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Hepatobiliary Disposition in Primary Cultures of Dog and Monkey Hepatocytes

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Abstract: Hepatobiliary transporters are a major route for elimination of xenobiotics and endogenous products. In vitro hepatobiliary models have been reported for human and rat, but not for the other preclinical species used in safety evaluation. We have established methodologies for culturing dog and monkey hepatocytes with optimal bile canalicular formation and function, using a sandwich culture comprising rigid collagen substratum and gelled collagen overlay. Hepatic uptake utilizing sinusoidal transporters and biliary excretion through canalicular transporters were assessed using the bile salt taurocholate, salicylate (negative control), and the Bsep inhibitors cyclosporin A (CsA) and glyburide. There was significant taurocholate and salicylate canalicular efflux in dog and monkey hepatocytes, although the amount of salicylate transported was one thousandth that of taurocholate. Species differences were observed, as glyburide significantly inhibited taurocholate uptake in monkey (64% at 10 μM) but not dog hepatocytes, and inhibited taurocholate efflux in dog (100% at 10 μM) but not monkey hepatocytes. CsA did not inhibit bile salt uptake and significantly inhibited canalicular efflux in dog (at 0.1 μM) and monkey (at 1 and 10 μM) hepatocyte cultures. These results suggest that glyburide is a bile salt uptake inhibitor in monkey but not in dog hepatocytes and that CsA inhibits bile salt canalicular efflux but not basolateral uptake in these species. We have established dog and monkey hepatocytes in sandwich culture with intact bile canalicular formation and function. The differences observed in taurocholate transport between dog and monkey hepatocytes may be indicative of in vivo species differences.

Keywords: Hepatocytes; biliary excretion; hepatic uptake; drug transport model; Bsep

Introduction

Traditionally, biliary excretion has been studied in vivo using rat and dog or monkey mass balance studies, whereby bile and/or feces is collected and analyzed for the drug and the metabolites, or in situ using isolated perfused rat livers. More recently, in vitro models have been developed using primary rat and human hepatocytes.[1,2] Primary hepatocytes polarize and establish distinct canalicular and sinusoidal membranes in a collagen-sandwich configuration,[1] while remaining metabolically active. The extracellular matrix proteins help in the formation of intact bile canalicular networks between the hepatocytes and thereby maintain the liver-specific function of bile acid uptake. The expression and function of hepatic transporters such as Mrp2,[2] P-gp,[3]

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and Bsep[4] are maintained. Using this model, biliary excretion can be characterized at the cellular level and the hepatic disposition of drug candidates evaluated. Sandwiched primary hepatocytes allow for transport to be assessed across the sinusoidal membrane, take into consideration phase I and phase II metabolism, as the drug is being transported through the hepatocyte, and finally, quantify transport of the parent and metabolites (if any) across the canalicular membrane.

Drug disposition can be severely impacted and toxicities result from drug−transporter interactions in the liver both in the preclinical species and in the clinic. Adverse events are likely to occur only in cases where the liver is the primary organ involved in the uptake and elimination of a xenobiotic. A potent substrate of a hepatobiliary transporter would be expected to have high hepatic clearance, e.g., the statins.[5] A strong inhibitor of a major hepatic transporter could cause higher than expected levels of the drug (if it is also a substrate) or of coadministered medications, e.g., inhibition of OATP1B1 mediated cerivastatin uptake by cyclosporin A. This interaction contributes to myopathy and rhabdomyolysis.[6] An inhibitor may also affect the transport of endogenous substrates like bile acids, resulting in cholestasis due to bile salt buildup in the liver, e.g., the bosentan inhibition of the bile salt export pump, Bsep.[7] Bsep is a canalicular membrane transporter that is involved in the excretion of bile salts and some xenobiotics. Bsep is inhibited by drugs such as CsA,[8] sulindac,[9] rifampin, rifamycin, and glyburide.[10] Inhibition of this pump may contribute to cholestasis.[11] Since biliary excretion is a major elimination pathway for a range of xenobiotics and endogenous substances, it is important to assess the effect of a new chemical entity on hepatobiliary transporters.

The methodologies used to conduct canalicular efflux and hepatic uptake studies using sandwich-cultured rat and human hepatocytes utilize tight junction modulation[2,4,12] by incubating the cells with and without the divalent cations Ca^{2+} and Mg^{2+}. Bile canaliculi are flanked by tight junctions under physiological conditions. In the absence of Ca^{2+} the tight junctions lose their integrity, opening up the bile canaliculi and causing the contents to be released into the media.[2] The bile acid taurocholate is a model probe substrate to study transporter drug−drug interactions via Bsep.[2,4] Conversely, salicylate is used as a negative control as it is not excreted into the bile in rats and has been shown to not be transported into bile canaliculi in vitro.[13] While there are several reports on in vitro biliary transport in rat[2,13,14] and human,[4] there are none on dog or monkey sandwich-cultured hepatocytes. In preclinical drug development the nonrodent species used most often are dog or monkey. In order to better predict or understand an adverse hepatotoxic event in these species, or explain unexpected pharmacokinetic data, we have established methodologies to place dog and monkey hepatocytes in primary sandwich culture so as to establish a functional bile canalicular network. The present studies were conducted to demonstrate the utility of the collagen sandwich technique with dog and monkey hepatocytes. A second objective was to study uptake of taurocholate into the hepatocytes and evaluate the effect of Bsep inhibitors on taurocholate efflux in the sandwich-cultured dog and monkey primary hepatocytes.

Materials and Methods

Chemicals. Hepatocyte maintenance medium (HMM) was obtained from Cambrex (Baltimore, MD). ITS+ media supplement, CsA, and glyburide were obtained from Sigma (St. Louis). Penicillin/streptomycin, fetal bovine serum (FBS), and Hanks balanced salt solution (HBSS) were

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obtained from Invitrogen (Carlsbad, CA). Collagen (rat tail type I) was obtained from BD Biosciences (Bedford, MA). Biorad DC protein reagents were obtained from Biorad (Hercules, CA). Protein standards were obtained from Pierce (Rockford, IL). [^{3}H]Taurocholate (2−3.5 mCi/mmol) and [^{14}C]salicylate (55 mCi/mmol) were obtained from Perkin-Elmer Life Sciences (Boston, MA). Hepatocytes were purchased from CEDRA Corporation (Austin, TX) and CellzDirect (Pittsboro, NC). Six- and twelve-well plates coated with Biocoat type 1 collagen were purchased from Becton Dickenson (Bedford, MA).

Cell Culture. Freshly isolated dog and monkey hepatocytes (1.0−1.3 × 10^6/mL) were plated on Biocoat type I collagen or gelled collagen coated dishes under sterile conditions in HMM supplemented with 0.1 μM dexamethasone, 10 μg/mL insulin, 5 ng/mL selenium, 5.5 μg/mL transferrin, 0.5 mg/mL bovine serum albumin, 4.7 ng/mL linoleic acid, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% FBS. Hepatocyte viability was greater than 80% in all cases. Cells were placed into a humidified 5% CO_2/37 °C incubator for 2−3 h to enable attachment, after which medium containing dead/unattached cells was replaced and cells were put back into the incubator. The next day, cell culture medium was replaced to remove any cell debris or floating cells and the cells were allowed to equilibrate while collagen was prepared for the overlay. Collagen was diluted to 1.5 mg/mL with ice-cold HMM, and the solution was placed on ice, as described previously with modifications.[1,15] After the medium was completely removed from cells, collagen (50 μL/12 well, 100 μL/6 well) was added to wells and the plate gently rotated until surfaces were completely coated with collagen. Plates were placed into the incubator for ∼60 min to gel, after which warm supplemented HMM was added to each well. The extracellular matrix was tested for each species at pH 7.4 and 9.0; tissue culture medium was at pH 7.4 in all cases. Cells were examined daily under a microscope to verify formation of bile canaliculi, and cell culture medium was changed daily. The cells typically took 4 days to form functional bile canaliculi.

Transport of Taurocholate and Salicylate. Hepatocytes were sandwich cultured for 4 days, and formation of canaliculi was verified via microscope. Experiment was initiated by equilibrating cells with standard HBSS for 10 min at 37 °C in a humidified incubator. Cells were loaded with HBSS containing radiolabeled substrate (1 μM taurocholate or 3.6 μM salicylate) for 10 min at 37 °C, and cells were washed 3× with ice-cold standard HBSS to stop uptake of substrate. HBSS was added to the first set of replicate wells, and Ca/Mg^{2+}-free HBSS with 1 mM EDTA was added to a second set of replicate wells, to open up tight junctions and initiate efflux from bile canaliculi.[15] Aliquots of the HBSS and Ca/Mg^{2+}-free HBSS were taken at 2−90 min and counted for 5 min in liquid scintillant. The remaining medium was aspirated and 0.1 N NaOH/0.1% SDS added to each well to digest protein. Hepatocytes were removed and aliquots stored at −80 °C for protein analysis, following manufacturer's instructions (Biorad DC protein assay). Data were normalized using milligrams of protein per well. A set of blanks with only appropriate substratum and overlay (no cells) were also run to check for nonspecific binding.

Inhibition of Taurocholate Transport. Hepatocytes were sandwich cultured for 4 days, and formation of canaliculi was verified via microscope. Experiments to assess inhibition of taurocholate uptake and efflux were conducted consecutively in the same set of cells. Experiments were initiated by equilibrating cells with standard HBSS for 10 min at 37 °C in a humidified incubator. Cells were loaded with HBSS containing radiolabeled taurocholate (1 μM) and the potential inhibitors cyclosporin A or glyburide (0.1, 1, and 10 μM) for 10 min at 37 °C. Cells were washed 3× with ice-cold standard HBSS to stop uptake of substrate. Inhibitor was not added for the remainder of the study. Normal HBSS was added to the first set of replicate wells, and Ca/Mg^{2+}-free HBSS with 1 mM EDTA was added to a second set of replicate wells, to open up tight junctions and initiate efflux from bile canaliculi.[15] For efflux experiments, aliquots of normal HBSS and Ca/Mg^{2+}-free HBSS were taken at 10 min for monkey and 30 min for dog and counted as described above to measure radioactive content. These time points were based on optimal times of initial taurocholate efflux experiments. The remaining medium was aspirated and 0.1 N NaOH/0.1% SDS was added to each well. The samples were processed to lyse the hepatocytes and analyze for protein content. Aliquots of lysates were counted as described above and were used to generate uptake experiment data. A set of blanks with only appropriate substratum and overlay (no cells) were also run to check for nonspecific binding.

Statistical Analysis. A mixed model was used to analyze data. Measurements were taken at several time points, and the cells in each well were randomly selected from a larger population. Data are reported as pmol/mg and refer to amount of efflux of substrate per milligram of hepatocyte protein.

The model used was

$$\text{pmol/mg} = \mu + \text{CA}_i + d_{ij} + T_k + (\text{CA}\times T)_{ik} + e_{ijk}$$

where μ is the overall mean of the model, CA is the fixed effect of the calcium treatment ($i = 1, 2$), T is the fixed effect of time ($k = 1, 2, ..., 7$), CA×T is the interaction between the effects of calcium and time, d_{ij} is the random effect of the jth well in the ith calcium group ($j = 1, 2, 3$), and e_{ijk} is the random effect of the jth well in the ith calcium group at time k.

The data consist of unequally spaced time points, and the spatial power law was used to model the covariance structure of each well within a group. The normality of residuals was checked for each analysis. The Mixed procedure in SAS was used for all analyses.

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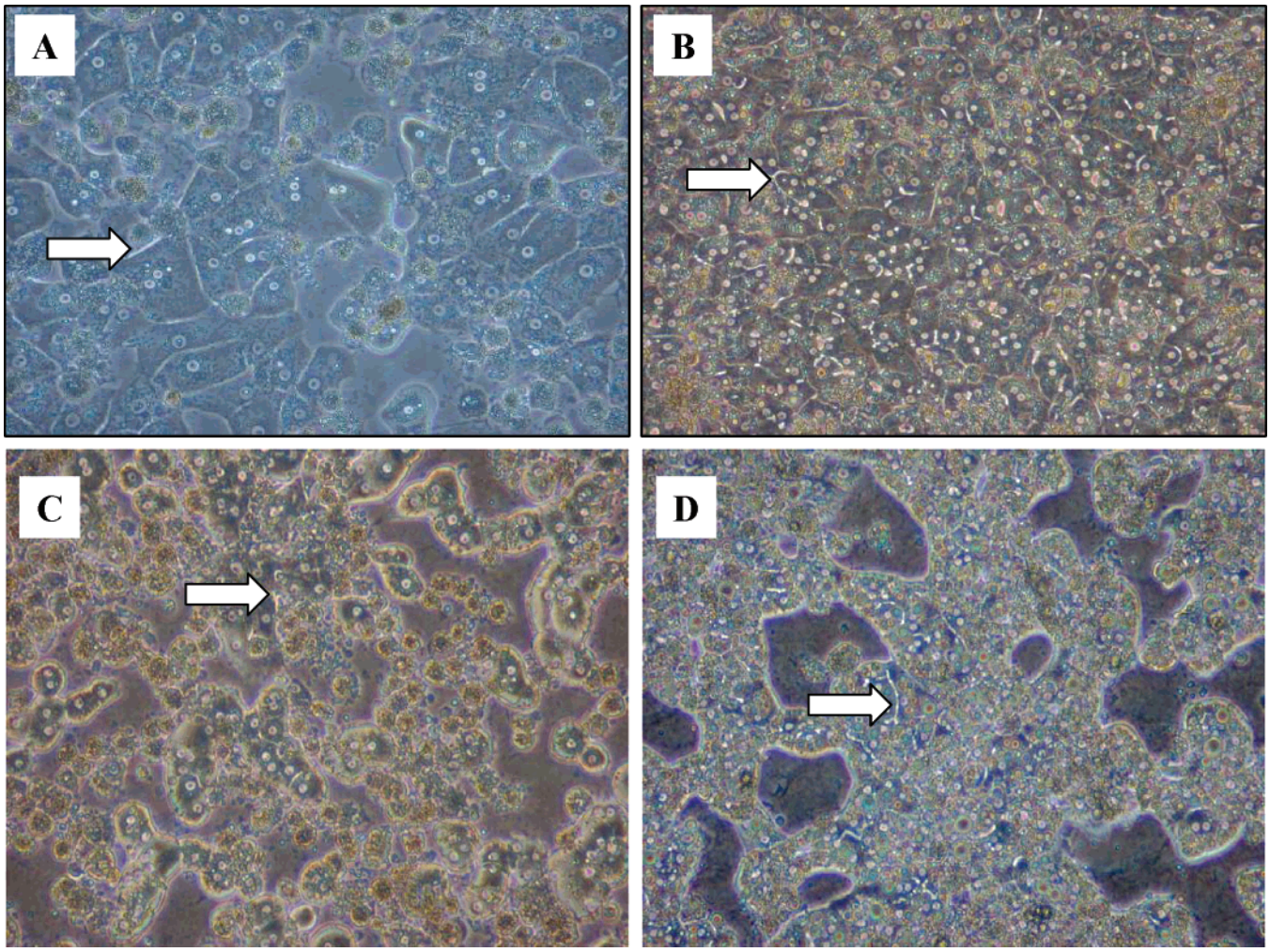

Figure 1. Dog and monkey hepatocytes cultured for 4 days in a sandwich configuration (10× magnification). Phase contrast micrographs of dog (A and C) and monkey (B and D) hepatocytes. Hepatocytes tend to form more flattened structures after 4 days in Biocoat type I BC/GC sandwich-culture configuration (A and B for dog and monkey hepatocytes, respectively), while in GC/GC sandwich culture (C and D for dog and monkey hepatocytes, respectively) chordlike formations are apparent in both species. Bile canaliculi are formed in both sets of matrices (see arrows, A−D).

To compare effects of inhibitors on uptake of taurocholate, 2 sample unpaired equal variance *t*-test was used.

Results

Morphology. Dog (Figure 1A,C) and monkey hepatocytes (Figure 1B,D) form visible canaliculi between adjoining hepatocytes after 4 days in sandwich culture. Dog and monkey hepatocytes plated on BC/GC (Figure 1A,B) formed a monolayer, while cells plated on GC/GC were in chordlike formations (Figure 1C,D). However, dog hepatocytes did not remain healthy on GC/GC and deteriorated rapidly, as is evidenced by the rounded cells in Figure 1C.

Optimization of ECM. Dog and monkey hepatocytes were tested on gelled collagen/gelled collagen (GC/GC) and Biocoat type I collagen/gelled collagen (BC/GC) sandwich configurations in 6-well and 12-well plates for formation of dilated bile canaliculi, which were then measured for functionality to transport taurocholate. The gelled collagen was tested at a physiological pH (7.4) and an alkaline pH of 9.0. The alkaline pH was produced by mistakenly overcorrecting the acidity of the collagen in order to form a gel and was subsequently found to result in a higher taurocholate efflux in dog hepatocytes compared to physiological pH (data not shown). The medium was at pH 7.4 in all cases. The canalicular efflux of taurocholate, i.e., the difference in efflux measured in the presence and absence of divalent cations, was used to assess the impact of ECM on the transport of taurocholate. For dog hepatocytes cultured on 6-well plates in gelled collagen, canalicular efflux could not be assessed on GC/GC as this matrix did not support healthy cell growth. However, on BC/GC, at 30 min, a canalicular efflux of 20.4 pmol/mg of protein was observed. For monkey hepatocytes, BC/GC sandwich conditions also resulted in optimal function, as shown in Table 1. Twelve-well plates worked successfully for both species in these configurations (data not shown). For monkey hepatocytes, the optimal canaliculi formation (both morphological and functional) was obtained when the pH of the extracellular matrix was adjusted to 7.4,

Table 1. Effect of Extracellular Matrix on Taurocholate Efflux in Primary Cultures of Dog and Monkey Hepatocytes[a]

	GC/GC		BC/GC	
	+ cations	− cations	+ cations	− cations
dog pH 7.4	no data[b]	no data	10.6 ± 5.6	19.8 ± 13.7
dog pH 9.0	no data	no data	9.9 ± 1.1	30.3 ± 3.6
monkey pH 7.4	50.3 ± 10.2	61.2 ± 4.9	32.3 ± 8.1	89.8 ± 5.0

[a] Data in pmol/mg of protein. Primary dog and monkey hepatocytes were sandwich cultured for 4 days using either gelled collagen as both substratum and overlay (GC/GC) or Biocoat type 1 collagen as the substratum and gelled collagen as the overlay (BC/GC). The gelled collagen was used at a pH of 7.4 or 9.0. Two different preparations of hepatocytes were used for these studies, which were performed in triplicate. The data shows taurocholate efflux at 10 min in monkey and at 30 min in dog hepatocytes. [b] No data: The hepatocytes were not healthy in this matrix, rounded up by day 2, and were dead by day 5.

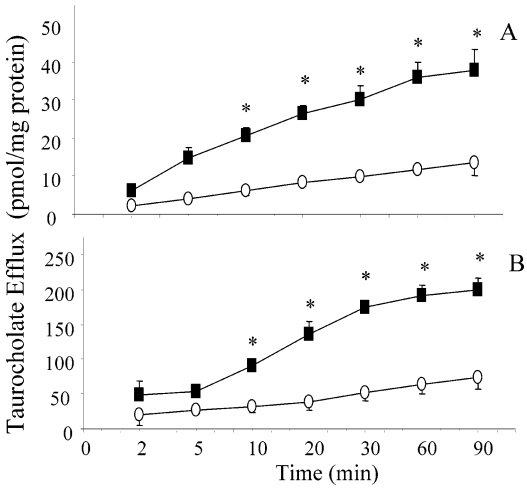

Figure 2. Taurocholate efflux in dog and monkey hepatocytes. Taurocholate efflux in dog (A) and monkey (B) hepatocytes sandwich cultured for 4 days, as per conditions outlined in Materials and Methods. Cells were incubated with 1 μM [^{3}H]taurocholate for 10 min and washed with ice-cold buffer to stop taurocholate uptake, cation containing (open circles) or cation-free (filled squares) buffer was added, and aliquots were removed at designated times. The difference in taurocholate efflux under the two conditions (bile canaliculi intact, open circles; bile canaliculi open, filled squares) indicates the amount of biliary excretion of taurocholate. Significance is indicated by the asterisk symbol (*), $p < 0.05$, $n =$ 2−3 wells for each time point. Each graph represents one of a total of three experiments conducted in triplicate wells.

and dog hepatocytes demonstrated greater taurocholate efflux when the pH was more alkaline, i.e., at 9.0 (Table 1). These conditions were used for all further studies.

Taurocholate Efflux. Dog and monkey hepatocytes (Figure 2A,B) exhibited efflux of taurocholate in sandwich culture. The difference in taurocholate efflux between hepatocytes incubated in the presence of the cations Ca^{2+} and Mg^{2+} (bile canaliculi intact) and the absence of the cations (bile canaliculi open) was significant at all time points

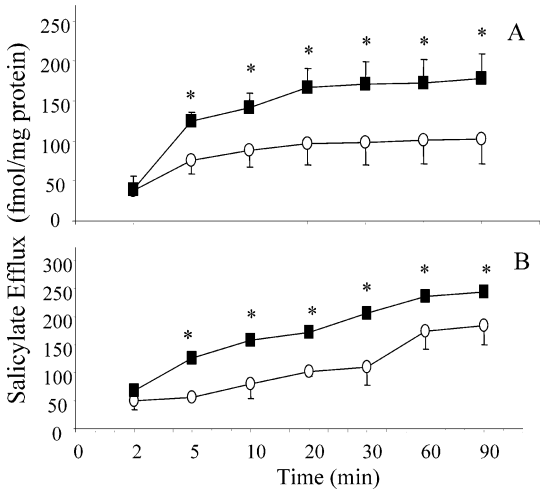

Figure 3. Salicylate efflux in dog and monkey hepatocytes. Salicylate efflux in dog (A) and monkey (B) hepatocytes sandwich cultured for 4 days, as per conditions outlined in Materials and Methods. Cells were incubated with 3.6 μM [^{3}H]salicylate for 10 min. After washing with ice-cold buffer to stop uptake of salicylate, divalent cation containing (open circles) or divalent cation-free (closed squares) buffer was added and aliquots were removed at designated times. The difference in salicylate transport between the two conditions (bile canaliculi intact, open circles; bile canaliculi open, filled squares) indicates the amount of biliary excretion of salicylate. Significance is indicated by the asterisk symbol (*), $p < 0.05$. Each graph represents one of two experiments conducted in triplicate wells.

from 10 min onward, indicating functional canaliculi in both species. Hepatocytes from both species exhibited time-dependent taurocholate efflux. Taurocholate efflux in dog hepatocytes (Figure 2A) increased over time, ranging on average from (2.2 ± 0.3)−(6.0 ± 0.6) pmol/mg of protein at 2 min up to (13.6 ± 3.6)−(37.9 ± 5.3) pmol/mg of protein at 90 min. Similarly, in monkey hepatocytes (Figure 2B), taurocholate efflux varied from an average of (20.6 ± 15.1)−(48.7 ± 20.1) pmol/mg of protein of taurocholate efflux at 2 min up to (73.0 ± 15.4)−(199.5 ± 17.1) pmol/mg of protein of taurocholate efflux at 90 min. On the basis of these data, the difference between the efflux in the presence and absence of cations at 30 min efflux time was used for dog inhibition studies and 10 min was used for the monkey inhibition studies.

Salicylate Efflux. Dog (Figure 3A) and monkey (Figure 3B) hepatocytes demonstrated small but statistically significant salicylate efflux from 5 min onward. The overall difference in efflux was in the fmol/mg of protein range, i.e., $^1/_{1000}$ that of taurocholate, as would be expected for a nonbiliary substrate. In dog hepatocytes the amount of salicylate efflux at 90 min was 103.0 ± 30.9 fmol/mg of protein in the presence of cations and 178.8 ± 30.4 fmol/mg of protein in the absence. Salicylate efflux in monkey hepatocytes at 90 min was 184.3 ± 34.4 fmol/mg of protein and 245.3 ± 1.7 fmol/mg of protein in the presence and absence of divalent cations, respectively.

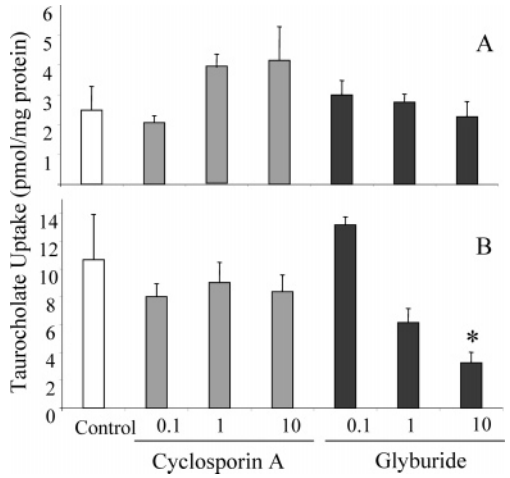

Figure 4. Effect of inhibitors on taurocholate uptake in dog and monkey hepatocytes. Taurocholate efflux in dog and monkey hepatocytes sandwich cultured for 4 days, as per conditions outlined in Materials and Methods. Cells were incubated with 1 μM [^{3}H]taurocholate and the Bsep inhibitors CsA or glyburide for 10 min. After washing with ice-cold HBSS to stop uptake of taurocholate, Ca^{2+}/Mg^{2+} containing buffer was added and aliquots were removed after 10 (monkey) or 30 min (dog). Cells were lysed with 0.1 N NaOH/0.1% SDS, and aliquots removed to assess uptake of taurocholate into the cell. Graph indicates amount of taurocholate present in hepatocyte lysates of dog (A) and monkey (B) hepatocytes. The data is the average of two separate experiments conducted in triplicate wells. Significance for the taurocholate efflux (%) data is indicated by the asterisk symbol (*), $p < 0.1$.

Effect of Cyclosporin A and Glyburide on Taurocholate Uptake. In dog hepatocytes no significant changes in taurocholate uptake were seen with CsA or glyburide treatment, although there was a dose-dependent increase in uptake with 1 and 10 μM CsA (58% and 67% increase respectively) (Figure 4A). In monkey hepatocytes, CsA treatment did not affect taurocholate transport, while with glyburide there was an increase (nonsignificant) in taurocholate uptake with 0.1 μM glyburide. Taurocholate uptake decreased with 1 μM glyburide (43%) and significantly with 10 μM glyburide (64%) (Figure 4B).

Effect of Cyclosporin A and Glyburide on Taurocholate Efflux. In dog hepatocytes (Figure 5A,B), CsA was the more potent inhibitor, with 100% inhibition at 0.1 μM. Treatment with 1 and 10 μM CsA resulted in 44% and 53% inhibition, respectively. Glyburide treatment resulted in dose-dependent inhibition of taurocholate efflux into the canaliculi: 16% inhibition with 0.1 μM, 36% inhibition with 1 μM, and complete inhibition of efflux with 10 μM (Figure 5B). CsA was the more potent of the two inhibitors in monkey hepatocytes (Figure 6), causing a moderate decrease (13%) in taurocholate efflux over control at the lowest dose (0.1 μM) and significant 86−89% inhibition at 1 and 10 μM. In monkey hepatocytes, glyburide (Figure 6A,B) increased taurocholate efflux at 0.1 μM, to 153% of control, but

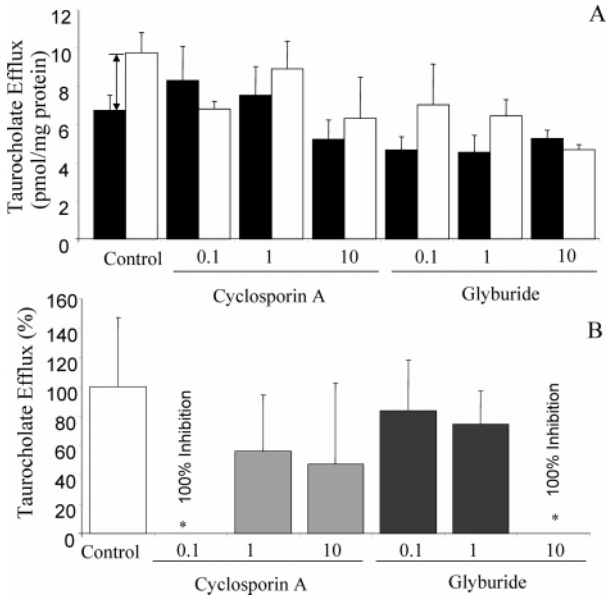

Figure 5. Inhibition of taurocholate efflux in dog hepatocytes. Taurocholate efflux in dog hepatocytes sandwich cultured for 4 days and incubated with 1 μM [^{3}H]taurocholate and Bsep inhibitors for 10 min, as per conditions outlined in Materials and Methods. After washing with ice-cold buffer to stop uptake of taurocholate, cation containing or cation-free buffer was added and aliquots were removed at 30 min of efflux. Panel A shows the amount of taurocholate efflux in pmol/mg of protein in the presence (black bars) and absence (open bars) of cations, with the two-headed arrow indicating the amount of canalicular efflux. In panel B this canalicular efflux is graphed as a percent of control and indicates amount of taurocholate that was transported into bile canaliculi. Groups with no visible bars indicate 100% inhibition. Control: open bar. CsA: shaded bars. Glyburide: filled bars. The data is the average of two separate experiments conducted in triplicate wells. Significance for graph B taurocholate efflux (%) is indicated by the asterisk symbol (*), $p \leq 0.05$.

inhibited efflux by 21% and 23% of control at doses of 1 and 10 μM.

Discussion

Transporters involved in uptake and efflux of xenobiotics and endogenous substances in the rat and human liver are well-characterized. The transporters believed to be relevant to drug transport in the human liver are OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, NTCP, MRP1, and MRP3 in the basolateral/sinusoidal membrane and BSEP, MRP2, MDR1, MDR3 and BCRP in the apical/canalicular membrane.[16−18] Drugs, their metabolites, and bile salts are transported into the hepatocytes via the sinusoidal membrane transporters. Some of these proteins can transport substrates in both

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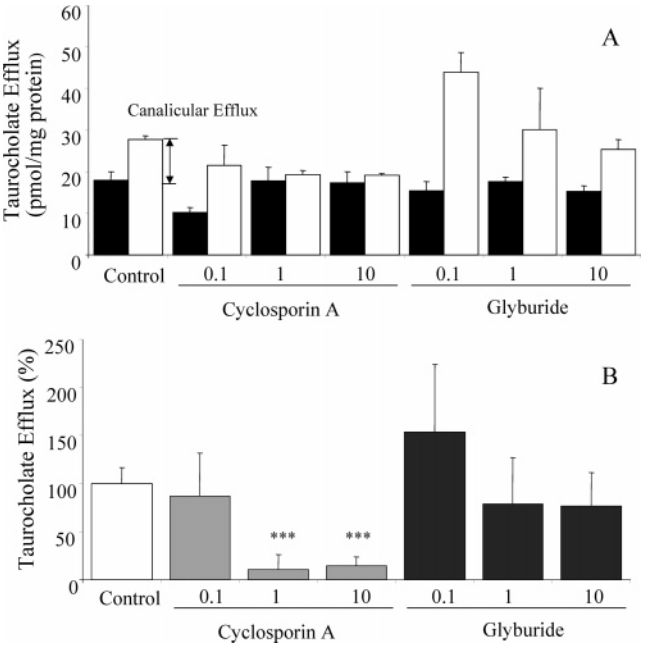

Figure 6. Inhibition of taurocholate efflux in monkey hepatocytes. Taurocholate efflux in monkey hepatocytes sandwich cultured for 4 days and incubated with 1 μM [^{3}H]taurocholate and Bsep inhibitors for 10 min, as per conditions outlined in Materials and Methods. After washing with ice-cold buffer to stop uptake of taurocholate, cation containing or cation-free buffer was added and aliquots were removed at 10 min of efflux. Panel A shows the amount of taurocholate efflux in pmol/mg of protein in the presence (black bars) and absence (open bars) of cations, with the two-headed arrow indicating the amount of canalicular efflux. In panel B this canalicular efflux is graphed as a percent of control and indicates amount of taurocholate that was transported into bile canaliculi. Groups with no visible bars indicate 100% inhibition. Control: open bar. CsA: shaded bars. Glyburide: filled bars. The data is the average of two separate experiments conducted in triplicate wells. Significance for graph B taurocholate efflux (%) is indicated by three asterisks (***), $p \leq 0.01$.

directions, depending upon the concentration gradient.[16] The transporters involved with efflux from liver back into the blood are MRP1 and MRP3. On the apical membrane, the hepatobiliary efflux transporters are responsible for the passage of therapeutics, bile acids, and metabolites from the liver into the gut, and can play an important role in the pharmacokinetics/toxicokinetics of drug candidates.

Some of the in vitro tools available to assess drug transport are immortalized/transformed cell lines, cells transfected with human or rodent transporters, and membrane vesicles isolated from cell lines.[19] The interaction between a compound and the transporter of interest can be assessed effectively and efficiently using these systems. Transporter panels can be generated by using stably or transiently transfected cell lines and the mechanism of interaction of compounds with specific transporters evaluated using the cells or vesicles derived from these. These systems provide for high-throughput studies and can be used for early screening of drug candidates. The major disadvantage of these systems is that the transfections result in variable levels of a transporter and quantitative results may be deceptive. Moreover, a majority of drug metabolizing enzymes are absent in these systems, so only parent drug transport can be evaluated. For highly metabolized drugs, erroneous/misleading predictions can be made. While the sandwich-culture primary hepatocyte model we have used is relatively labor intensive and has lower throughput, the data generated can lead to better predictions. This is because, in addition to the relevant transporters being present, the primary hepatocytes also contain drug metabolizing enzymes and form functional bile canaliculi, resulting in a more complete picture of hepatic transport.[1,15] Our experiments established models for studying transport into the hepatocytes, efflux into the bile canaliculi, and inhibition of bile salt transporter(s), likely Bsep in dog and monkey hepatocytes, so as to enable valid predictions and/or explain preclinical in vivo safety data.

We found that optimal cell morphology and bile canalicular formation required different extracellular matrix (ECM) for the two species, and these conditions were different from what we have reported earlier for rat[12] hepatocytes. The rat hepatocytes required a gelled collagen sandwich,[12] while human,[4] dog, and monkey hepatocytes established intact canaliculi with nongelled collagen as the substratum. To establish functional bile canaliculi in the two species, we had to use different pH values for the extracellular matrix, while medium and assay conditions remained identical.

In drug development it is helpful to know whether the compound being studied is a substrate for a hepatic uptake transporter, as the liver is the primary site of metabolism and detoxification of xenobiotics. In the case of some compound classes, e.g., the statins, this becomes more important as the site of pharmacological activity is also the liver. Our results suggest that in vivo data must be extrapolated to human with caution, as there are distinct species differences. While taurocholate uptake was not changed over basal in the presence of glyburide in dog hepatocytes, a significant dose-dependent decrease was observed in the monkey hepatocytes at 10 μM concentrations. This suggests that, in monkey hepatocytes, glyburide is an inhibitor of a basolateral membrane uptake transporter. It is also possible that glyburide activates an efflux transporter, causing taurocholate to be transported out of the cells. Since certain sinusoidal membrane transporters can function in both directions depending on concentration gradients (e.g., the OAT family) and there are several canalicular membrane efflux transporters (e.g., members of the MDR and MRP families), this efflux could be in either direction. Specific transporters involved in bile salt transport in monkey have

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not yet been identified. In human hepatocytes, taurocholate is transported into cells via NTCP and OAT.[20] A species difference was also observed in the taurocholate uptake in response to CsA treatment. Uptake was not changed in monkey hepatocytes, and a 58−67% increase in taurocholate uptake was observed in the dog hepatocytes. While we were unable to decipher these mechanisms of decreased uptake in the case of glyburide in monkey hepatocytes, or increased uptake with CsA in dog hepatocytes, these results suggest differences in hepatic transporter function between the species.

We also studied the effect of these two inhibitors on biliary excretion, by examining the elimination of taurocholate into the bile canaliculi, using the principle that the divalent cations Ca^{2+} and Mg^{2+} are required to maintain the integrity of the intercellular tight junctions. Removal of these cations disrupts the junctional complexes, resulting in leaky bile canaliculi, allowing compounds access across the tight junctions.[2,21] The activity of the in vitro hepatobiliary transport was assessed by quantitating the bile acid taurocholate collected in canaliculi, after disrupting the canalicular junctions, by incubating in a Ca^{2+}/Mg^{2+}-free buffer. The difference between bile acid released in the absence and in the presence of these cations represents the amount of taurocholate accumulated in canaliculi. Salicylate was selected as the negative control, as this compound is not excreted via bile in vivo in rat and does not get transported into the canaliculi in rat hepatocytes in sandwich culture.[2] Prior literature indicates that in the dog there is minor salicylate clearance (1−2%) via the bile (Rutyshauser et al., 1974). There is no data available for the monkey in vivo. We obtained similar results in earlier studies with the rat and with human hepatocytes,[4,12] i.e., there was no salicylate detected in canaliculi after a 1.5 h incubation. In dog and monkey hepatocytes, salicylate was transported into the canaliculi, although at levels of magnitude (fmol vs pmol) lower than for taurocholate. The dog and monkey hepatocytes appear to have a minor transport mechanism for salicylate that is absent in the rat and human, suggesting further interspecies differences in hepatic transporters. This indicates that extrapolation of hepatic transport between species should be avoided. This data also shows that our in vitro model appropriately reflects the in vivo situation in the case of dog. On the basis of our in vitro data, it is likely that there is minor biliary excretion of salicylate in the monkey in vivo, similar to the dog.

Transporter−drug interactions in the dog and monkey sandwich-cultured hepatocytes were evaluated using two known inhibitors of the Bsep, a transporter implicated in cholestasis. In humans, mutations in the Bsep gene result in progressive familial intrahepatic cholestasis type 2 disorder, characterized by high serum bile acid concentrations[22] and less than 1% of normal levels of bile salts in the bile.[23] In preclinical drug development, the safety profile of a drug on the liver is studied in vivo, most often using the rat, dog, and/or monkey as surrogates for human. Hepatic liability is assessed by examining the liver of the preclinical species for pathology, analyzing liver enzymes for changes, and collecting serum for liver enzyme tests. These models are not always predictive of the clinical outcome, and a contributing cause could be interspecies differences in hepatic transport systems. Human and rat uptake transporters are known to have vastly differing substrate specificities as well as amino acid composition and gene sequence and are not real orthologues.[16] For example, OAT transporter-mediated glucuronide transport is somewhat similar in rat and human livers while that of other anionic compounds is different.[24] In monkey hepatocytes, CsA inhibited taurocholate canalicular efflux in a dose-dependent manner. In dog hepatocytes interestingly, CsA caused complete inhibition of taurocholate efflux at 0.1 μM, while there was efflux at the two higher doses, albeit less than half of basal levels (Figure 5). Since there is increased taurocholate uptake into the hepatocytes via the basolateral membrane with the two higher concentrations of cyclosporin (Figure 4A), it is possible that the higher efflux into the bile canaliculi at these concentrations is related to increased taurocholate concentrations in the hepatocytes. Bsep can be inhibited from either the cytosolic side (e.g., CsA, rifampin, and rifamycin) or the bile side (e.g., estradiol-17β-glucuronide).[10] In the latter case, the drug has to be transported into the cells (via Oatp1 and/or Oatp2 and/or Oatp4) and then the drug and/or drug metabolites need to be transported out of the cell via the efflux pump Mrp2, thus entering the bile canaliculus and being available to inhibit Bsep. Since we have conducted our studies in a system that likely has these transporters as well as drug metabolizing enzymes functional, our observations are the result of the interplay between multiple transporters.

Glyburide treatment resulted in dose-dependent inhibition of taurocholate efflux in dog hepatocytes, with 100% inhibition obtained when 10 μM glyburide was administered. This is similar to what we have earlier observed in human

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and rat hepatocytes.[4] However, in monkey hepatocytes, glyburide increased, rather than inhibited, taurocholate efflux over control at the 0.1 μM dose. Taken together with the uptake data that indicate an inhibition of taurocholate uptake into the monkey hepatocytes in the presence of the higher doses of glyburide, it is clear that glyburide affects more than Bsep. There is likely inhibition of a sinusoidal transporter, causing less taurocholate to enter the monkey hepatocytes, at those doses. There also appears to be a stimulation of one or more of the canalicular transporters, resulting in the increased efflux observed with 0.1 μM glyburide (Figure 6B). Our data suggests that there is a species difference in the effects of glyburide and cyclosporin on both sinusoidal and canalicular transport. Since the glyburide metabolites formed in these four species are similar,[25] this is not due to a metabolite generated and most likely is due to different transporter systems in these species. Our results indicate that it is advisable to conduct in vitro studies in the species of interest to get a valid in vivo prediction.

In summary, this is the first report on establishment of conditions for formation of functional bile canaliculi in dog and monkey primary hepatocytes. In this configuration, hepatocytes establish a functional hepatobiliary system, transporting the bile salt taurocholate. Using this system, it is possible to assess inhibition and substrate potential of compounds and to measure the effect of a compound on inhibition of biliary efflux as an assessment of cholestatic potential within a species. This will help in predictions of potential biliary efflux and hepatotoxicity due to inhibition of bile salt efflux in the preclinical species and also in understanding the in vivo preclinical safety and pharmacokinetic data. Our data suggests that extrapolating results on hepatobiliary disposition or cholestatic potential due to Bsep inhibition obtained in one species to another is not recommended. Our data further strengthens the literature regarding cross-species differences in transporter activities and provides for an effective in vitro model to use for predictions and understanding of preclinical data.

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Abbreviations Used

GC, gelled collagen; BC, Biocoat type I collagen; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; HMM, hepatocyte maintenance medium; ECM, extra cellular matrix; CsA, cyclosporin A.

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