Cholyllysyl Fluroescein and Related Lysyl Fluorescein Conjugated Bile Acid Analogues

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There have been attempts to couple bile acids to fluorescein to permit their visualization during studies of physiology and pathophysiology. Although conjugation has been achieved by many, the product differed in many respects from the parent bile acid congener. We describe lysylfluorescein conjugated bile acid analogues (LFCBAA) synthesized in our laboratory as model divalent "unipolar" molecules. We have determined LFCBAA properties including their water:octanol partition coefficient, HPLC retention time and critical micellar concentration and compared them with their parent bile acid congeners. Cholyl lysylfluorescein (CLF) and lithocholyl lysylfluorescein (LLF) have properties similar to cholyglycine (CG) and glycolithocholate (GLC), respectively. In human and rat hepatocytes uptake of CLF follows Michaelis-Menten kinetics with K_m and V_max similar to CG. Biliary excretion rates of CLF and LLF closely resemble those of CG and GLC in both normal and mutant TR^- rats which lack the multiorganic anion transporter (MOAT), strongly supporting the notion that CLF and LLF are substrates for the canalicular bile salt transporter (cBST).

The close similarity of hepatocyte uptake and biliary secretion of these LFCBAA and their parent bile acid congeners makes them potentially useful probes for the intracellular visualization of bile salt movement and deposition in various models of bile formation and secretion.

INTRODUCTION

Bile salts are amphiphilic acidic steroids with detergent properties [1]. Their transcellular transport in the liver and subsequent canalicular secretion is associated with their unique physicochemical properties determined by the interplay of the effects of the number and orientations of the steroid ring hydroxyl groups [2], side chain length and charge [3], as well as any conjugate or charged species on the steroid ring [4]. These numerous structural variations contribute to the hydrophilic-hydrophobic balance of each bile salt molecule [5]. A good indication of this balance can be obtained from their relative retention times on high performance liquid chromatography (HPLC)b or water:octanol partition coefficient (WOPC), which represents the probable distribution of a bile salt molecule between membranes and the aqueous milieu [6]. Any fluorescent bile salt analogue synthesized should have this amphiphilic property and incorporate at least one negative

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b Abbreviations: LFCBAA, lysylfluorescein conjugated bile acid analogues; HPLC tr, high performance liquid chromatography retention time; CG, cholyglycine; CLF, cholyl lysylfluorescein; LLF, lithocholyl lysylfluorescein; CMC, critical micellar concentration, GLC, glycolithocholate; MOAT, multiorganic anion transporter; cBST, canalicular bile salt transporter; WOPC, water-octanol partition coefficient; FITC-CG, fluorescein isothiocyanate-glycocholate; Ntcp, Na^+ taurocholate-cotransporting polypeptide; Oatp1, organic anion transporting polypeptide; TC, taurocholate; CDCLF, chenodeoxycholyl lysylfluorescein; IPRL, isolated perfused rat liver; CVA, canalicular vacuole accumulation; NBD, 7β-nitroazoxoazadiol.
charge on the side chain to make it unipolar [7]. The hydrophilic-hydrophobic balance must be similar to that of natural bile salts because each molecule's physicochemical properties so precisely determines its biological properties. Previous attempts to produce fluorescein conjugates of bile salts, which preserve these essential bile salt characteristics, have been only partially successful. These include fluorescein isothiocyanate-glycocholate (FITC-CG) employed by Kitamura et al. [8] and the fluorescent bile acid analogues based on 7β-nitroazoxadiol (NBD) [9, 10]. The chemical structures of different fluorescent bile acid analogues are shown in Figure 1. In this communication, we report on the physical and biological properties of cholylylsylfluorescein (CLF) and related lysylfluorescein conjugated bile acid analogues (LFCBAA) synthesized in our laboratory by C.O. Mills [11]. We report for the first time the results of uptake experiments for CLF in human hepatocytes and review other recently published data pertaining to LFCBAA.

![Chemical structures of glycolithocholic acid and various fluorescent bile salts.](image)

Figure 1. Chemical structures of glycolithocholic acid and various fluorescent bile salts.
Table 1. Physicochemical properties of LFCBAA.

| Bile salt       | WOPC (molar ratio) | HPLC tr (min) | CMC (mM) |
|-----------------|--------------------|---------------|----------|
| $[^{14}\text{C}]$-CG | ND                 | 8.492         | 6.5      |
| CLF             | ND                 | 8.775         | 6.5      |
| $[^{14}\text{C}]$-GLC | 3.5±0.5*          | 26.617*       | 2.9*     |
| LLF             | 6.5±2.2*           | 27.950*       | 3.8*     |

Abbreviations: ND, not done; HPLC tr, high performance liquid chromatography retention time; $[^{14}\text{C}]$-CG, carbon -14 cholyglycine; CLF, cholyl lysylfluorescein; LLF, lithocholyl lysylfluorescein; CMC, critical micellar concentration; $[^{14}\text{C}]$-GLC, carbon-14 glycolithocholate; WOPC, water-octanol partition coefficient. * Ref. [12].

**PHYSICAL PROPERTIES OF LYSYL FLUORESCEIN CONJUGATED BILE ACID ANALOGUES**

Lysyl fluorescein bile acid analogues are freely soluble in water. They represent the analogues of glycine conjugates of bile salts with the least soluble, lithocholyl lysyl fluorescein (LLF) having a water:octanol partition coefficient, similar to glycolithocholate [12]. LFCBAA synthesized in our laboratory also share similar hydrophobic-hydrophilic balance with their respective natural congeners as judged by their relative HPLC retention times (Rt) (Table 1).

Another physical characteristic of bile salts is their tendency to form polymolecular aggregates or micelles in water when present at concentrations above their critical micellar concentration (CMC). Using the Du Nouy ring detachment method [13, 14] and the dye solubilization measurement as described by Roda et al. [2], we found the CMC of CLF and LLF to be similar to cholyglycine (CG) and glycolithocholate (GLC) respectively (Table 1).

The similarity between the physical characteristics of each lysylfluorescein bile salt analogue and its natural congener with respect to their partition coefficients and HPLC retention times suggests that attachment of the fluorophore within LFCBAA has a minor effect on the physico-chemical properties which characterize bile salts.

**BIOLOGICAL PROPERTIES OF LFCBAA**

Several studies have shown that bile salts such as taurocholate and glycocholate are transported within the liver by ATP dependent transporter systems at the sinusoidal (or basolateral) and canicular plasma membrane domains of hepatocytes [15-18].

Their basolateral uptake is mediated by Na$^+$ taurocholate-cotransporting polypeptide (Ntcp), which is a 51 kDa glycoprotein, selectively localized to the sinusoidal membrane [19, 20]. A second transporter system, multispecific and Na$^+$ independent is mediated by a 80 kDa protein called organic anion transporting polypeptide (Oatp1) [21, 22] and is responsible for the transport of unconjugated bile acids as well as many other, differently charged substrates including anionic steroid conjugates or even some amphipathic organic cations [21, 23, 24]. At the canicular membrane the liver secretes into bile a variety of organic anions besides hydrophilic bile salts. These include fluorescent dyes, glucuronide conjugates of bilirubin and sulfate and glucuronide conjugates of hydrophobic bile salts like lithocholate [25]. These anions are secreted via an ATP-dependent mechanism different from that which secretes hydrophilic bile salts. The mediator of this transport mechanisms is called multi-specific organic anion transporter (MOAT). A
hereditary defect in MOAT probably accounts for the conjugated hyperbilirubinemia present in the human Dubin-Johnson syndrome as well as in mutant TR\textsuperscript{−} rats, the equivalent animal model [26].

The following subsections will report for the first time uptake, transcellular transport and biliary secretion of LFCBAA in vitro and in vivo studies.

**Uptake of LFCBAA into human hepatocytes in vitro**

The relative uptake kinetics of CLF and CG were studied in human hepatocytes isolated from tissue leftover after trimming donor livers utilized in "reduced-size" transplantation in pediatric recipients [27]. Viability of hepatocyte preparations ranged between 95 and 99 percent by Trypan blue exclusion [28]. Hepatocytes were incubated with 5 to 200 \( \mu M \) solutions of either CLF or \([^{14}C]-CG \) at different time intervals (15 sec-30 min) at 37°C in Krebs-Hensleit buffer and uptake of CLF was measured by a Perkin-Elmer LS 5B fluorimeter and \([^{14}C]-CG \) by liquid scintillation counter.

Results were taken as the mean of triplicate readings using isolates from 4 donor organs. Velocity of initial uptake (\( V \)) was calculated from the slope of uptake versus time curve at each concentration (Figures 2A and B). The Michaelis Menten constant (\( K_m \)) and maximum velocity (\( V_{max} \)) were then calculated from intercepts of Lineweaver-Burke plots (Figure 2C). Uptake of both CLF and \([^{14}C]-CG \) was similar, \( (K_m = 37.8 \pm 13.1 \mu M \) and \( 30 \pm 5.8 \mu M \), respectively) as was the maximal transport velocity \( (V_{max} = 11.8 \pm 1.2 \) and \( 16.1 \pm 6.4 \mu M/10^6 \) cells/min, respectively). Co-incubation of CLF with \([^{14}C]-CG \) at 20 \( \mu M \) and 40 \( \mu M \) concentration, respectively, resulted in 87 percent inhibition of uptake (Figure 3). These uptake kinetics of CLF and CG suggest that they share a common sinusoidal transport mechanism and may be competing ligands for the Ntcp transporter. This demonstration of competitive inhibition of hepatocyte uptake may also explain the published observation of reduced canalicul vacuole accumulation of CLF when hepatocyte cuplets were incubated in the presence of both taurocholate (TC) with CLF [29, 30].

The external sodium chloride employed in the uptake kinetic study of CLF and CG was at physiological concentration (0.15 M) to reflect the sodium dependence of Ntcp [31], and it would be anticipated that like TC decrease in the extracellular sodium chloride concentration would impair the uptake of CLF and other LFCBAA for Ntcp on the basolateral plasma membrane of hepatocytes.

**Transcellular transport of LFCBAA**

The transcellular route by which bile salts proceed from portal blood to bile has been investigated in a series of collaborative experiments. Using CLF and chenodeoxycholyl lysylfluorescein (CDCLF), it was shown that the partitioning of these fluorescent bile acid analogues into intracellular compartments, and their canalicul secretion proceeds as simultaneous rather than sequential process [30]. In a further series of experiments employing LFCBAA, there is preliminary evidence that choleretic (CLF, CDCLF) and cholestatic (LLF) bile salts follow different transcellular pathways as CLF and CDCLF moved rapidly through the cytoplasm, unaffected by microtubular inhibition [30, 32]. On the other hand, plasma-bile transport of LLF and ursodeoxycholyl lysyl fluorescein was significantly delayed by colchicine treatment indicating that these bile acids were probably involved in a microtubule dependent vesicular system of transport within the hepatocyte [32]. The results indicate that LFCBAA can serve as probes in the study of hepatic transcellular transport and may shed light on unresolved mechanisms by which ligand trafficking is effected, involving cytosolic proteins, intracellular membranes and/or tubulovesicular movement.
Figure 2A. Mean uptake vs time plot for cholyl lysyl fluorescein (CLF). Normal human hepatocytes isolated from human liver (n = 4) by a modified collagenase perfusion technique was resuspended in Kreb’s-Henseleit buffer. Cells with a viability between 92-97 percent (by trypan blue exclusion) were used. Cells were incubated at 37°C with shaking at 60 oscillations/min for 15-90 sec, at concentrations of 5, 10, 20, 50, 100 or 200 μM. Uptake fluorescence of CLF was measured with a Perkin-Elmer LS 5B fluorimeter. Only plots up to a concentration of 20 μM are shown. Concentration of 5 μM (*), 10 μM (■) and 20 μM (▲). Each point represents the mean of 4 experiments in which each analysis was performed in triplicate.

Figure 2B. Mean uptake vs Time plot for [14C]-cholyl-glycine ([14C]-CG). Normal human liver leftover after “cutdowns” used for [14C]-CG, n = 3. The procedure for [14C]-CG was similar to that of CLF in figure 2A except that measurement of [14C]-CG radioactivity was by Beckman liquid scintillation counter. Concentration of 5 μM (*), 10 μM (■) and 20 μM (▲).
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![Graph](image)

**Figure 2C.** Lineweaver-Burke plot for CLF (●) and [14C]-CG (■): Velocity of initial uptake (V) for CLF and [14C]-CG was calculated from slope of uptake vs time (Figures 2A and 2B respectively) curves at each concentration. Michaelis-Menten $K_m$ and maximum velocity, $V_m$ were calculated from intercepts of Lineweaver-Burke plot ($1/V$ vs $1/S$) for CLF and [14C]-CG.

![Bar chart](image)

**Figure 3.** Inhibition study: Human liver leftover after "cutdowns" was processed as described under figure 2A. Hepatocytes with a viability between 92-97 percent (by trypan blue exclusion) were incubated with both CLF (closed symbols) and [14C]-CG (open symbols) at 20 and 40 μM concentrations respectively ($n=5$) gave 90 percent inhibition of CLF uptake at all the time points investigated between 15-90 sec.
Table 2. Biliary output of lipids and bile salt recovery.

| Bile salt   | Cholesterol/bile acid (0-20 percent) of dose | Phospholipid/bile acid | Cum. excr. in bile |
|-------------|---------------------------------------------|------------------------|-------------------|
| [14C]-CG    | 0.019*                                      | 0.30*                  | 93.1 ± 1.2†       |
| CLF         | 0.20*                                       | 0.27*                  | 94.4 ± 0.3†       |

Abbreviations: [14C]-CG, carbon-14 cholyglycine, CLF, choly lysylfluorescein, cum. excr., cumulative excretion. *Ref. [34]. †Ref. [11].

Biliary secretion of LFCBAA

Bile formation is a highly regulated process [15] involving the canalicular transporter proteins including MOAT and cBST, which represent the molecular basis of bile salt-dependent bile flow [33] and are responsible for biliary clearance of unipolar bile salts. Following bolus intravenous injection in the bile-fistula rat model and in the isolated perfused rat liver (IPRL) model, biliary clearance of CLF was similar to that of [14C]-CG with near complete excretion of the unchanged molecule occurring within 20 min [11]. During their investigation of bile salt interaction with lipids, Baxter et al. [34] found that at high infusion rates that exceeded the normal flux of bile salt through the liver in rats, CLF was transferred to bile less efficiently than glycocholate but that, within the bile, the ratio of cholesterol:bile acid or phospholipid:bile acid was not different for CLF and CG in the IPRL model (Table 2). In experiments with rat hepatocyte couplets, CLF canalicular vacuole accumulation was rapid with optimum canalicular vacuole accumulation observed within 1-2 min [35] and contrasted sharply with the finding of slow canalicular secretion of carboxy dichloro-fluorescein diacetate (CFDA), which is a MOAT substrate [36]. A further series of experiments were reported in control and mutant TR− rats that lack a functional multispecific organic anion transporter MOAT/mrp2 [37]. We found the secretion of CLF (65 percent in 30 min) [38] in mutant TR− rats to be similar to chenodeoxycholyl taurine (67 percent in 30 min) reported previously [4] suggesting that CLF utilizes the bile salt transporter which is responsible for ATP dependent secretion of bile salts [19, 39-41], thus further confirming its biological functionality as that of a divalent fluorescent unipolar bile salt analogue.

On the basis of these systematic studies LFCBAA appear to have a significant potential application to studies of hepatocyte function and dysfunction. Thus CLF retention is used as a marker of integrity of canaliculi between hepatocyte couplets [32]. Couplet canalicular accumulation of CLF may be a sensitive indicator of bile canalicular secretion and canalicular vacuole accumulation (CVA) within the sealed canalicular space, formed by hepatocyte couplets has been utilized as a sensitive indicator of canalicular dysfunction and cholestasis in studies with menadione and cyclosporin [42-44] as recently reviewed by Coleman et al. [45].

POSSIBLE APPLICATIONS OF LFCBAA

Plasma clearance studies

In preliminary studies involving six healthy human volunteers, the plasma clearance of CLF appeared to obey a three exponential model of elimination [46], similar to that of 14C-glycocholic acid as described previously by Engelking et al. [47].
Half lives for the first, second and third phase were 1.7 ± 0.9 min, 6.7 ± 1.6 min and 68 ± 17 min, respectively [46], as compared to biexponential values of 1.7 ± 0.1 and 7.0 ± 0.3 min for radiolabelled cholyglycine found by others [48]. The volume of distribution of CLF and plasma retention expressed as residual fluorescence after 60 min were similar to the data obtained by others for conventional or radiolabelled bile acids [49, 50].

This study showed that, in normal human subjects, CLF clearance is similar to the clearance of natural bile acids and may potentially offer a new, dynamic test of liver function.

In vivo visualization of the biliary tree.

We have conducted preliminary studies to evaluate application of CLF for intraoperative visualization of the biliary tree. In rabbits [51], we found that within 2 min of a single intravenous injection of CLF, the entire extrahepatic biliary tree and gallbladder of the rabbit became brightly fluorescent when viewed under Woods' light. Excellent visualization persisted for up to 45 min. Iatrogenic bile leaks could be seen and located with ease. Further developmental work is required to determine whether this technique could be applied for intraoperative visualization of the biliary tree in humans.

Limitations of LFCBAA

Intensity of fluorescence decreases for LFCBAA in acidic solution whereas the intensity increases in alkaline solution. Therefore, fluorescent bile salts are dissolved in buffered solutions (pH 7.4) [11] to overcome the pH-dependent variability in fluorescence measurement. Because of this pH dependence of the fluorescence quantum yield of LFCBAA no direct quantitative assumptions regarding concentrations of LFCBAA should be based on the intensity of in vivo fluorescence. Protein binding of LFCBAA, such as binding of CLF or CDCLF to albumin, also decreased fluorescence intensity [30]. Because of these constraints, in the CLF serum clearance study [46] CLF was extracted from serum by using methanol to precipitate serum proteins followed by measurement of fluorescence of CLF in the supernatant.

Despite the need for cautious interpretation of the experimental findings, the numerous studies carried out with CLF and other LFCBAA in a variety of experimental models including hepatocyte couplets, isolated perfused livers or mutant TR rats have provided information on their hepatocellular deposition, as well as the plasma and biliary kinetics of bile salt transport.

CONCLUSION

Further studies are required to define the role and limitations of LFCBAA as probes of bile salt transport in health and disease. On the basis of our early studies, we believe they have a potentially useful role as long as their properties and movement can be shown to be consistent with those of their naturally occurring bile salt congeners. The similar physical and biological properties of CLF to that of CG justify its utilization in bile acid transport studies [32, 34, 35, 42-44, 52].

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REFERENCES

1. Hofmann, A.F. and Small, D.M. Bile salts are amphipatic compounds with distinctive detergent properties. Ann. Rev. Med. 18:333-376, 1967.

2. Roda, A., Hofmann, A.F., and Mysels, K.J. The influence of bile salt structure on self-association in aqueous solutions. J. Biol. Chem. 258:6362-6370, 1983.

3. Hardison, W.G.M., Heasley, V.L., and Shellhammer, D.F. Specificity of the hepatocyte Na⁺ dependent taurocholate transport: influence of side chain length and charge. Hepatology 13:68-72, 1991.

4. Kuipers, F., Enserink, M., Havinga, R., van der Steen, A.B., Hardenk, M.J., Fevery J., and Vonk, R.J. Separate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. J. Clin. Invest. 81:1593-1599, 1988.

5. Heuman, D.M. Quantitative estimation of the hydrophobic-hydrophilic balance of mixed bile salt solutions. J. Lipid Res. 30:719-730, 1989.

6. Roda, A., Minutello, A., Angelotti, M.A., and Fini, A. Bile acid structure-activity relationship: evaluation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC. J. Lipid Res. 31:1433-1443, 1990.

7. Oude Elferink, R.P., Ottenhoff, R., Radominska, A., Hofmann, A.F., Kuipers, F., and Jansen, P.L. Inhibition of glutathione-conjugate secretion from isolated hepatocytes by dipolar bile acids and other organic anions. Biochem. J. 274:281-286, 1991.

8. Kitamura, T., Gatamaitan, Z., and Arias, I.M. Serial quantitative image analysis and confocal microscopy of hepatic uptake, intracellular distribution and biliary secretion of a fluorescent bile acid analog in rat hepatocyte doublets. Hepatology 12:1358-1364, 1990.

9. Weiman, S.A., Graf, J., Veith, C., and Boyer, J.L. Electroneutral uptake and electrogenic secretion of a fluorescent bile salt by rat hepatocyte couplets. Am. J. Physiol. 264:G220-230, 1993.

10. Maglova, L.M., Jackson, A.M., Meng, X.J., Carruth, M.W., Schteingart, C.D., Ton-Nu, H.T., Hofmann, A.F., and Weiman, S.A. Transport characteristics of three fluorescent conjugated bile acid analogs in isolated rat hepatocytes and couplets. Hepatology 22:637-647, 1995.

11. Mills, C.O., Rahman, K., Coleman, R., and Elias, E. Cholyl-lysylfluorescein: synthesis, biliary excretion in vivo and during single-pass perfusion of isolated perfused rat liver. Biochim. Biophys. Acta 1115:151-156, 1991.

12. Mills, C.O., Milkieicz, P., Molloy, D.P., Baxter, D.J., and Elias, E. Synthesis, physical and biological properties of lithocholyllysylfluorescein: a fluorescent bile salt analogue with cholestatic properties. Biochim. Biophys. Acta 1336:485-496, 1997.

13. Du Nour, P.L. Surface equilibria of biological organic colloids. Am. Chem. Soc. 1926, pp. 35-70.

14. Padday, J.F. The measurement of surface tension. In: Matejevic, E. and Eirich, F., eds. Surface and Colloid Science. Vol. 1. New York; 1969, pp. 101-149.

15. Boyer, J.L., Graf, J., and Meier, P.J. Hepatic transport systems regulating pH, cell volume, and bile secretion. Ann. Rev. Physiol. 54:415-438, 1992.

16. Gatmaiten, Z.C., Leveille-Webster, C.R., and Arias, I.M. The biology of the bile canaliculus. In: Arias, I.M. and Boyer, J.L., eds. The Liver, Biology and Pathobiology. New York: Raven; 1994, pp. 665-676.

17. Meier, P.J. Canicular membrane transport processes. In: Hepatic Transport and Bile Secretion: Physiology. Tavoloni, N. and Berk, P.D., eds. New York: Raven; 1993, pp. 587-596.

18. Boelsterli, U.A., Zimmerli, B., and Meier, P.J. Identification and characterization of a basolateral dicarboxylate/cholate antiporport in rat hepatocytes. Am. J. Physiol. 268:G797-805, 1995.

19. Stieger, B., O’Neill, B., and Meier, P.J. ATP-dependent bile-salt transport in canalicular rat liver plasma-membrane vesicles. Biochem. J. 284:67-74, 1992.

20. Ananthanarayanan, M., Ng, C., Boyer, J.L., and Suchy, F.J. Characterization of cloned rat liver Na⁺-bile acid cotransporter using peptide and fusion protein antibodies. Am. J. Physiol. 267:G637-643, 1994.

21. Jacquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A.W., and Meier, P.J. Expression cloning of a rat liver Na⁺-independent organic anion transporter. Proc. Natl. Acad. Sci. USA 91:133-137, 1994.

22. Shi, X., Bai, S., Ford, A.C., Burk, R.D., Jacquemin, E., Hagenbuch, B., Meier, P.J., and Wolkoff, A.W. Stable inducible expression of a functional rat liver organic anion transport protein in HeLa cells. J. Biol. Chem. 270:25591-25595, 1995.

23. Kullak-Ublick, G.A., Hagenbuch, B., Stieger, B., Wolkoff, A.W., and Meier, P.J. Functional characterization of the basolateral rat liver organic anion transporting polypeptide. Hepatology 20:411-416, 1994.
24. Bossuyt, X., Muller, M., Hagenbuch, B., and Meier, P.J. Polyspecific drug and steroid clearance by an organic anion transporter of mammalian liver. J. Pharmacol. Exp. Ther. 276:891-896, 1996.
25. Zimniak, P. and Awasthi, Y.C. ATP-dependent transport systems for organic anions. Hepatology 17:330-339, 1993.
26. Zimniak, P. Dubin-Johnson and Rotor syndromes: Molecular basis and pathogenesis. Sem. Liv. Dis. 13:248-260, 1993.
27. Broelsch, C.E., Emond, J.C., Whittington, P.F., Thistlewaite, J.R., Baker, A.L., and Lichtor, J.L. Application of reduced size liver transplants and split grafts, auxiliary orthotopic grafts and living related transplants. Ann. Surg. 212:368-372, 1990.
28. Berry, M.N. and Friend, D.S. High yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell. Biol. 43:506-520, 1969.
29. Wilton, J.C., Chipman, J.K., Lawson, C.J., Strain, A.J., and Coleman, R. Periportal- and perivenous-enriched hepatocyte couplets: differences in canalicular function and in response to oxidative stress. Biochem. J. 292:773-779, 1993.
30. El-Saidy, A.Z., Mills, C.O., Elias, E., and Crawford, J.M. Lack of evidence for vesicle trafficking of fluorescent bile salts in rat hepatocyte couplets. Am. J. Physiol. 272(Gastrointest. Liver Physiol. 35):G298-G309, 1997.
31. Hegenbuch, B., Steiger, B., Fuguet, M., Lubbert, H., and Meier, P.J. Functional expression cloning and characterization of the hepatocyte Na/bile co-transport system. Proc. Natl. Acad. Sci. USA 88:10629-10633, 1991.
32. Wilton, J.C., Matthews, G.M., Burgoyne, R.D., Mills, C.O., Chipman, J.K., and Coleman, R. Fluorescent choleric and cholestatic bile salts take different paths across the hepatocyte: transcytosis of glycolithocholate leads to an extensive redistribution of annexin II. J. Cell. Biol. 127:401-410, 1994.
33. Wolters, H., Kuipers, F., Sloor, M.J.H., and Vonk, R.J. ATP dependent taurocholate transport in human liver plasma membranes. J. Clin. Invest. 90:2321-2326, 1992.
34. Baxter, D.J., Rahman, K., Bushell, A.J., Mills, C.O., Elias, E., and Billington, D. Biliary lipid output by isolated perfused rat livers in response to cholylystfluorescein. Biochem. Biophys. Acta 1256:374-380, 1995.
35. Wilton, J.C., Coleman, R., Lankester, D.J., and Chipman, J.K. Stability and optimization of canalicular function in hepatocyte couplets. Cell. Biochem. Funct. 11:179-185, 1993.
36. Kitamura, T., Jansen, P., Hardenbrook, C., Kamimoto, Y., Gatmaitan, Z., and Arias, I.M. Defective ATP-dependent bile canalicular transport of organic anions in mutant (TR-) rats with conjugated hyperbilirubinemia. Proc. Natl. Acad. Sci. USA 87:3557-3561, 1990.
37. Paulusma, C.C., Bosma, P.J., Zaman, G.J., Bakker, C.T., Otter, M., Scheffer, G.L., Scheper, R.J., Borst, P., and Oude Elferink, R.P. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 271:1126-1128, 1996.
38. Mills, C.O., Milkiewicz, P., Muller, M., Havinga, R., Kuipers, F., Jansen, P.L.M. and Elias, E. Canalicular secretion of lysylfluorescein bile acid analogues in normal and mutant TR- rats: sulfation of lithocholyl-lysyl fluorescein makes it a MOAT substrate. J. Hepatol. (Suppl 1)26:161A, 1997.
39. Adachi, Y., Kobayashi, H., Kurumi, Y., Shouji, M., Kitano, M., and Yamamoto, T. ATP-dependent taurocholate transport by rat bile canalicular membrane vesicles. Hepatology 14:655-659, 1991.
40. Muller, M., Ishikawa, T., Berger, U., Klunemann, C., Lucka, L., Schreyer, A., Kannicht, C., Reutter, W., Kurz, G., and Keppler, D. ATP-dependent transport of taurocholate across the hepatocyte canalicular membrane mediated by a 110-kDa glycoprotein binding ATP and bile salt. J. Biol. Chem. 266:18920-18926, 1991.
41. Nishida, T., Hardenbrook, C., Gatmaitan, Z., and Arias, I.M. ATP-dependent organic anion transport system in normal and TR- rat liver canalicular membranes. Am. J. Physiol. 262:G629-635, 1992.
42. Stone, V., Johnson, G.D., Wilton, J.C., Coleman, R., and Chipman, J.K. Effect of oxidative stress and disruption of Ca2+ homeostasis on hepatocyte canalicular function in vitro. Biochem. Pharmacol. 47:625-632, 1994.
43. Stone, V., Chipman, K.J., and Coleman, R. Disruption of hepatocyte canalicular function by depletion of cytosolic glutathione. Hum. Exp. Toxicol. 13:175, 1994.
44. Roman, I.D. and Coleman, R. Disruption of canalicular function in isolated rat hepatocyte couplets caused by cyclosporin A. Biochem. Pharmacol. 48:2181-2188, 1994.
45. Coleman, R., Wilton, J.C., Stone, V., and Chipman, J.K. Hepatobiliary function and toxicity in vitro using isolated hepatocyte couplets. Gen. Pharmacol. 26:1445-1453, 1995.
46. Milkiewicz, P., Baiocchi, L., Mills, C.O., Ahmed, M., Khalaf, H., Keogh, A., Baker, J., and Elias, E. Plasma clearance of choly lysyl fluorescein: a pilot study in humans. J. Hepatol. 27:1106-1109, 1997.

47. Engelking, L.R., Barnes, S., Dasher, C.A., Naftel, D.C., and Hirschowitz, B.I. Radiolabelled bile acid clearance in control subjects and patients with liver disease. Clin. Sci. 57:499-508, 1979

48. Cowen, A.E., Korman, M.G., Hofmann, A.F., and Thomas, P.J. Plasma disappearance of radioactivity after intravenous injection of labeled bile acids in man. Gastroenterology 45:1567-1573, 1975.

49. Horak, W., Waldram, R., Murray-Lyon, I.M., Schuster, E., and Williams, R. Kinetics of $^{14}$C cholic acid in fulminant hepatic failure: a prognostic test. Gastroenterology 71:809-813, 1976.

50. Thjodleifsson, B., Barnes, S., Chitrakukroh, A., Billing, B.H., and Sherlock, S. Assessment of the plasma disappearance of choly$^{14}$C-glycine as a test of hepatocellular disease. Gut 18:697-702, 1977.

51. Oddi, A., Mills, C.O., Custureri, F., Di Nicola, V., Elias, E., and Di Matteo, G. Intraoperative biliary tree imaging with choly-lysyl-fluorescein: an experimental study in the rabbit. Surg. Laparos. Endos. 6:198-200, 1996.

52. Fentem, J.H., Foster, B., Mills, C.O., Coleman, R., and Chipman, K.J. Biliary excretion of fluorescent cholephiles in hepatocyte couplets: an in vitro model for hepatobiliary and hepatotoxicity studies. 4:452-457, 1990.