 3 independent experiments. *$p < 0.05$ compared with that of untreated cells.

Figure 2. Effects of tested drugs on endogenous BA production and profiles in HepaRG cell cultures incubated with BA-free charcoal stripped serum. BA contents were measured in both supernatants and cell layers by HPLC–MS/MS (**A)** Endogenous BAs **(B)** Total CDCA (TCDCA and GCDCA) **(C)** TCA. **(D-F)** Total BAs after **(D)** 4h, (**E)** 24h and **(F)** 6-day treatment with cholestatic drugs. Both individual and total BAs were calculated in µg/10^{6} hepatocytes. Values represent the mean ± SEM of 3 independent experiments. **(A-C)** *$p<0.05$ compared with the values after 4h. **(C-D) ***$p<0.05$ compared with the values in cell layers of untreated cells, $^{\#}p<0.05$ compared with the values in supernatants of untreated cells and $^{\$}p<0.05$ compared with the values in cells+supernatant of untreated cells.

Figure 3. Effects of tested drugs on BA profiles in HepaRG cell cultures incubated with 1X-BA mixture. **(A-C)** HepaRG cells were incubated for 4h, 24h and 6 days in the presence of 1X-BA mixture alone. **(A)** Total unconjugated BAs **(B)** total conjugated BAs and (**C)** sulfated

LCA (S-LCA) in supernatants and cell layers. **(D-E)** HepaRG cells were co-exposed to cholestatic drugs and 1X-BAs for 4h. **(D)** Total BAs in supernatants and cell layers. **(E)** Fold changes in cellular accumulation of conjugated and unconjugated BAs after treatment with tested drugs compared to untreated cells arbitrarily set at a value of 1. BAs were calculated in µg/10^6 hepatocytes. Values represent the mean ± SEM of 3 independent experiments. **(A-C)** *p<0.05 compared with the values in the non-exposed media. **(D-E)** *p<0.05 compared with the values in cell layers of untreated cells and $^{\#}$p<0.05 compared with the values in supernatant of untreated cells.

Figure 4. Effects of 24h treatment with tested drugs on BA profiles in HepaRG cell cultures incubated with 1X-BAs. **(A-H)** The cells were co-exposed to cholestatic drugs and 1X-BAs for 24h. **(A)** Total conjugated BAs and **(B)** total unconjugated BAs in supernatants and cell layers. **(C)** Fold changes in cellular accumulation of conjugated and unconjugated BAs after treatment with tested drugs compared to untreated cells arbitrarily set at a value of 1. **(D)** Unconjugated CDCA, **(E)** unconjugated DCA, **(F)** unconjugated LCA, **(G)** sulfated LCA and **(H)** unconjugated CA content in supernatants and cell layers after 24h treatment. Both individual and total BAs were calculated in µg/10^6 hepatocytes/24h. Values represent the mean ± SEM of 3 independent experiments. *p<0.05 compared with the values in cell layers of untreated cells, $^{\#}$p<0.05 compared with the values in supernatant of untreated cells and $^{\$}$p<0.05 compared with the values in cells + supernatants of untreated cells.

Figure 5. Effects of tested drugs on BA amidation and sulfation and BA profiles in HepaRG cell cultures incubated with 1X-BAs. **(A)** Effects of tested drugs on BA amidation after 4h, 24h and 6 days of treatment. Amidation activity was based on content of total conjugated BA species in each condition and data are expressed in percent relative to the amount present in untreated cells arbitrarily set at a value of 100. **(B)** Effects of tested drugs on BA sulfation after 4h, 24h and 6 days of treatment. Sulfation activity was based on content of total sulfated LCA in each condition and data are expressed in percent relative to the amount

present in untreated cells arbitrarily set at a value of 100. **(C-G)** The cells were treated with cholestatic drugs for 6 days in presence of 1X-BAs. **(C)** Total BAs **(D)** total conjugated BAs and **(E)** total unconjugated BAs in supernatants and cell layers. **(F)** Fold changes in cellular accumulation of conjugated and unconjugated BAs after treatment with tested drugs compared to untreated cells arbitrarily set at a value of 1. **(G)** Amounts of unconjugated BAs accumulated in cell layers after 6 days compared to 24h treatment with tested drugs. BAs were calculated in µg/10^6 hepatocytes/24h. Values represent the mean ± SEM of 3 independent experiments. *p<0.05 compared with the values in cell layers of untreated cultures, $^{\#}$p<0.05 compared with the values in supernatants of untreated cultures and $^{\$}$p<0.05 compared with the values in cells + supernatants of untreated cultures.

Figure 6. Effects of tested drugs on cell viability and BA profiles in HepaRG cell cultures incubated with 60X-BAs. (A) HepaRG cells were treated with 5µM CsA, 15µM CPZ, 10µM Tro, 10µM Tol, 10µM TVX and 5µM Tac in absence or presence of 60X-BAs for 6 days. Cytotoxicity was measured using the MTT colorimetric assay. **(B)** Cellular accumulation of total BAs after 4h, 24h and 6 days of incubation with 60X-BAs compared to 1X-BAs. **(C)** Percent of conjugated and unconjugated BAs in cell layers of cultures incubated with 1X- and 60X-BAs. **(D)** Amounts of conjugated and unconjugated BAs in cell layers after 4h, 24h and 6 days of treatment with cholestatic drugs in presence of 60X-BAs. **(E)** Total CDCA **(F)** total LCA and **(G)** total CA content accumulated in cell layers after 6 days of treatment with cholestatic drugs compared to 24h in presence of 60X-BAs. Both individual and total BAs were calculated in µg/10^6 hepatocytes. Values represent the mean ± SEM of 3 independent experiments. (**C)** *p<0.05 values in the cell layers in 60X-BAs compared to their corresponding values in cell layers in 1X-BA condition and (**D-G)** compared with the values in cell layers of untreated cells.

Figure 7. Schematic representation of the diverse effects of the tested cholestatic drugs. The 3 potent cholestatic drugs CsA, CPZ and Tro inhibit BA amidation either by inhibition of BA CoA ligation activity of BACs **(1)**, CoA-BA translocation to the peroxisomes **(2)**, and/or

DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

BAAT activity leading to increase of total unconjugated BAs **(3)**. CsA, CPZ and Tro also inhibit LCA sulfation **(4)**. TVX induces similar effects particularly after 6 daily (repeated) treatments and Tol and Tac are ineffective. The 3 potent cholestatic drugs also alter BA transporter activities: CsA and CPZ inhibit BSEP activity **(5)** and CsA and Tro inhibit MRP2 **(6)** and NTCP activity **(7)**.

Table 1: Concentration and corresponding quantity of individual BAs measured in unexposed media supplemented with 1X- or 60X-BA mixture compared to their concentration in human plasma

Bile acids	Human plasma	1X-BA mixture		60X-BA mixture	
	Concentration (μM)	Concentration measured in 1X (μM)	Corresponding quantity (μg/10^6 hepatocytes)	Concentration measured in 60X(μM)	Corresponding quantity (μg/10^6 hepatocytes)
TCDCA	0.21	0.2±0.01	0.3±0.02	10.71±0.67	15.29±0.95
TCA	0.048	0.06±0.01	0.08±0.01	2.24±0.02	3.3±0.03
GCDCA	1.71	1.64±0.28	2.11±0.36	82.43±1.42	105.86±1.82
GDCA	0.38	0.36±0.03	0.46±0.04	14.58±1.33	18.72±1.71
GCA	0.41	0.3±0.01	0.4±0.02	18.65±0.92	24.81±1.23
LCA	0.03	0.04±0.01	0.05±0.01	1.63±0.11	2.25±0.15
CDCA	0.34	0.37±0.04	0.42±0.04	19.81±0.93	22.21±1.04
DCA	0.48	0.4±0.02	0.45±0.02	19.75±0.61	22.14±0.69
CA	0.2	0.18±0.02	0.21±0.02	8.83±1.29	10.31±1.51

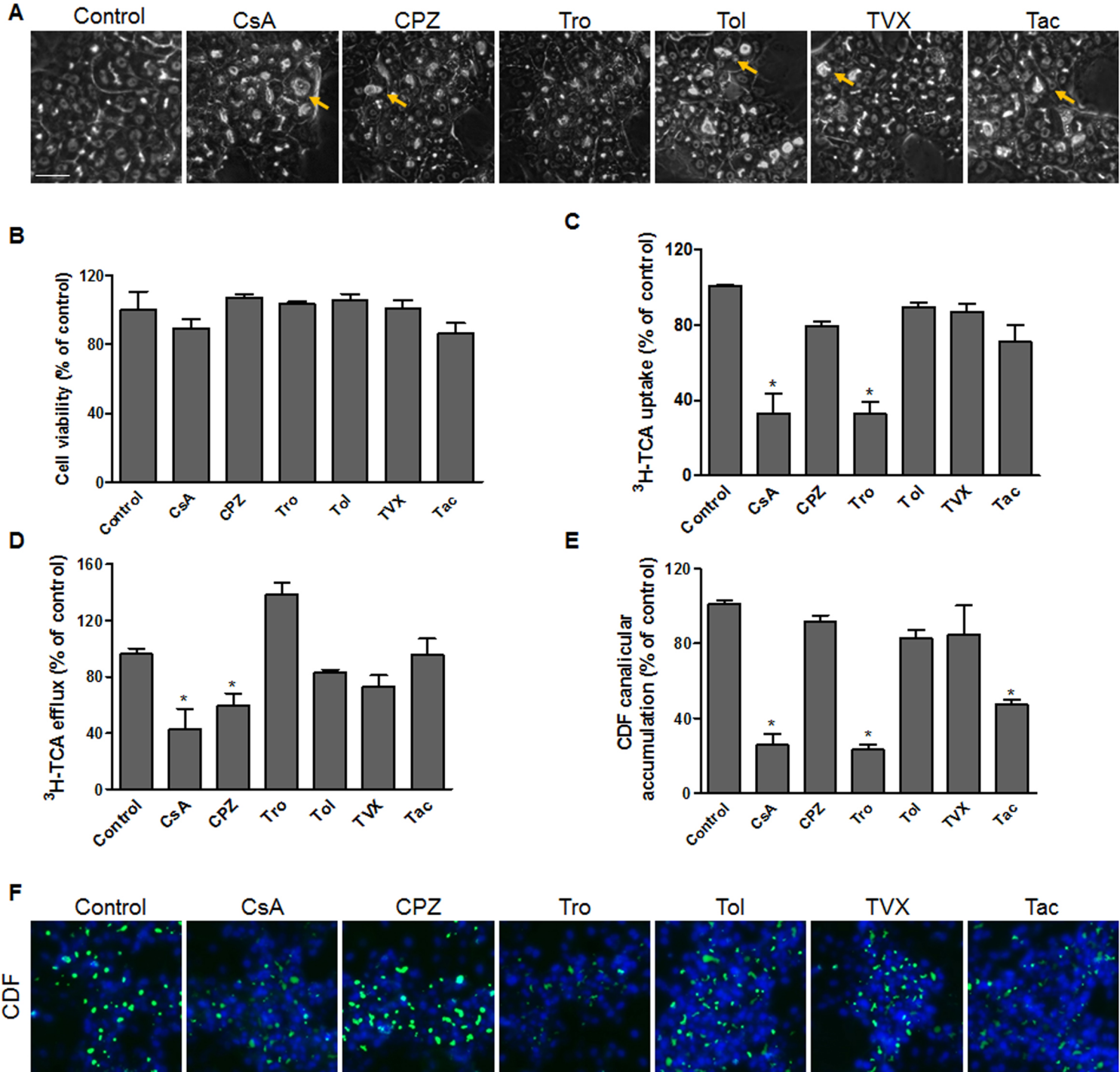
Corresponding quantity of each individual BA available for 10^6 hepatocytes was calculated from the concentration measured using the following formula: Quantity (μg/10^6 hepatocytes) = Concentration* molecular weight* volume of medium / number of hepatocytes. Based on medium volume= 10ml and number of hepatocytes=3.5*10^6 hepatocytes per dish.

Table 2: Effects of tested drugs on expression of mRNAs encoding genes related to hepatobiliary transporters, BA metabolizing and conjugating enzymes, ROS and ER stress markers in HepaRG cells after 6-day treatment in absence and presence of 1X- and 60X- mixtures.

	1X-BA	60X-BA	CPZ	CPZ 1X-BA	CPZ 60X-BA	CsA	CsA 1X-BA	CsA 60X-BA	TRO	TRO 1X-BA	TRO 60X-BA	TOL	TOL 1X-BA	TOL 60X-BA	TVX	TVX 1X-BA	TVX 60X-BA	Tac	Tac 1X-BA	Tac 60X-BA
BSEP	1.6±0.2	**4.3±0.9**	**0.5±0.1**	**0.6±0.1**	**4.6±0.9**	**0.8±0.0**	1.5±0.1	**5.9±1.1**	0.7±0.1	1.5±0.2	**3.8±0.1**	1.2±0.3	1.4±0.1	**4.4±0.2**	0.7±0.1	1.1±0.1	**4.5±0.7**	**0.4±0.1**	1.1±0.0	**3.7±0.2**
NTCP	1.1±0.1	**0.7±0.1**	**0.7±0.1**	0.8±0.1	0.8±0.1	**2.4±0.2**	**2.2±0.5**	0.8±0.1	1.1±0.3	1.1±0.2	0.9±0.2	1.0±001	1.4±0.1	1.0±0.1	**0.7±0.1**	**0.7±0.1**	0.8±0.1	1.4±0.3	1.4±0.1	1.0±0.2
MRP2	1.4±0.1	1.0±0.1	1.0±0.1	1.0±0.1	1.3±0.1	1.2±0.0	1.2±0.0	1.4±0.1	1.2±0.1	1.1±0.0	1.3±0.1	1.3±0.1	1.2±0.0	1.4±0.0	1.4±0.1	**1.5±0.1**	**1.6±0.1**	1.2±0.1	1.2±0.0	1.3±0.1
MRP3	1.3±0.0	**1.6±0.1**	**0.7±0.0**	**0.7±0.0**	0.9±0.1	1.0±0.1	1.1±0.0	**2.1±0.5**	1.0±0.1	1.1±0.0	1.2±0.2	1.4±0.1	1.2±0.1	1.3±0.1	1.1±0.0	1.1±0.0	1.3±0.1	1.2±0.1	1.4±0.1	1.2±0.1
MRP4	1±0	1.1±0.1	1.2±0.01	1.2±0.01	**1.8±0.1**	1.5±0.1	**1.8±0.1**	**1.8±0.3**	1.1±0.1	1.1±0.1	1.5±0.2	1.1±0.1	1.3±0.01	1.5±0.2	1.3±0.1	1.4±0.01	**1.8±0.1**	1.3±0.1	1.3±0.1	1.2±0.1
MDR1	1.2±0.1	**1.9±0.2**	1.1±0.2	1.4±0.2	1.5±0.2	1.4±0.2	**1.9±0.2**	**1.7±0.2**	1.5±0.3	**1.8±0.2**	1.5±0.3	1.2±0.1	1.6±0.2	1.4±0.2	1.2±0.1	1.4±0.1	1.4±0.1	1.5±0.2	1.5±0.2	1.3±0.1
MDR3	1.4±0.1	**3.5±0.3**	**0.5±0.1**	**0.6±0.1**	**2.4±0.3**	0.7±0.1	1.6±0.1	4±0.5	1±0.1	2.5±0.6	4.7±0.4	1.4±0.4	**1.7±0.1**	**3.3±0.6**	**0.5±0.1**	1±0.1	**2.2±0.4**	0.8±0.2	1.5±0.3	**2.9±0.4**
CYP7A1	0.9±0.01	**0.7±0.1**	**0.7±0.1**	**0.7±0.01**	**0.6±0.01**	0.8±0.1	**0.7±0.01**	0.8±0.01	**0.7±0.1**	**0.7±0.1**	**0.7±0.01**	1±0.1	1.1±0.01	0.9±0.1	0.8±0.1	0.8±0.1	**0.7±0.1**	**0.7±0.1**	**0.7±0.1**	**0.7±0.1**
CYP8B1	1±0.01	**0.8±0.01**	**0.6±0.02**	**0.6±0.01**	**0.5±0.03**	**0.8±0.04**	0.8±0.2	**0.7±0.04**	**0.7±0.1**	**0.8±0.02**	**0.7±0.03**	**0.7±0.1**	1.1±0.02	0.9±0.1	0.8±0.1	**0.8±0.01**	**0.5±0.05**	**0.7±0.1**	**0.8±0.02**	**0.7±0.03**
CYP27A1	0.8±0.01	**0.6±0.1**	**0.7±0.03**	**0.6±0.2**	0.7±0.2	0.8±0.1	0.8±0.2	**0.7±0.1**	**0.7±0.01**	0.8±0.2	**0.6±0.1**	1±0.01	0.9±0.2	1.2±0.1	**0.8±0.02**	0.7±0.2	**0.7±0.03**	0.8±0.2	0.8±0.1	0.8±0.2
ATF4	1.3±0.05	1.2±0.01	1±0.01	1.2±0.03	1.1±0.05	**2.4±0.15**	**2.6±0.13**	**2.9±0.2**	1.4±0.03	1.4±0.03	1.3±0.03	1.3±0.05	1.5±0.1	1.4±0.02	**2.2±0.05**	**2.3±0.23**	**2.7±0.01**	1.3±0.03	1.1±0.05	1.1±0.03
ATF6	1.4±0.03	1.4±0.08	1.1±0.05	1.3±0.03	1.5±0.13	1.3±0.05	**1.5±0.01**	**1.9±0.1**	1.3±0.05	1.2±0.01	1.5±0.1	1.3±0.08	1.4±0.03	1.6±0.2	**1.5±0.08**	1.7±0.25	**1.94±0.05**	1.4±0.08	1.2±0.03	1.3±0.08
CHOP	**1.6±0.03**	1.3±0.05	1.4±0.05	**1.6±0.03**	1.5±0.02	**3.4±0.02**	**3.6±0.02**	**4.1±0.01**	1.5±0.05	1.5±0.03	1.3±0.04	1.3±0.13	1.5±0.14	1.4±0.05	**2.3±0.08**	**2.3±0.3**	**2.7±0.13**	1.2±0.18	1.2±0.11	1.4±0.08
GRP78	0.85±0.03	0.9±0.02	1.05±0.13	1±0.15	1.2±0.25	**2±0.02**	**2.3±0.03**	**2.9±0.01**	1.15±0.03	1.2±0.14	1.2±0.2	0.95±0.08	1.35±0.13	1.5±0.25	1.25±0.08	1.4±0.18	**1.7±0.25**	1.35±0.18	1.3±0.15	1.5±0.25
MnSOD	0.8±0.03	0.7±0.05	0.9±0.05	0.8±0.03	0.9±0.03	0.9±0.03	0.8±0.03	0.7±0.01	0.9±0.03	1±0.03	0.8±0.05	1.1±0.05	1.1±0.03	0.8±0.13	0.9±0.02	0.9±0.05	0.8±0.02	0.8±0.05	0.7±0.13	0.7±0.01
HO-1	1.1±0.01	1±0.03	**1.8±0.03**	**1.9±0.02**	**2.4±0.13**	0.9±0.02	0.8±0.03	0.9±0.05	**1.9±0.03**	**2.3±0.03**	**2.9±0.01**	1.1±0.08	1.1±0.01	1±0.02	1.4±0.3	0.9±0.03	1±0.01	1.1±0.05	1±0.05	1.1±0.04
BAAT	**1.7±0.1**	**1.9±0.13**	**0.7±0.01**	**0.5±0.01**	**0.6±0.01**	0.8±0.02	0.8±0.09	**0.7±0.01**	0.8±0.04	**0.8±0.01**	**0.8±0.01**	1.1±0.15	1.2±0.14	1.2±0.16	0.9±0.03	**0.8±0.01**	**07±0.03**	0.9±0.1	1±0.1	**0.7±0.09**
FXR	1.1±0.06	**0.5±0.02**	1±0.07	**0.7±0.06**	**0.5±0.08**	1.1±0.03	0.8±0.03	**0.7±0.02**	1.1±0.08	1.1±0.08	0.9±0.08	1.6±0.03	1±0.08	0.8±0.02	0.9±0.06	0.8±0.1	**0.6±0.02**	0.8±0.07	0.9±0.06	**0.6±0.04**
ALB	1±0.06	1.1±0.06	1.2±0.07	1.1±0.2	1.0±0.06	1.1±0.06	1±0.04	0.8±0.07	1.3±0.06	1.2±0.16	1.1±0.03	1±0.06	1.2±0.05	0.9±0.04	0.7±0.02	0.9±0.04	1±0.07	1±0.1	1.2±0.16	0.9±0.03
SUL2A1	1.4±0.1	**2.0±0.13**	**0.7±0.01**	**0.5±0.01**	**0.6±0.01**	0.8±0.02	0.8±0.09	**0.7±0.01**	0.8±0.04	**0.8±0.01**	**0.8±0.01**	1.1±0.15	1.2±0.14	1.2±0.16	0.9±0.03	**0.8±0.01**	**0.7±0.03**	0.9±0.1	1±0.1	**0.7±0.09**

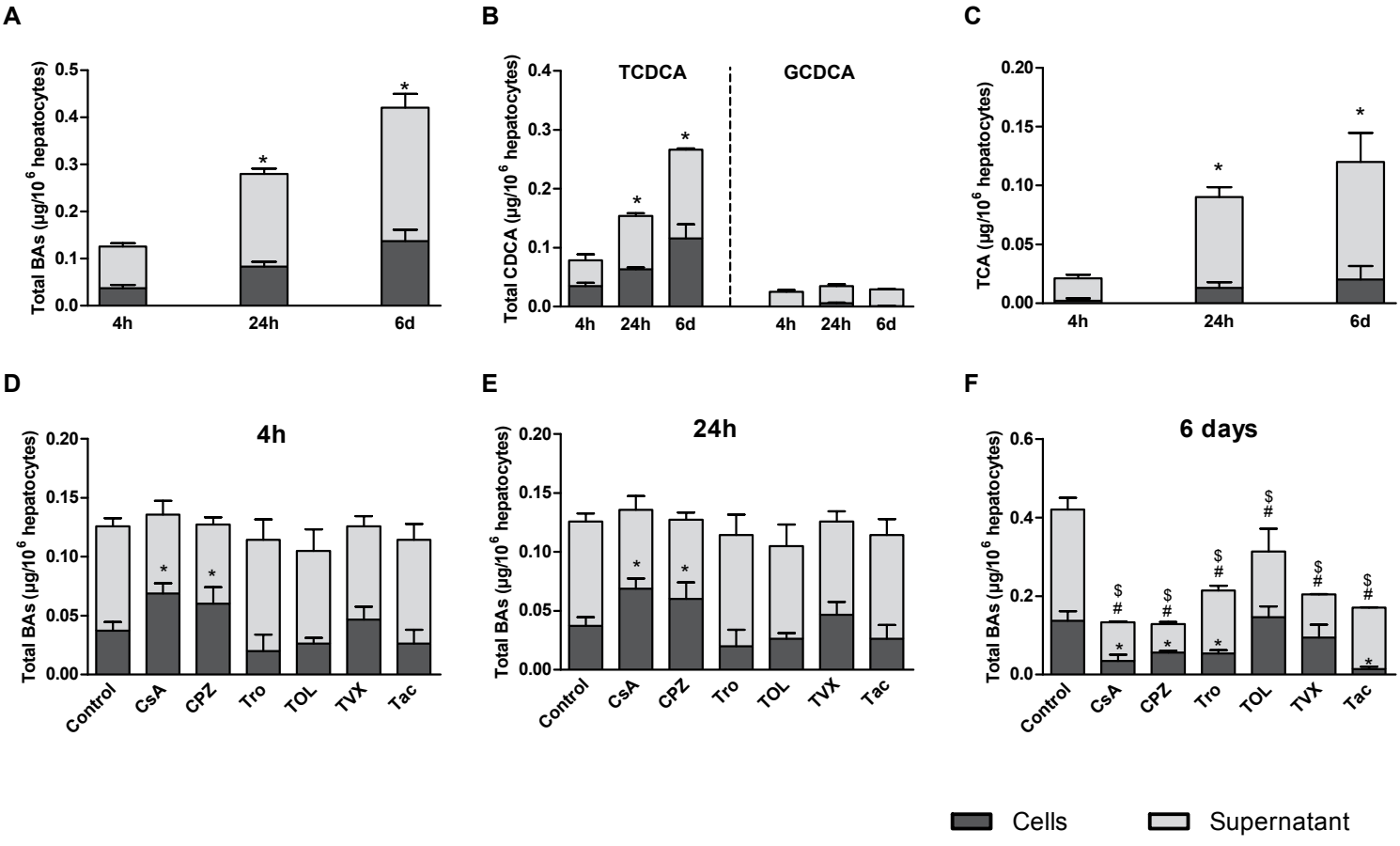
Data represent the means ± SEM of 3 independent experiments. All results are expressed relative to the levels found in untreated cells arbitrarily set at 1. Bold and underlined data are statistically significant ($p<0.05$) compared to untreated cells

Figure 1


A
Control
CsA
CPZ
Tro
Tol
TVX
Tac
B
Cell viability (% of control)
120
80
40
0
Control
CsA
CPZ
Tro
Tol
TVX
Tac
C
^{3}H-TCA uptake (% of control)
120
80
40
0
*
*
Control
CsA
CPZ
Tro
Tol
TVX
Tac
D
^{3}H-TCA efflux (% of control)
160
120
80
40
0
*
*
Control
CsA
CPZ
Tro
Tol
TVX
Tac
E
CDF canalicular accumulation (% of control)
120
80
40
0
*
*
*
Control
CsA
CPZ
Tro
Tol
TVX
Tac
F
Control
CsA
CPZ
Tro
Tol
TVX
Tac
CDF

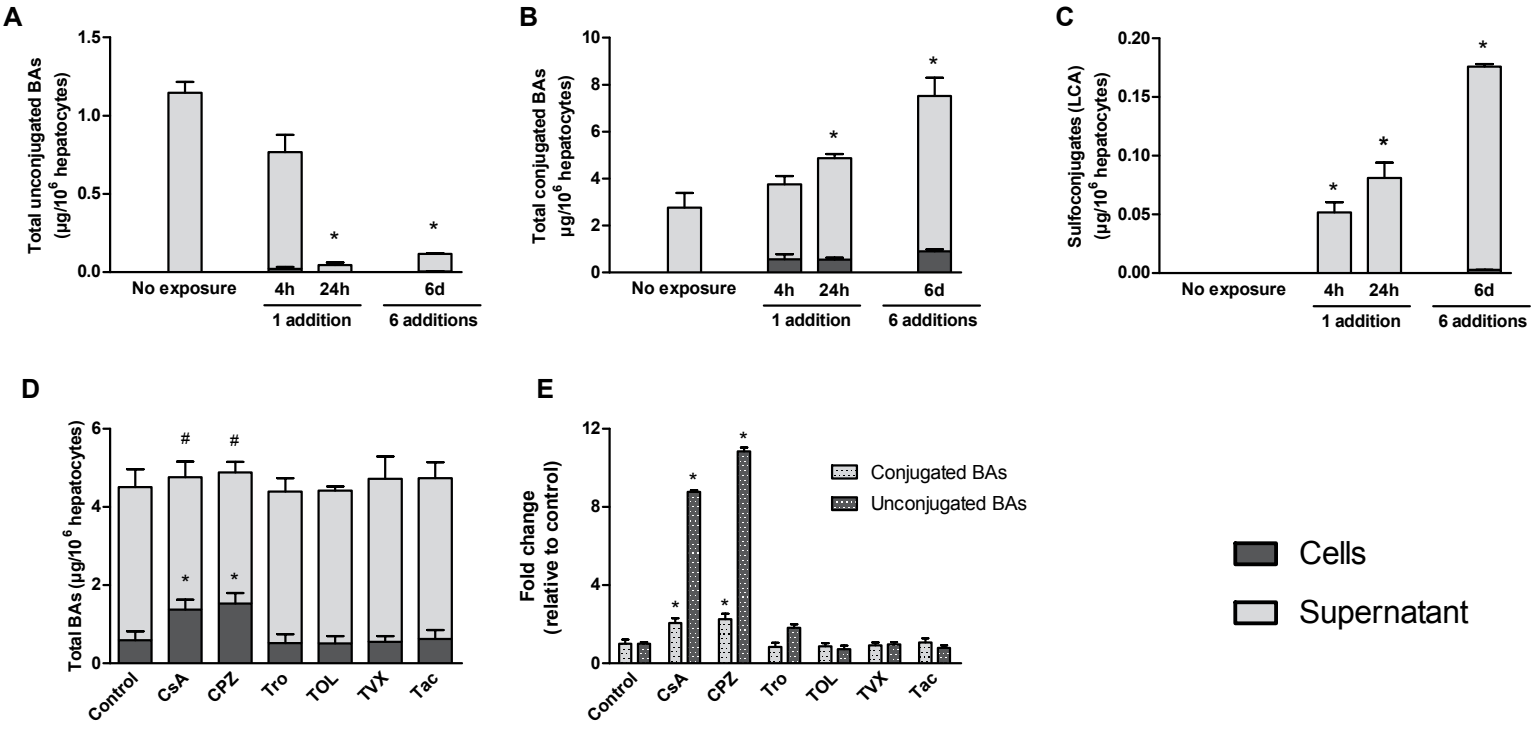
DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4

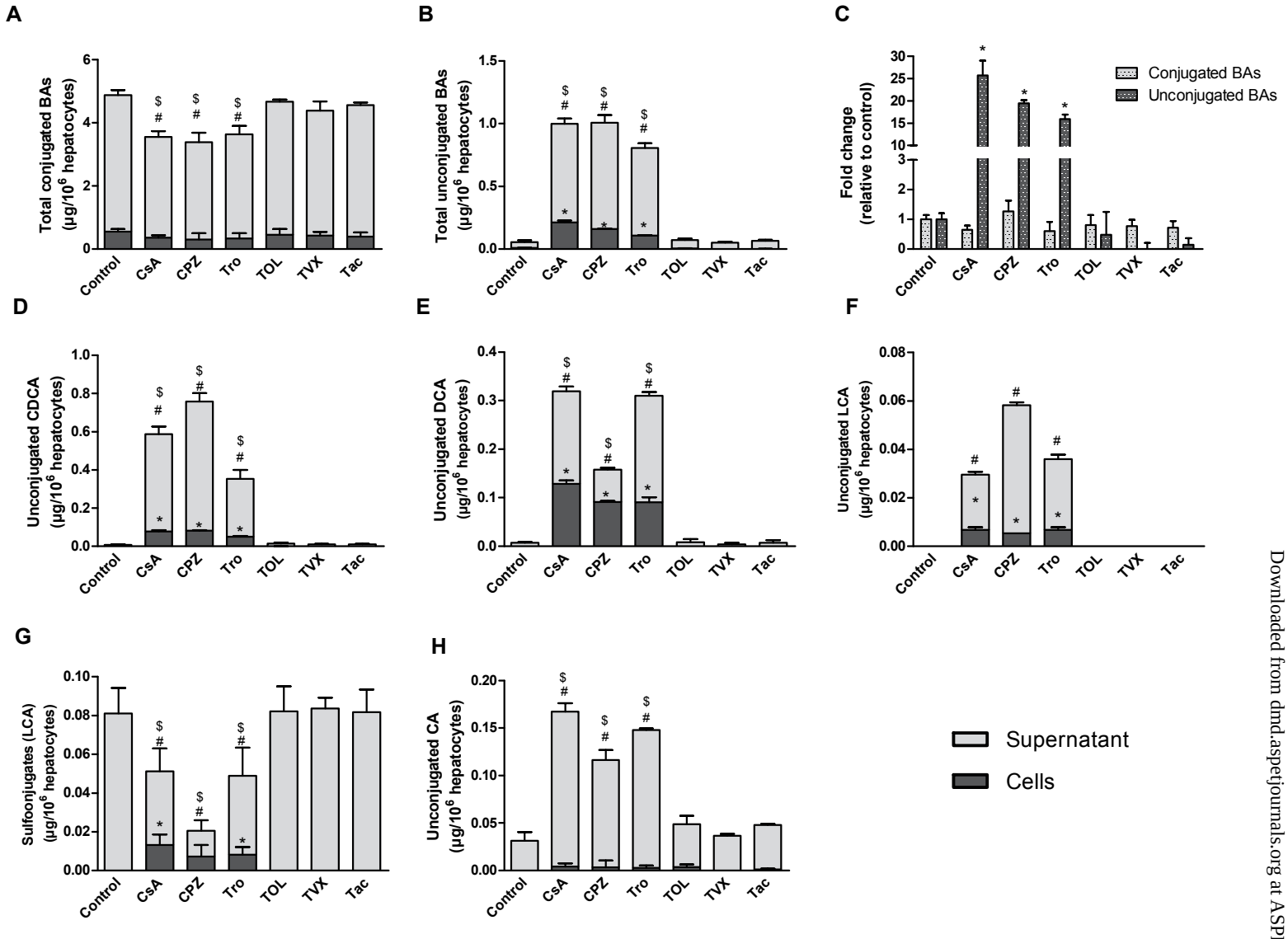
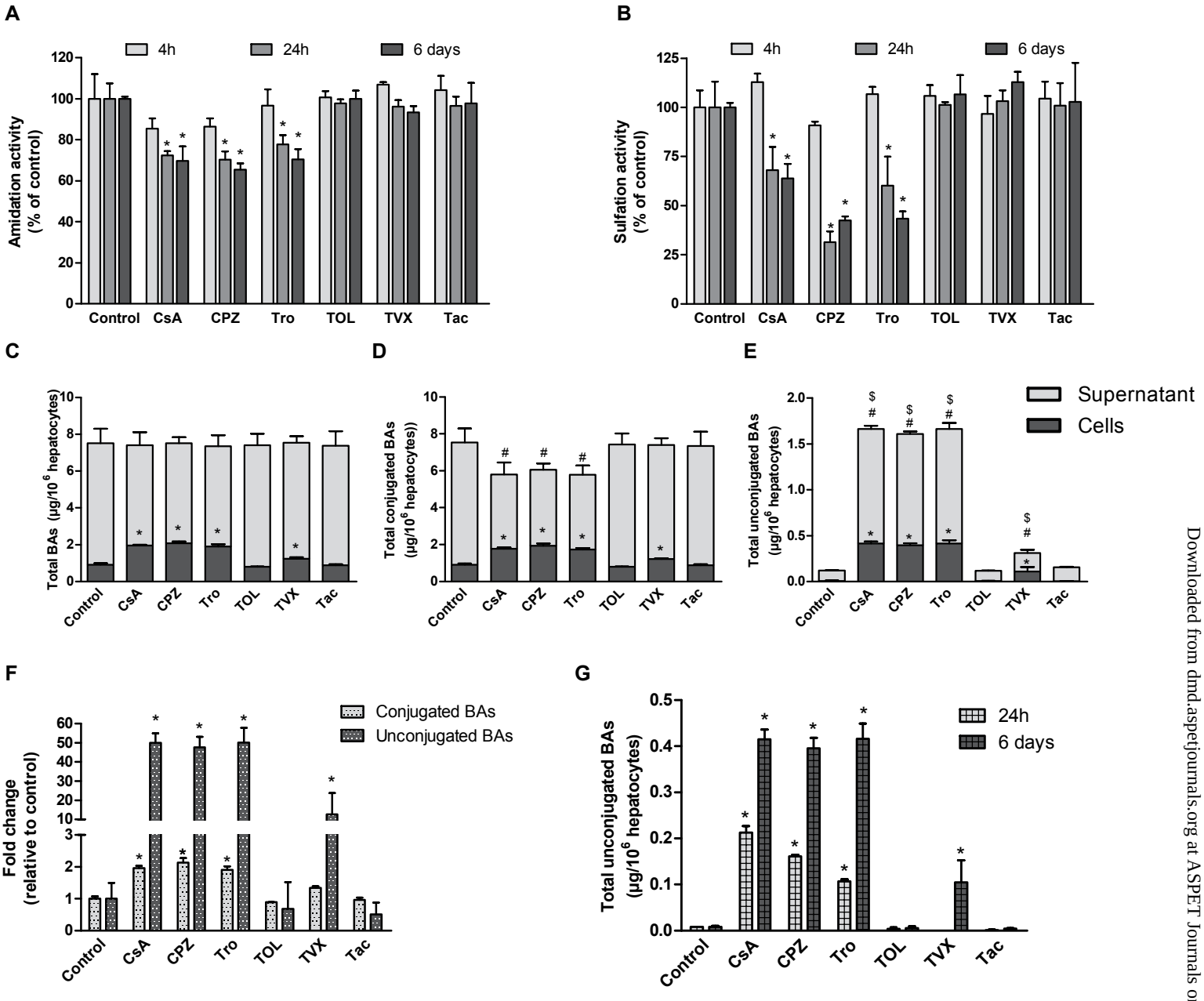


Figure 5

DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.



DMD Fast Forward. Published on September 19, 2017 as DOI: 10.1124/dmd.117.077420
This article has not been copyedited and formatted. The final version may differ from this version.

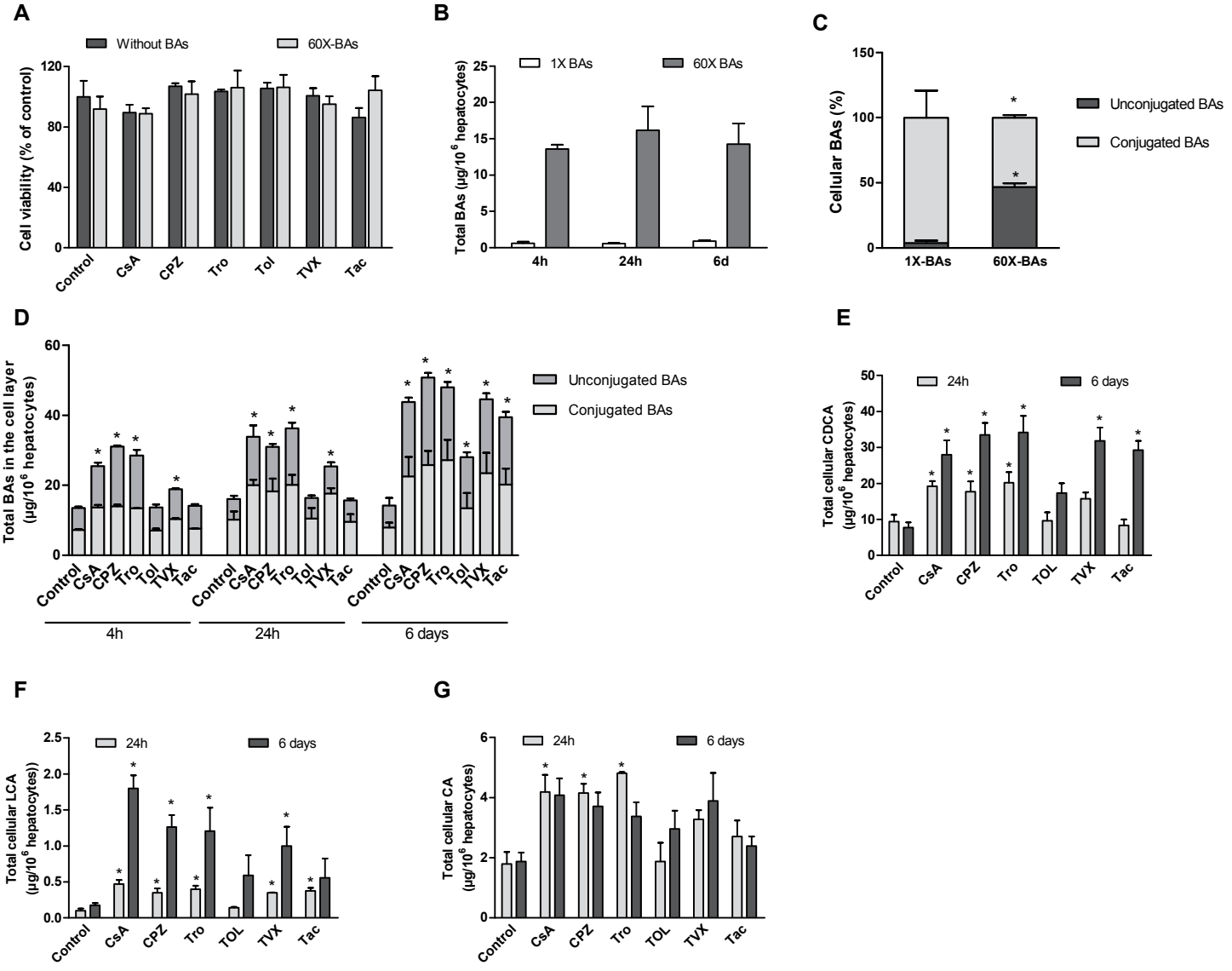


Figure 7