Title

Link between toxicity and intracellular accumulation of bile acids in sandwich-cultured rat hepatocytes: role of glycine conjugates

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List of abbreviations:

BA, bile acids; BEI, biliary excretion index; BSEP/Bsep, bile salt export pump (human/rat); DMSO, dimethyl sulfoxide; CDCA, chenodeoxycholic acid; CA, cholic acid; FBS, fetal bovine serum GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HBSS, Hanks' Balanced Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; NOAEL, no observed adverse effect level; NTCP/Ntcp, sodium taurocholate cotransporting polypeptide (human/rat); PBS, phosphate buffered saline; SCH, sandwich-cultured hepatocytes; SCRH, sandwich-cultured rat hepatocytes; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid

1. Introduction

Bile acids (BA) are the major organic solutes of bile. They are amphiphilic molecules facilitating the intestinal absorption of fat-soluble compounds such as lipophilic vitamins and dietary lipids. Under normal physiological conditions, BA are actively taken up from portal blood into the hepatocytes primarily by sodium taurocholate cotransporting polypeptide [NTCP (human) or Ntcp (rat); *SLC10A1/Slc10a1* gene] in a sodium-dependent manner. In addition, sodium-independent uptake by hepatic isoforms of the organic anion transporting polypeptide (OATP/Oatp; *SLCO/Slco* gene family) also plays a role. Subsequently, BA are conjugated in the liver with glycine and taurine (at the C24 carboxylic acid moiety) and may also be metabolized by different liver enzymes such as cytochrome P450s, glucuronosyl- and sulfotransferases. They finally undergo efflux into the bile canaliculi by the bile salt export pump (BSEP/Bsep, an ATP binding cassette (ABC) transporter; *ABCB11/Abcb11* gene) (Kullak-ublick *et al.* 2004). Multidrug resistance-associated protein-2 (MRP2/Mrp2; *ABCC2/Abcc2* gene) is another canalicular ABC transporter that mediates the canalicular efflux of divalent BA such as the sulfated glycine or taurine conjugates. MRP3/Mrp3 (*ABCC3/Abcc3*) and MRP4 (*ABCC4*), localized at the basolateral side of the hepatocytes, are responsible for the efflux of the BA back into the sinusoidal blood (Alrefai and Gill 2007).

BSEP is the transport protein primarily responsible for the elimination of monovalent BA at the canalicular membrane. Its inhibition is implicated in the pathogenesis of estrogen-induced intrahepatic cholestasis of pregnancy. Genetic defects in BSEP can lead to progressive familial intrahepatic cholestasis type 2 (Dawson *et al.* 2009). Bsep (or BSEP) function can also be affected by endo- (e.g. estradiol-17 β -D-glucuronide) and xenobiotics leading to impaired canalicular BA excretion and subsequent excessive intracellular accumulation of BA (Stieger *et al.* 2000). These clinical adverse events appear to be associated with disturbed BA homeostasis due to altered BA elimination as key underlying toxicity mechanism. This

underscores the need for a more fundamental understanding of the exact role of BA accumulation mediated toxicity at the cellular level and in a quantitative manner.

Serum BA concentrations of about 3 - 7 μM are considered as physiological (Scherer *et al.* 2009; Tribe *et al.* 2009). When BA accumulate in hepatocytes above their physiological concentration range (which may or may not be associated with increased serum BA concentrations), they are associated with clinical hepatotoxicity. Higher BA concentrations (in the millimolar range) are thought to exert cytotoxicity by causing necrosis. However, in the lower range (μM range) of supra-physiological BA concentrations, bile acid-induced apoptosis is supposed to be the predominant mechanism (Perez and Briz 2009). Several mechanisms have been suggested to explain bile acid-induced apoptosis, such as: (i) direct activation of the death receptors Fas and TRAIL-R2, (ii) mitochondrial permeability transition induction and subsequent cytochrome c release and, (iii) mitochondrial and endoplasmic reticulum-mediated oxidative stress (Perez and Briz 2009). The toxicity associated with excessive BA accumulation results in impaired liver functions as observed in case of cholestasis. Higher plasma as well as hepatic bile acid concentrations has indeed been reported in case of cholestasis in both humans and rodents. In chronic cholestatic patients, hepatic bile acid concentrations as high as 430 μM have been estimated (Fischer *et al.* 1996; Rolo *et al.* 2003). Therefore, to gain insight into the toxicity exerted by BA in cholestasis, a thorough mechanistic investigation of concentration-dependent toxicity of BA in hepatocytes is of prime importance.

Various experimental animal models are available for studying the pathophysiological alterations during intrahepatic cholestasis (Rodríguez-Garay 2003). However, it is not possible to accurately control and measure the (hepatic) bile acid concentrations *in vivo*. In contrast, cell-based *in vitro* systems can provide insight into the role of individual bile acid

species (and/or their metabolites) in causing hepatotoxicity when their concentrations are elevated.

Bile acid-mediated toxicity has been studied in different *in vitro* systems including isolated rat liver mitochondria, cell lines (as HepG2), and isolated hepatocytes in suspension and in conventional primary cultures of rat hepatocytes (Hillaire *et al.* 1995; Martinez-Diez *et al.* 2000; Rolo *et al.* 2004; Rolo *et al.* 2002; Spivey *et al.* 1993). However, these *in vitro* systems only partly reflect the intricate interplay between uptake and efflux transporters and the metabolizing enzymes that takes place inside the liver, to regulate the intracellular bile acid concentration. Sandwich-cultured hepatocytes (SCH) represent an *in vitro* model that maintains this expression of relevant basolateral and canalicular transporters. In addition, cultured hepatocytes have been reported to contain the necessary enzymes such as BAAT (bile acid-N-acetyl transferase), which mediates conjugation of unconjugated BA with either glycine or taurine (Falany *et al.* 1994; Rembacz *et al.* 2010). SCH are therefore an excellent model to provide further evidence for the link between bile acid-mediated toxicity and intracellular hepatic exposure to these BA or their conjugates.

In sandwich-cultured rat hepatocytes (SCRH), the expression/activity level of the different enzymes and transporters changes with culture time: while it has been reported that Ntcp expression gradually decreases, Bsep expression gradually increases (De Bruyn *et al.* 2013; Kotani *et al.* 2011). Functional bile canaliculi are fully developed in SCRH by day-3 after seeding (Liu *et al.* 1998). Hence, it is expected that Bsep-mediated bile acid efflux will constitute an elimination mechanism in day-3 but not in day-1 SCRH. Therefore, we hypothesized that the (apparent) toxicity of the BA will decrease with culture time because of more effective elimination (and/or less uptake). This implies that intracellular BA concentrations are expected to be lower in day-3 compared to day-1 SCRH.

In the present study, the BA toxicity in SCRH [as determined with an assay reflecting (loss of) hepatocyte-specific function] was linked for the first time to the *in vitro* hepatobiliary disposition profiles of these BA (and their metabolites/conjugates). A comparison of hepatic disposition and toxicity of BA was made between day-1 and day-3 SCRH. This revealed novel insight regarding a putative link between toxicity of certain bile acid species and the intracellular levels they reach in cultured hepatocytes.

2. Materials and methods

2.1. Materials

William's E Medium (WEM), L-glutamine, penicillin-streptomycin mixture (contains 10,000 IU penicillin and 10,000 IU of streptomycin per ml), Fetal Bovine Serum (FBS), Hanks' Balanced Salt Solution (HBSS), $\text{Ca}^{++}/\text{Mg}^{++}$ -free HBSS, Phosphate Buffered Saline (PBS; 1x and 10x), Trypan blue solution (0.4%) were purchased from Lonza Verviers SPRL (Verviers, Belgium). ITS +^{TM} Premix (contains insulin 6.25 mg/l, transferin 6.25 mg/l, selenious acid 6.25 mg/l, bovine serum albumin 1.25 g/l and linoleic acid 5.35 mg/l) was purchased from BD Biosciences (Erembodegem, Belgium). Sulfuric acid (95-97%) was purchased from Chem-Lab NV (Zedelgem, Belgium). All the BA, collagenase type IV (from *Clostridium histolyticum*), recombinant human insulin, Triton X-100, dexamethasone, urea, diacetyl monoxime, thiosemicarbazide, iron (III) chloride hexahydrate, ortho-phosphoric acid, ethylene glycol-bis(2-aminoethylether)-N,N,N', N'-tetraacetic acid (EGTA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from MP Biochemical (Illkirch, France). Thermostable 96-well plates were kindly provided by Greiner Bio-One BVBA (Wommel, Belgium). Collagen was prepared in-house from rat tails according to established procedures. For incubations with sandwich-cultured hepatocytes described below, 'standard buffer' consisted of HBSS adjusted to pH 7.4 and ' $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer' consisted of $\text{Ca}^{++}/\text{Mg}^{++}$ -free HBSS containing 1 mM EGTA (adjusted to pH 7.4).

2.2. Animals

The rats were housed according to the Belgian and European laws, guidelines and policies for animal experiments, housing and care in the Central Animal Facilities of the university.

Approval for this project was granted by the Institutional Ethical Committee for Animal Experimentation.

2.3. Isolation of rat hepatocytes

Hepatocytes were isolated from male Wistar rats (170-200 g) based on a two-step collagenase perfusion method, as described previously (Annaert *et al.* 2001), without adding trypsin inhibitor. After isolation, cells were centrifuged (50 g) for 3 min at 4°C and the pellet was re-suspended in William's E medium containing 5 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, 4 µg/ml insulin, and 1 µM dexamethasone. Hepatocytes were counted using a hemocytometer and cell viability was determined using Trypan blue. Cells were re-suspended in WEM and diluted to a final concentration of 1×10^6 cells/ml. Hepatocytes used in experiments had a viability after isolation of at least 85%.

2.4. Sandwich-cultured rat hepatocytes

24-well/6-well plates were coated with ice-cold collagen solution (~ 1.5 mg/ml final concentration: 50 µl/well for 24-well; 150 µl/well for 6-well plates) prepared by neutralizing a mixture of 4 ml of rat-tail collagen, 4 ml of deionized water, and 1 ml of 10 x Dulbecco's modified Eagle's medium with ~ 1 ml of 0.2 N NaOH (final pH ~ 7.4), and placed overnight at 37°C in a humidified incubator. Three hours before seeding, each well was hydrated with 500 µl (2 ml/well for 6-well plates) of PBS at 37°C. In 24-well plates, hepatocytes were seeded at a density of 0.5×10^6 cells/well ($0.26 \text{ million cells/cm}^2$) while in 6-well plates 1.75×10^6 cells/well in seeding medium (for 24-well 500 µl/well, while for 6-well plates 2 ml/well), which consists of WEM containing 5% FBS, 100 IU/ml penicillin, 100 IU/ml streptomycin, 4 µg/ml insulin, and 1 µM dexamethasone. After incubating the cells at 37°C in a humidified atmosphere with 5% CO₂ (Binder CO₂ incubator, Binder GmbH) for 1-2 h, unattached cells were removed by gently swirling the plate and immediately aspirating the

medium. To obtain a “sandwich” configuration the cells were overlaid with 50 μ l and 200 μ L of rat tail collagen solution ($\sim$ 1.5 mg/ml, pH 7.4) for 24 and 6-well plates respectively, (“day 0”). One hour later, completed cell culture medium at 37°C was added onto the cultures which were kept in a humidified atmosphere with 5% CO₂. The medium was changed every day with culture medium consisting of, WEM containing 1% (v/v) ITS+™ Premix, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 0.1 μ M dexamethasone.

2.5. Determination of urea formation in SCRH

To determine the urea synthesis capacity in SCRH, culture medium was aspirated and hepatocytes were rinsed twice with HBSS at 37°C. The cells were then incubated with HBSS containing 10 mM HEPES, 2 mM glutamine, 10 mM ammonium chloride and 3 mM ornithine (250 μ l/well for 24-well plates) for 1 h at 37°C in a humidified atmosphere with 5% CO₂. After 1 h of incubation, 60 μ l of the incubation medium/well was mixed with 240 μ l of color reagent (see below) in a 96-well thermostable plate. The mixture of color reagent and incubate was heated at 85°C for 20 min in a water bath and subsequently cooled down by keeping the plate at 4°C for 10 min. Absorbance was measured at 525 nm using a Tecan Infinite M200 plate reader (Austria). The color reagent was prepared by mixing the following solutions A and B in 2:1 ratio (v/v) just before the measurement. Solution A: 30 ml of concentrated sulfuric acid and 10 ml orthophosphoric acid added slowly to 60 ml of ferric chloride solution (160 mg/l FeCl₃.6H₂O) and mixed gently; the solution was kept on ice. Solution B: A 1:1 mixture of 60 mg thiosemicarbazide in 100 ml of ELGA water and 1200 mg diacetyl monoxime dissolved in 100 ml ELGA water.

2.6. Bile acid toxicity determination

Toxicity determination of the selected BA was carried in two stages. First, toxicity of the BA was evaluated in day-1 SCRH. Subsequently, the BA showing toxicity in day-1 SCRH, were further evaluated for culture-time dependent toxicity (day-1 *versus* day-3 SCRH).

2.6.1. Toxicity of BA in day-1 SCRH

Toxicity of the BA in SCRH was determined based on their influence on the ability of SCRH to convert toxic ammonia to urea. BA at different concentrations (5, 50, 100, 500 and 1000 μ M for CDCA, GCA; 5, 50, 100, 250 and 500 μ M for DCA and the equimolar mixture of CA, CDCA and DCA; 5, 50, 100, 200, 350, 500 and 1000 μ M for UDCA and GCDCA) were incubated with day-1 SCRH (24 h after seeding). After 24 h of incubation with the BA, urea formation was determined (at day-2).

2.6.2. Comparison of bile acid toxicity between day-1 and day-3 SCRH

Bile acid toxicity was compared between day-1 and day-3 SCRH in two batches of rat hepatocytes. For this purpose, CDCA and DCA at 5, 50, 100, 150, 200, 250, and 500 μ M and 5, 50, 100, 200, 350, 500 and 1000 μ M of UDCA and GCDCA were incubated with day-1 and day-3 SCRH. After 24 h of incubation with BA, urea formation was measured at day-2 and day-4. DMSO content was normalized to 0.3% for each condition.

2.7. Measurement of accumulation of CDCA, DCA and their taurine and glycine conjugates

To reveal a possible relation between BA toxicity and intracellular exposure of hepatocytes to BA, CDCA and DCA were incubated for 30 min (CDCA only) or 4 h with day-1 and day-3 SCRH. Following 30 min / 4 h incubation, the incubation medium was aspirated and the hepatocytes were rinsed twice with either standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer at 37°C.

Subsequently, hepatocytes were incubated for 10 min (37°C) with either standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer (B-CLEAR® procedure, Qualyst Transporter Solutions, Durham, NC). Next, incubation buffer was aspirated (collected for further analysis in one of efflux studies, see below for sample preparation) and cells were immediately rinsed four times with ice-cold standard buffer. Hepatocytes were then harvested with a chilled (-20°C) 7:3 mixture of methanol:water, containing 1 μM d^4 -cholic acid (d^4 -CA) as internal standard, and centrifuged at 14000 rpm for 15 min. After centrifugation, supernatants were collected and kept at -20°C till analysis by LC-MS (/MS). For determination of the total formation and sinusoidal efflux of GCDCA and TCDCA (Figure 3), the culture medium was collected immediately after incubation of the day-1 and day-3 SCRH with CDCA. Subsequently, the cells were rinsed three times with standard buffer at 4°C, following harvesting the SCRH according to the above mentioned procedure. The collected medium samples were diluted with two volumes of a 7:3 mixture of methanol:water, containing 1 μM d^4 -CA as internal standard, and centrifuged at 14000 rpm for 15 min. After centrifugation, supernatants were collected and kept at -20°C till analysis by LC-MS (/MS).

The supernatants collected after 10 min incubation with either standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer were diluted two times with 7:3 mixture of methanol:water, containing 1 μM d^4 -CA as internal standard, and centrifuged at 14000 rpm for 15 min. After centrifugation, supernatants were collected and kept at -20°C till analysis by LC-MS (/MS).

2.8. LC-MS analysis

Bile acid analysis was performed either with a Waters LC-MS system (Milford, MA, USA) or a Thermo Fisher Scientific LC-MS/MS system (Thermo Fischer, Breda, The Netherlands). The Waters system consisted of an HT alliance HPLC component and Micromass ZQ MS component with ESI source. The Thermo Fisher Scientific system consisted of a TSQ

Quantum Access^{TM} mass spectrometer coupled with Accela^{TM} U-HPLC system (Thermo Fischer, Breda, The Netherlands). For analysis in the Waters LC-MS system, separation was achieved with a Phenomenex Gemini C18 column (3 μ , 150 x 4.6 mm) with matching guard and pre-column, at a flow rate of 400 μ l/min. The injection volume was 25 μ l. The mobile phase was 15% methanol, 15% ammonium formate with formic acid (pH adjusted to 3.5) and 70% acetonitrile for 12 min. For analysing BA in the Thermo Fisher Scientific LC-MS/MS system, a Kinetex C18 column (1.7 μ , 100A, 50 x 3 mm) with a KrudKatcher ultra HPLC in-line filter (Phenomenex^R, The Netherlands) was used to achieve optimum separation. The injection volume was 10 μ l. The chromatographic conditions were: 300 μ l/ min flow rate for 3 min, with mobile phase consisting of 80:20 methanol:5 mM ammonium formate buffer (pH adjusted to 3.5 with formic acid). Analytical methods were validated for reproducible quantification of (i) CDCA and its conjugates, and (ii) DCA and its conjugates, with nominal QC sample concentrations of 0.05, 0.5 and 2 μ M. Analysis was done in the negative electrospray ionization mode. The interday inaccuracy varied between +0.31 % and -5.97 % and +1.96 % and +4.78 % for analysis of CDCA + conjugates and DCA + conjugates, respectively. The reproducibility as measured by % CV was 0.55 -9.19 % for CDCA and conjugates, and 3.4-5.22 % for DCA and conjugates. The interday inaccuracy of the analysis using the Thermo LC-MS/MS system for CDCA + conjugates group varied between -4.87 % to +7.87 %, while that for DCA + conjugates was +1.21 % to -7.08 %. The reproducibility (% CV) for analysis at different days was 3.77 - 13.41 % for BA in the CDCA group, while it was 1.96 to - 10.12 % for BA in the DCA group. BA concentrations in samples were determined by calculating the area ratio of analyte to internal standard. The monitored mass/charge ratios and transitions are included in Table 1a and Table 1b, respectively.

2.9. Data analysis

The Emax model was used to describe the concentration-dependency of the inhibitory effect of various BA on the capacity of hepatocytes to convert urea:

$$E = E_{max} - \left[(E_{max} - E_0) \cdot \frac{C^n}{C^n + IC_{50}^n} \right]$$

Where E is the urea production by hepatocytes, E_{max} is the urea production under control condition (without inhibitor), E_0 is the urea production at the maximum inhibitory effect of inhibitor, $(E_{max} - E_0)$ is the maximum inhibitory effect. The IC_{50} is the bile acid concentration causing a 50% inhibition. The parameter “n” denotes the Hill factor. The best fits of the above equation to the individual urea formation data sets were obtained by non-linear regression analysis with the NLS2 package in R version 2.15.1. The inverse of the experimentally obtained standard deviations were used for weighing.

In the absence of Ca^{++}/Mg^{++} the tight junctions between hepatocytes are disrupted, resulting in release of biliary content from the canalicular space (Liu *et al.* 1999). The accumulation values obtained from wells treated with Ca^{++}/Mg^{++} -free buffer were considered to reflect intracellular BA accumulation only. The amounts excreted in bile canaliculi was determined by subtracting the amounts of BA in cell lysates prepared from cultures treated with Ca^{++}/Mg^{++} -free buffer from that measured in cultures treated with standard buffer. Intracellular bile acid concentrations were estimated by dividing amounts of intracellularly accumulated bile acid by intracellular hepatocytes volume. The hepatocyte volume used to calculate intracellular BA concentrations (6.34 pl/cell) was based on the mean rat hepatocyte cellular volume as previously reported in two different studies (Brouwer 2010; Uhal and Roehrig 1982).

For calculating the biliary excretion of BA, a Biliary Excretion Index (BEI) was calculated according to the following equation (Liu *et al.* 1999), (B-CLEAR® procedure, Qualyst Transporter Solutions, Durham, NC) :

$$BEI = \frac{Accumulation_{standard\ buffer} - Accumulation_{Ca^{++}/Mg^{++}-free\ buffer}}{Accumulation_{standard\ buffer}} \times 100\%$$

Canalicular Efflux Index (CEI) was calculated according to the following equation:

$$CEI = \frac{Efflux_{Ca^{++}/Mg^{++}-free\ buffer} - Efflux_{standard\ buffer}}{Efflux_{Ca^{++}/Mg^{++}-free\ buffer}} \times 100\%$$

2.10. Statistics

ANOVA F-test (in MS Excel version 2007) was used to evaluate statistical significance of differences between day-1 and day-3 toxicity profiles (Figure 1) as well as accumulation (Figure 2, and 4) profile curves of BA, by comparing separately fit profiles for each culture day *versus* the simultaneous fit obtained with day-1 and day-3 measurements combined (Gibson *et al.* 1994). The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Comparison of bile acid toxicity between day-1 and day-3 SCRH

The effect of culture time (day-1 *versus* day-3 cultures) on BA toxicity was evaluated for CDCA, DCA, UDCA and GCDCA in two batches of hepatocytes (Figure 1). Higher BA concentrations were required in day-3 cultures, as compared to day-1 cultures, to decrease the urea formation to the same extent. This implied that BA were significantly more toxic in day-1 SCRH than day-3 SCRH ($p < 0.05$ for the difference of the toxicity profiles between day-1 and day-3 SCRH). The cytotoxicity differences are reflected by the significantly higher IC₅₀ values obtained for day-3 *versus* day-1 SCRH in both batches of SCRH (Table 2).

3.2. Accumulation of CDCA, DCA and their conjugates upon extracellular exposure of day-1 and day-3 SCRH to CDCA and DCA

As differences in bile acid toxicity between day-1 and day-3 hepatocyte cultures are possibly related to different intracellular accumulation and thus exposure to BA, concentration dependent accumulation of CDCA and DCA (including their formed conjugates) was determined. For this purpose, hepatocytes were incubated with CDCA and DCA at 4 different concentrations covering the NOAEL based on the toxicity data presented in Figure 1. Figure 2 depicts mean ($\pm$ SEM) values for bile acid accumulation after 4 h exposure to 5, 25, 50 or 100 μ M of CDCA or DCA, in three different batches of SCRH. Note that the 4 h incubation with bile acids in culture medium was followed by a rapid rinsing of the cells and subsequent 10 min incubation with standard buffer. This particular incubation design was used for 2 reasons: (i) it allowed to directly compare the obtained accumulation data to those obtained following incubation of the hepatocytes for 4 h + 10 min in Ca⁺⁺/Mg⁺⁺-free buffer (see 3.4); (ii) it allowed to focus on those bile acid(s) (metabolites) showing substantial retention in hepatocytes (i.e. not subject to efficient sinusoidal efflux).

Figure 2 illustrates that the hepatocytes converted the administered unconjugated BA (CDCA and DCA) to corresponding glycine and taurine conjugates. The combined amounts of CDCA and its two conjugates GCDCA and TCDCA that had accumulated in cells (+ bile canaliculi in case of day-3 cultures) after 4 h exposure to 5, 25, 50 or 100 μ M of CDCA (and followed by 10 min efflux in either standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer) are shown in Figure 2A. The corresponding data following exposure to DCA are shown in Figure 2E. The combined bile acid levels did not differ significantly between day-1 and day-3 SCRH for both CDCA and DCA group of BA. However, as illustrated in Figure 2B for CDCA and Figure 2F for DCA, significantly higher levels ($p < 0.05$) of these unconjugated BA were observed in day-3 compared to day-1 hepatocytes, for all incubation concentrations (except 5 μ M). Interestingly, the opposite was observed for the respective glycine and taurine conjugates, that reached higher accumulation in day-1 SCRH compared to day-3 SCRH, when exposed to the respective unconjugated BA (Figure 2 C-D, for CDCA conjugates and Figure 2 G-H for DCA conjugates). Figure 2D and 2H further illustrate that 4 h exposure to (C)DCA results in ~ 10 - 20 times lower taurine conjugate levels as compared to glycine conjugate levels.

3.3. Effect of culture time on formation and sinusoidal efflux of GCDCA and TCDCA in day-1 and day-3 SCRH

Subsequent incubations were conducted to delineate the exact roles of bile acid conjugation and/or sinusoidal efflux of bile acids as underlying mechanisms in culture time-dependent bile acid disposition. For this purpose, day-1 and day-3 hepatocytes were exposed to different concentrations of CDCA for 4 h, before measuring CDCA (and conjugate) levels in both hepatocyte cultures and the medium. Importantly, the 10 min incubation with standard buffer (which was included for the data presented in Figure 2), was now omitted. Instead, not only intracellular bile acid levels but also bile acid levels in the medium were measured at the end

of the 4 h incubation period. Thus, total conjugating capacity towards BA could be assessed for the various CDCA concentrations in both day-1 and day-3 cultures (Figure 3A and 3C for GCDCA and TCDCA). Note that total conjugate levels were plotted against intracellular CDCA concentrations (X-axis) as these constitute the driving force for BA conjugation. Intracellular concentrations were calculated based on measured intracellular CDCA amounts and estimated total intracellular hepatocyte volume (Brouwer 2010; Uhal and Roehrig 1982). Figure 3A/C confirms that after 4h exposure to CDCA, glycine and taurine conjugate levels in day-1 hepatocytes clearly exceeded those in day-3 hepatocytes for intracellular CDCA concentrations above 20 μ M. Furthermore, the conjugate formation rates (essentially total amounts formed/4h, Figure 3A/C) exhibited saturation within the studied concentration range, irrespective of the days in culture of the hepatocytes. Figure 3B/D depicts corresponding medium concentrations (μ M) of GCDCA and TCDCA as measured immediately after this 4h exposure. These profiles essentially reflect sinusoidal conjugate efflux that occurred during the 4h incubation period. It is important to note that these medium data reveal a similar saturation pattern as compared to the total formation data. Also similar to their total formation profiles, GCDCA and TCDCA concentrations were about 2 times higher in the medium of day-1 SCRH compared to day-3 SCRH, at intracellular CDCA concentrations of 20 μ M onwards.

3.4. In vitro biliary excretion of CDCA/ DCA and their conjugates

Apart from elimination by conjugation (and subsequent sinusoidal efflux), BA added to day-3 (but not day-1) SCRH may also undergo in vitro biliary excretion. To further explore the contribution of this elimination mechanism, SCRH were first exposed to CDCA or DCA for 4h; subsequently, the medium was rinsed off and hepatocytes were incubated for 10 min with either standard or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer. For a selected batch of day-3 SCRH, the combined

mean ($\pm$ SD) amounts of CDCA and its two conjugates (TCDCA and GCDCA) accumulating in cells and bile networks (standard buffer), were measured to be higher than in cells only ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer) (Figure 4A, $p < 0.05$). Also, the individual amounts of CDCA and GCDCA (Figure 4B-C) were found to be higher in cells and bile pockets, compared to cells only ($p < 0.05$). Combined and individual amounts of DCA and GDCA (Figure 4 D-F) were higher in cell and bile pockets compared to cells only ($p < 0.05$). It should be recognized that bile acid accumulation in standard *versus* $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer treated cells did not differ significantly in two other batches of SCRH (data not shown).

3.5. Biliary Disposition of CDCA and DCA in day-3 SCRH

Results presented in Figures 2-4 indicate that in vitro elimination of the bile acids CDCA and DCA in SCRH occurs via two distinct pathways: (i) bile acid conjugation (Figure 2); (ii) and direct biliary excretion. In order to illustrate the role of latter elimination route for CDCA and DCA, biliary excretion data for the batch of SCRH presented in Figure 4 were plotted, as shown in Figure 5. In contrast to the clear saturation of conjugate formation (Figure 2), biliary excretion of unconjugated (C)DCA did not reveal saturation.

3.6. Disposition of GCDCA and GDCA in day-3 SCRH

After formation, the conjugates of CDCA and DCA also get excreted into the bile networks. Figure 6A illustrates that upon 4 h exposure to CDCA (followed by 10 min efflux phase), the intracellular levels of GCDCA were found to increase with extracellular CDCA concentration up to 100 μM . In contrast, the biliary excretion of GCDCA did not increase proportionally beyond 25 μM of CDCA (Figure 6A). The maximum amount of GCDCA in bile was detected at 25 μM of extracellular CDCA exposure. Accordingly, the BEI of GCDCA was found to decrease with increasing intracellular concentration of GCDCA (Figure 6B). For GDCA, the amount excreted into the bile was also measured to be lower than the intracellular

amount of GDCA at each extracellular DCA concentration (Figure 6C-D), but no clear saturation was observed. For GDCA, the BEI appeared to remain more or less constant (27-35%) (Figure 6D).

3.7. Disposition of CDCA and conjugates in day-4 SCRH

As mentioned before, the extent of biliary excretion of (C)DCA and their conjugates in day-3 SCRH exhibited considerable interbatch variability. Therefore, an adapted incubation design was used to confirm the biliary excretory capacity for CDCA and its conjugates in day-4 SCRH. For this purpose, sinusoidal and canalicular efflux (10 min) of CDCA (and GCDCA) was determined in day-4 SCRH (seeded in 6-well plates) that had been exposed to various concentrations of CDCA for 4 h. Amounts of CDCA and GCDCA that were effluxed in the extracellular buffer during the 10 min incubation were much higher for $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer as compared to standard buffer throughout the concentration range 5-100 μM (Figure 7). A canalicular efflux index (CEI), representing the relative contribution of canalicular efflux to overall efflux was calculated based on the efflux data shown in Figure 7. The CEI values varied between 76-82 % for CDCA, while for GCDCA it varied between 78 and 81 %. In line with accumulation data, efflux of TCDCA was about 10 times less than the efflux of GCDCA.

3.8. Effect of incubation time on disposition of CDCA in day-1 and day-3 SCRH

In a final set of experiments, the time dependency of CDCA accumulation and conjugation in day-1 versus day-3 SCRH was determined. Relative (and absolute) amounts of TCDCA and GCDCA that accumulated (and remained) in the hepatocytes were found to be different after 30 min as opposed to 4 h incubation (always followed by 10 min efflux) with CDCA. After 30 min incubation (followed by 10 min efflux) with CDCA, higher levels of TCDCA were seen as compared to GCDCA, while after 4 h incubation, the opposite was observed (see also

Figure 2-3). This phenomenon was observed at each concentration of extracellular exposure (5, 25, 50 and 100 μ M) of CDCA and in both day-1 (column A of Figure 8) and day-3 (column B of Figure 3) SCRH. The combined accumulated amounts of CDCA and its conjugates (CDCA+GCDCA+TCDCA) in cell and bile networks decreased with time (30 min accumulation > 4 h accumulation), at all concentrations. Compared to levels observed at day-1, much higher levels of unconjugated CDCA were observed in day-3 cultures, irrespective of incubation time.

4. Discussion

The array of cellular and molecular mechanisms involved in the development cholestatic disease remains elusive. However, excessive intrahepatic accumulation of BA and/or their metabolites is thought to play a major role in this respect. As discussed in the introduction, intracellular bile acid levels in the liver are the result of intricate influences of hepatic BA metabolism along with hepatic uptake and efflux processes. Using sandwich-cultured rat hepatocytes, we presently provide novel evidence regarding the link between (excessive) intracellular bile acid accumulation and compromised hepatocyte viability and function. Moreover, in view of the dynamic changes in (relative) activities of enzymes and transporters involved in BA disposition, we hypothesized that exogenously added BA would exert their cytotoxic effect in SCH in a culture time-dependent fashion. The fact that SCH require several days for re-establishing functional bile canalicular networks, strongly suggests that this bile acid-eliminating capacity will be higher in day-3 *versus* day-1 SCRH. Our present observation (Figure 1) that bile acid-induced cytotoxicity in SCRH was indeed significantly lower in biliary excretion-competent (i.e. day-3) hepatocytes corroborates this concept of culture time-dependent capacity in bile acid handling. At the same time, quantifying intracellular bile acid levels following extracellular exposure of day-1 *versus* day-3 SCRH to exogenously added BA becomes an attractive approach to further explore the putative bile acid exposure-toxicity association. A particular advantage of this approach includes the fact that the use of transporter and/or enzyme inhibitors (with often questionable specificity) of hepatic bile acid disposition processes can be circumvented.

In the present work, compromised urea formation capacity was chosen as indicator to evaluate the concentration-dependency of *in vitro* cytotoxicity of BA - added individually or as a mixture - in SCRH. Determination of urea formation by bile acid-exposed hepatocytes enables detecting (sub-lethal) reduction in hepatocyte functionality (Dabos *et al.* 2004; Poll *et*

al. 2006). To efficiently measure urea formation in SCRH for our purpose, the well-known urea assay (Goeyens *et al.* 1998) was first miniaturized to a higher throughput 96-well format. We presently show that among all BA evaluated, glycine-conjugated BA were most potent in reducing the urea formation rate in day-1 SCRH upon 24 h exposure (Supplementary Figure 1). Consistently, several earlier *in vitro* observations in rat hepatocytes (Patel *et al.* 1994; Spivey *et al.* 1993) illustrated that glycine-conjugated BA were more toxic than their unconjugated or taurine-conjugated counterparts. This result supports the validity of the urea assay for the purpose of the present study.

Concentration-dependent accumulation (Figure 2) of CDCA and DCA (including their glycine/taurine conjugates) was studied, both after 1 day and 3 days in culture, in three batches of SCRH, at concentrations around the *in vitro* NOAEL for these BA (5-100 μ M). For this part of the study, a 4 h exposure period (rather than 24 h used for toxicity assessments) was selected to achieve low/moderate toxicity but avoid excessive cell death as this could mask the hepatic BA disposition processes. When “total” accumulation of BA was compared between day-1 and day-3 SCRH (Figure 2A/E), no statistically significant differences were found. This may appear contrary to the knowledge that there is a culture time-dependent decrease in uptake transporter activity and simultaneous increase in sinusoidal efflux transporter activity (De Bruyn *et al.* 2013; Liu *et al.* 1998). Moreover, accumulation values of the unconjugated BA species CDCA and DCA were actually higher in day-3 *versus* day-1 SCRH. This indicates that CDCA and DCA do not play a major direct role in the culture time-dependent decrease in toxicity (Figure 1) that was observed when SCRH were exposed to these BA. While (C)DCA accumulation increased with culture time, the opposite was observed for the corresponding glycine conjugates [accumulation in day-1 > day-3]; and this compensated more or less for the differences in accumulation observed for the “parent” (unconjugated) BA. Interestingly, the more than proportional increase in CDCA

and DCA accumulation (Figure 3B/F) with increasing extracellular exposure to these BA (5-100 μ M), was consistent with the saturation kinetics in the formation of the glycine conjugates. This is illustrated by the simultaneously fitted accumulation profiles obtained for unconjugated BA and their corresponding glycine conjugates (Supplementary Figure 2). Taken together, the substantially higher levels of glycine conjugates of CDCA and DCA in day-1 as compared to day-3 cultures (Figure 2C and 2G) suggests that these glycine conjugates play an important role in the toxicity differences when SCRH of different days in culture are exposed to the unconjugated CDCA and DCA.

The reduced glycine conjugate levels in day-3 SCRH may result from: (i) culture time-dependent decrease in overall formation of glycine conjugates, and/or (ii) increased basolateral efflux of these conjugated BA in day-3 cultures. In order to delineate between these two mechanisms, total conjugate levels in SCRH were assessed by analyzing both the culture medium and the hepatocytes (including bile networks, in case of day-3 SCRH) following a 4 h exposure to 5-100 μ M CDCA (Figure 3 A/C). These data clearly illustrate that total amounts (cells + medium) of formed GCDCA and TCDCA were higher in day-1 compared to day-3 SCRH for intracellular CDCA concentrations above 25 to 100 μ M. A very similar pattern was observed when only medium conjugate concentrations were plotted against intracellular CDCA concentration (Figure 3 B/D). This is consistent with non-restrictive efflux of BA conjugates via basolateral efflux transporters. This also means that the lower intracellular levels of BA conjugates in day-3 SCRH cannot be explained in terms of a culture time-dependent increase in sinusoidal efflux. It is noteworthy that the elevated glycine conjugate levels were observed for CDCA incubation concentrations above 25 μ M. This corresponds to the concentration range around which CDCA becomes toxic in day-1 (but not day-3) SCRH (Figure 1). Therefore, decreased formation of glycine conjugates (but

not increased sinusoidal efflux) in day-3 cultures appears to be the major mechanism explaining reduced glycine conjugate levels in day-3 cultures as shown in Figure 2 C/D.

Further to BA metabolism by conjugation, biliary excretion of BA will also contribute to their hepatic elimination. As mentioned earlier, both these elimination pathways are taking place in SCH. Intracellular and intracanalicular bile acid accumulation were determined via the B-CLEAR® method as explained in the materials and methods and results section (Annaert and Brouwer 2005; Annaert *et al.* 2001; Liu *et al.* 1999). As illustrated in Figure 4A-C for CDCA and in Figure 4D-F for DCA, up to about 50% of the accumulated amounts of BA (including conjugates) were residing in bile canalicular compartments. Figure 5 provides a direct comparison of the importance of CDCA and DCA biliary excretion in hepatic elimination. For both CDCA and DCA, biliary excretion rates continue to increase up to intracellular concentrations of 300 μ M. Also GDCA showed non-saturable biliary excretion for intracellular GDCA concentrations up to 100 μ M (Biliary excretion index, BEI = 27-35%; Figure 6D). In contrast, clear saturation of biliary excretion was observed for the glycine conjugate of CDCA, as reflected by decreasing BEI values (from 62% down to 21%) for intracellular GCDCA concentrations between 3 and 80 μ M.

Although conjugated bile acid levels were found to be very similar in three batches of SCRH, considerable interbatch variability was observed with respect to biliary excretion of CDCA, DCA and their conjugates. The inter-batch variability in biliary excretion may result from different factors. Pulsatile and/or cyclic release of canalicular contents by sandwich-cultured hepatocytes may be one of these factors (Brouwer *et al.* 2007). Indeed, within the 4 h bile acid exposure time frame, different batches may show dissimilar bile canalicular release profiles. This implies low values for measured excretion in at least some batches of SCRH. Another factor causing interbatch variability in biliary excretion might involve variable extents of sinusoidal BA efflux in SCRH (Jemnitz *et al.* 2010), in turn affecting biliary

excretion rates. Efficient sinusoidal efflux during and immediately after the 4 h incubation (cfr. glycine conjugate medium concentrations shown in Figure 2), may have prevented accurate and consistent assessment of biliary excretion. Hepatocytes may indeed upregulate sinusoidal efflux in situations where canalicular excretion clearance becomes insufficient to get rid of rising intracellular bile acid levels; under those conditions, biliary excretion of BA is likely to become more variable across different batches of SCRH (Donner and Keppler 2001). A third factor potentially contributing to variable BA excretion relates to the incubation design used in the present study. A 4 h exposure of the SCRH to BA was followed by a 10-min incubation with either standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer. This allowed releasing canalicular contents specifically in hepatocyte cultures exposed to $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer. However, during this 10-min incubation periods concomitant sinusoidal efflux is continuing, which further contributes to decreasing differences in residual bile acid levels between hepatocytes exposed to standard *versus* $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer.

The BA efflux data (Figure 7) obtained after exposure of SCRH to various CDCA concentrations, showed higher sinusoidal + canalicular BA efflux (SCRH exposed to $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer) as opposed to sinusoidal efflux (SCRH exposed to standard buffer). Moreover, corresponding BA accumulation data for these day-4 SCRH revealed some biliary excretion, mainly of GCDCA when higher CDCA concentrations were used (supplementary Figure 3). Taken together, these data suggest that direct measurement of differential bile acid efflux in addition to differential bile acid accumulation provides a reliable approach for determining *in vitro* biliary excretion of these BA in SCRH. Figure 7 illustrates that biliary excretion of CDCA increases more than proportional with increasing extracellular CDCA concentrations (consistent with concentration-dependent changes in intracellular levels; see Figure 2 and Figure 4). In contrast, GCDCA biliary excretion appears to be somewhat

reduced for CDCA concentrations $> 50 \mu\text{M}$, which is consistent with intracellular GCDCA concentrations that are leveling off after exposure to the higher CDCA concentrations.

As discussed above, intracellular exposure to BA was first studied only after a 4 h exposure to CDCA or DCA, revealing much higher levels of glycine as compared to taurine conjugates. However, these values may be largely dependent on the incubation time and initial accumulation and metabolism of these BA may differ from the 4 h values. In this context, intracellular accumulation and metabolism of CDCA was compared for incubation times of 30 min and 4 h in SCRH (Figure 8). These data revealed that, irrespective of the culture time (day-1 or day-3), total levels of CDCA + glycine and taurine conjugates were higher after 30 min of incubation as compared to 4 h incubation. Depletion of extracellular CDCA by the end of the 4h incubation may explain this finding. Indeed, extracellular CDCA concentrations in the culture medium after 4 h incubation were reduced to between 20 % (5 μM) and 50 % (100 μM) of the initial concentration (data not shown). The reduced intracellular accumulation after 4 h (as compared to 30 min) may also reflect adaptive response of the SCRH, e.g. upregulation of sinusoidal efflux mechanisms (Mrp3/4, Ost α/β) to protect hepatocytes in response to supraphysiological BA concentrations or decrease of uptake transporters by short-term feedback regulation (Alnouti 2009; Mühlfeld *et al.* 2012). Furthermore, bile acid metabolites may be formed that are not measured in our assay. Both sulfation and CYP-mediated metabolism have been reported to play a part in hepatic bile acid disposition (Alnouti 2009). Another remarkable finding was that the absolute and relative levels of the taurine conjugates of CDCA were much higher after 30 min incubations than after 4 h exposure. For instance, after exposure of day-1 SCRH to 25 μM CDCA, TCDCA accounted for more than 50% of total intracellular exposure (TCDCA>CDCA>GCDCA), whereas TCDCA levels were negligible following the 4 h exposure time. In line with the higher 4 h exposure values observed for GCDCA in day-1 versus day-3 cultures, 30 min

exposure values of particularly TCDCA were much higher in day-1 as compared to day-3 cultures. As the incubation medium is composed primarily of Williams' E Medium, containing 50 mg/L of glycine (but no taurine), it is possible that the intracellular source of taurine was depleted by 4 h, thus leading to increased formation of glycine conjugates. This has been suggested for cholic acid (Rembacz *et al.* 2010). However, this does not refute our explanation for the observed culture time-dependent toxicity, as the same composition of culture medium was used for day-1 and day-3 SCRH.

To our knowledge, this is the first time that a possible link is revealed between bile acid toxicity and intracellularly formed glycine conjugate exposure in sandwich-cultured rat hepatocytes. We have demonstrated that the intrinsic toxicity of glycine conjugates is higher than for the corresponding taurine conjugates of CDCA, DCA. Toxicity of several BA in SCRH is clearly culture time-dependent, with toxicity at day-3 being less pronounced than at day-1. The lower intracellular exposure to the glycine conjugates (formed from CDCA and DCA) appears to be a key factor in the culture-time dependent decline in sensitivity of SCRH towards bile acid-mediated toxicity. The decreased intracellular exposure to glycine conjugates in older cultures can first be attributed to a decreased formation of glycine conjugates. In addition, day-3 SCRH benefit from the relative advantage of canalicular efflux of these formed glycine conjugates, thus further reducing the effective intracellular exposure to these toxic bile acid species. Assuming that glycine conjugates play a similar role in bile acid-mediated toxicity in human liver, our work has diagnostic and therapeutic implications for conditions of intrahepatic cholestasis in man.

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Figure captions:

Figure 1: Culture-time dependent toxicity of bile acids: Day-1 (○) *versus* day-3 (Δ) toxicity profiles of CDCA, DCA, UDCA and GCDCA in two batches of SCRH (column A and B for batch-1 and 2 respectively), following 24 h incubation with these BA. The solid and the dotted lines represent the best fit of the sigmoid $E_{\max}$ model to the day-1 and day-3 toxicity in SCRH. Urea formation is expressed as the % control (hepatocytes treated with 0.3 % DMSO). The toxicity profiles of the four BA are significantly ($p < 0.05$) different between day-1 and day-3 SCRH.

Figure 2, Culture-time dependent accumulation of CDCA, DCA and their formed conjugates: **A-D:** Intracellular accumulation of: CDCA and its conjugates (A), CDCA (B), GCDCA (C), and TCDCA (D), in day-1 (○) or day-3 (□) SCRH after incubating with 5, 25, 50 and 100 μM of CDCA for 4 h, followed by 10 min incubation with standard buffer. **E-H:** Intracellular accumulation of: DCA and its conjugates (E), DCA (F), GDCA (G), and TDCA (H), in day-1 (○) or day-3 (□) SCRH after incubating with 5, 25, 50 and 100 μM of DCA for 4 h, followed by 10 min incubation with standard buffer. Data represent the mean ($\pm$ SEM) accumulation in three different batches of SCRH ($n=3$, for TDCA $n=2$), each measured in triplicate. * indicates that the accumulation profiles are statistically significantly different from each other ($p<0.05$).

Figure 3, Total formation and culture-medium concentration of GCDCA and TCDCA in day-1 and day-3 SCRH, cultured in 6-well plates: Total amounts (in hepatocytes + medium) of GCDCA (3A) and TCDCA (3B) and concentration of GCDCA (3C) and TCDCA (3D) in the culture-medium after incubation of day-1 or day-3 SCRH with 5, 25, 50 and 100 μM of CDCA

for 4 h. The experiments were conducted in 6-well cell culture plates. Incubations were followed by analysis of BA in the medium and in the SCRH. Data represents mean ($\pm$ SD) of triplicate measurements in a single batch of SCRH. Note that the x-axis represents intracellular CDCA concentrations after 4 h of incubation.

Figure 4, Disposition of CDCA, DCA and their conjugates in day-3 hepatocytes: A-C:

Amounts of CDCA and its conjugates (A), CDCA (B), GCDCA (C), that accumulated in cells only ($\square$), or in cells + bile networks (o) of day-3 SCRH after incubating with 5, 25, 50 and 100 μ M of CDCA for 4 h, followed by 10 min incubations with either $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer (cells only) or standard buffer (cells + bile canaliculi). **D-F**: Amounts of DCA and GDCA (D), DCA (E), or GDCA (F), that accumulated in cells only ($\square$), or in cells + bile networks (o) of day-3 SCRH after incubating with 5, 25, 50 and 100 μ M of DCA for 4 h, followed by 10 min incubations with either $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer (cells only) or standard buffer (cells + bile canaliculi). Data represent the mean ($\pm$ SD) accumulation of triplicate measurements in a selected batch of SCRH. * indicates that the accumulation profiles are statistically significantly different from each other ($p < 0.05$). Taurine conjugate levels were around detection limit (see Figure 2) and therefore not plotted.

Figure 5, Comparison between biliary excretion of CDCA and DCA in day-3 SCRH:

Amount of unconjugated CDCA ($\square$), or DCA ($\blacksquare$), directly excreted in bile as a function of CDCA and DCA intracellular concentrations, after exposure to extracellular concentrations of 5, 25, 50 and 100 μ M of CDCA or DCA. After 4 h of incubation with CDCA/DCA, cellular and canalicular bile acid concentrations were determined following incubations with standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer for 10 min, as described in the materials and methods section. Points represent mean ($\pm$ SD) of triplicate measurements in a selected batch of SCRH.

Figure 6, Disposition of GCDCA and GDCA in day-3 SCRH: Cellular accumulation (o) and biliary excretion (□) of GCDCA (A and B), GDCA (C and D) after 4 h incubation of day-3 SCRH with 5, 25, 50 and 100 μ M of CDCA or DCA, followed by incubations with standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer for 10 min. BEI was calculated as described in materials and methods section. Data represents mean values ($\pm$ SD) of triplicate measurements in one batch of SCRH.

Figure 7, Efflux of CDCA and its conjugates by day-4 SCRH: Sinusoidal [in standard buffer (o)] or combined 'sinusoidal + canalicular' [in $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer (□)] efflux of CDCA and GCDCA by day-4 SCRH seeded in 6-well culture plates. Following a 4 h exposure of day-4 SCRH to 5-100 μ M CDCA, hepatocytes were rinsed (to remove residual extracellular BA) and immediately incubated for 10 min with standard or $\text{Ca}^{++}/\text{Mg}^{++}$ -free buffer. Data represents mean ($\pm$ SD) amounts of CDCA or GCDCA measured in triplicate.

Figure 8, Time-dependent accumulation of CDCA and its conjugates: Compositional bar charts for absolute amounts (nmol/cm^2) of CDCA and its conjugates (GCDCA and TCDCA) in cells and bile networks of SCRH, after incubating day-1(A) or day-3 (B) SCRH with 5, 25, 50 and 100 μ M of CDCA for 30 min or 4 h, followed by incubations with standard buffer for 10 min. Data represents mean values of triplicate measurements obtained from a single batch (30 min) or three batches (4 h) of hepatocytes for each time point.