Synthesis of phospholipid-conjugated bile salts and interaction of bile salt-coated liposomes with cultured hepatocytes[1]

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Abstract **To examine the possibility of targeting liposomes to hepatocytes via bile salts, the bile salt lithocholyltaurine was covalently linked to a phospholipid. The isomeric compounds disodium 3α-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy)-5β-cholan-24-oyl-2′-aminoethansulfonate and disodium 3β-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy-5β-cholan-24-oyl-2′-aminoethansulfonate (DSPE-3β-LCT) were synthesized and incorporated into liposomal membranes. Confocal laser scanning microscopy studies showed that bile salt-bearing liposomes (BSLs) attach to the surface of rat hepatocytes in culture. Studies with radioactively labeled liposomes revealed that the bile salt linked via the 3β-conformation resulted in a higher attachment efficiency than that with the 3α-derivative. In the presence of BSLs corresponding to 2 mM liposomal phosphatidylcholine, uptake of 50 μM cholyltaurine (CT) into hepatocytes was reduced by ∼40% by the 3β-derivative and by ∼17% by the 3α-derivative. When added simultaneously with the liposomes, CT up to 75 μM inhibited the binding of DSPE-3β-LCT-bearing liposomes. By contrast, increasing concentrations reversed this inhibition and resulted in an increased bile salt-mediated binding. The same was true when CT was added 10 min before the liposomes were added. The attachment of BSLs to the surface of hepatocytes opens up promising possibilities for hepatocyte-specific drug delivery. More generally, not only substrates for cellular endocytosing receptors but also substrates for cellular carrier proteins should be suitable ligands for the cell-specific targeting of nanoscale particles such as liposomes.**—Pütz, G., W. Schmider, R. Nitschke, G. Kurz, and H. E. Blum. **Synthesis of phospholipid-conjugated bile salts and interaction of bile salt-coated liposomes with cultured hepatocytes. *J. Lipid Res.* 2005.** 46: **2325–2338.**

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Hepatocyte pathology is central to many severe liver diseases. For optimal treatment and minimal adverse effects, drug concentrations in the target cell should be high while the systemic exposure to the drug should be as low as possible. For hepatocyte-specific drug delivery, hepatotropic liposomes represent an attractive strategy. Such hepatotropic liposomes need a signal on their surface targeting them selectively and specifically to hepatocytes. Hepatocyte-specific targeting of liposomes has been achieved by covalently linking the ligand of a hepatocyte-specific receptor to a lipid moiety. The most prominent targeted receptor is the hepatic asialoglycoprotein receptor, which has been targeted by various galactosyllipids (1, 2) or by asialofetuin, a glycoprotein (3). Glycyrrhizin (4) and a soybean-derived sterylglucoside mixture (5) have been used to target hepatocytes via receptors that have not yet been identified at the molecular level. To date, all of these receptors lead to the endocytosis of bound liposomes.

After targeting the liposomes to endocytosing receptors,

Abbreviations: BSL, bile salt-bearing liposome; CH, cholesterol; COL, control liposome; CT, cholyltaurine; DCCI, *N,N′*-dicyclohexylcarbodiimide; DPPC, 1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPS, 1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoserine; DSPE, 1,2-*O*-distearoyl-*sn*-glycero-3-phosphoethanolamine; DSPE-3-LCT, disodium 3-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy)-5β-cholan-24-oyl-2′-aminoethansulfonate; DSPE-3α-LCT, disodium 3α-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy)-5β-cholan-24-oyl-2′-aminoethansulfonate; DSPE-3β-LCT, disodium 3β-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy-5β-cholan-24-oyl-2′-aminoethansulfonate; DSPG, 1,2-*O*-distearoyl-*sn*-glycero-3-phospho-rac-(1-glycerol); LSM, laser scanning microscopy; NBD, 4-nitrobenzo-2-oxa-1, 3-diazol; MP, melting point; MS, mass spectrum; Ntcp, Na^+/cholyltaurine-cotransporting polypeptide; PC, phosphatidylcholine; Ph_2-DiOC18, 3,3′-dioctadecyl-5,5′-diphenyloxacarbocyanine chloride; PSL, phosphatidylserine-bearing liposome; RT, room temperature; SS, solvent system.

[1] This paper is dedicated to the memory of Prof. Dr. G. Kurz, deceased May 29, 2002.

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the physiological path of internalization usually ends up in the lysosomes, where most of the liposomal content is degraded. To avoid this problem, the ligand used for homing of the liposomes should target a cellular receptor that mediates only the binding of the liposomes to the hepatocytes but not endocytotic internalization. Binding of liposomes to the cells without endocytosis may allow the delivery of the liposomal content into the cytoplasm by diffusion over adjacent membranes (6) or by direct fusion of the liposomal membrane with the sinusoidal membrane of the hepatocytes. Cellular carrier proteins are one group of proteins that may act as receptors for modified liposomes without mediating the endocytosis of the bound liposomes.

A main problem of liposomes with a homing moiety is the immunogenicity of nonphysiological ligands. Any immune reaction to surface-modified liposomes does not allow their repeated administration. Linking a physiological substrate of a cellular carrier protein to the liposomal surface should avoid an immune response while introducing an appropriate homing device. To evaluate the concept of using a physiological ligand of hepatocyte-specific carrier proteins to target liposomes to hepatocytes, lithocholic acid was used targeting the hepatic bile salt transport system.

Bile salts are removed from the bloodstream specifically by the hepatocytes, which use certain well-defined transport systems (7–9). The bile salt uptake into hepatocytes is very effective and characterized by half-saturation transport constants in the micromolar range (10, 11). By covalently coupling a drug to a bile salt, the bile salt-transporting systems have been already used for hepatic drug delivery. Bile salt-drug conjugates are readily taken up by the hepatocytes, but uptake is followed by a fast excretion of the drug conjugate into the bile (12, 13).

Bile salt carrier systems have not been addressed as targets for liposome-based drug delivery. The half-saturation transport constant of a carrier protein does not equal its affinity for the substrate, and even though bile salt transport is very effective, it had to be proven whether bile salts linked to the liposomal membrane may allow an effective binding and anchoring of a huge particle like a bile salt-bearing liposome (BSL) to the hepatocytes. In this study, we addressed the interaction of liposomes bearing a covalently linked bile salt-lipid conjugate with cultured hepatocytes and the question of whether a substrate of a cellular carrier may be useful as a ligand for particle-based drug delivery.

MATERIALS AND METHODS

Animals

Male Wistar S 300 rats (Interfauna, Tuttlingen, Germany) weighing 200–300 g were used in all experiments. They had free access to tap water and the standard rat diet Altromin 300® (Altromin GmbH, Lage, Germany) and were housed under a constant temperature and an alternating 12/12 h light/dark cycle.

Materials

Phospholipids were obtained from Sygena, Ltd. (Liestal, Switzerland). Cholyltaurine (CT), *N,N′*-dicyclohexylcarbodiimide (DCCI), lithocholic acid, cholesterol (CH), Williams E medium, and supplements were from Sigma Chemie GmbH (Deisenhofen, Germany). *N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (triethylammonium salt), 3,3′-dioctadecyl-5,5′-diphenyloxacarbocyanine chloride (Ph_2-DiOC18), and calcein-blue-AM were from Molecular Probes (Leiden, The Netherlands). Fetal calf serum was from PAA Laboratories GmbH (Linz, Austria). Collagenase Worthington CLS II (specific activity of 220–230 U/mg protein) was obtained from Biochrom KG (Berlin, Germany). [1,2-^{3}H]cholesterylhexadecylether (1.9 TBq/mmol) was from NEN Life Science Products GmbH (Frankfurt, Germany), and [^{3}H-(G)]CT was from Biotrend Chemikalien GmbH (Cologne, Germany). All other chemicals were of the highest quality available commercially.

Determination of protein concentration and radioactivity

Protein content of cultured hepatocytes was determined by a modified Lowry procedure (14) using the DC protein determination assay (Bio-Rad Life Science Group, München, Germany) and BSA, dissolved in the solution used for dissolving the cells, as a standard. Radioactivity was determined with a Wallac 1411 (Berthold, Wildbad, Germany) liquid scintillation counter using Ultima Gold® (Canberra Packard GmbH, Frankfurt, Germany) as the liquid scintillation cocktail.

Preparation and characterization of liposomes

The liposomes were prepared by the extrusion method (15, 16) in the medium used for isolation of hepatocytes, omitting $CaCl_2$, D-glucose, and saturation with carbogen. The extrusion device (LiposoFast™; Milsch-Equipment, Laudenbach, Germany) used was equipped with polycarbonate filters (Milsch-Equipment); the samples were first extruded 21 times through one filter with a pore diameter of 200 nm and subsequently 21 times through two stacked filters having a pore diameter of 100 nm. BSLs were composed of 26% 1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 25% POPC, 40% CH, 8% 1,2-*O*-distearoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (DSPG), and 1% disodium 3α-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy)-5β-cholan-24-oyl-2′-aminoethansulfonate (DSPE-3α-LCT) or disodium 3β-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy) ethoxy-5β-cholan-24-oyl-2′-aminoethansulfonate (DSPE-3β-LCT). Control liposomes (COLs) were composed of 25% DPPC, 25% POPC, 40% CH, and 10% DSPG, and phosphatidylserine-bearing liposomes (PSLs) were composed of 17.5% DPPC, 17.5% POPC, 40% CH, and 25% 1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS) (given as molar ratios). As radioactive label, [1,2-^{3}H]cholesterylhexadecylether was used. Fluorescence-labeled liposomes were prepared by adding 0.3% Ph_2-DiOC18. Freshly prepared liposomes were stored at 4°C and used within 7 days. The size distributions and the zeta potentials of the liposomes were determined with a Zetasizer III (Malvern, UK). The phosphatidylcholine (PC) concentration in the liposomal suspension was measured by colorimetric determination using ammonium ferrothiocyanate (17). The CH concentration was controlled enzymatically (18, 19) using the CH reagent kit INFINITY™ (Sigma Diagnostics).

To evaluate the lamellarity of the liposomes, the amount of encapsulated lipid was estimated with liposomes containing 0.1 mol% 4-nitrobenzo-2-oxa-1, 3-diazol (NBD)-1,2-O-dipalmitoyl-sn-glycero-3-phosphoethanolamine by determining the decrease of NBD fluorescence attributable to reduction with dithionite (20). Fluorescence was measured with a Perkin-Elmer LS 50B fluorescence spectrophotometer (Perkin-Elmer, Überlingen, Germany) using 3 cm fluorescence cuvettes equipped with a stirring bar. The fluorescence intensities were measured at 540 nm using excitation at 470 nm and a slit width of 10 nm and were corrected for autobleaching. In all experiments, 20 μl of a 1 M solution of dithionite in 1 M Tris-HCl buffer, pH 10.0, was added to 2 ml of

the appropriate liposomal suspension kept at 25°C and stirred at a constant rate of 750 rpm. After the decay in intensity had stopped, 10 μl of 400 μM cholate was added. The amount of encapsulated lipid is given by the difference between the lipid in the liposomal membrane ($\Delta F_{membrane}$) and the total amount of liposomal lipid (ΔF_{total}). At a liposomal diameter of 122 or 118 nm, the ratio of lipid in the outer and inner membrane leaflet of the liposomal membrane is 1:0.85. The parameters ΔF_{total} and $\Delta F_{outside}$ are measured (Fig. 3), and the amount of encapsulated lipid is calculated by the following equation:

$$F_{encapsulated} = \Delta F_{total} - \Delta F_{membrane} = \Delta F_{total} - (\Delta F_{outside} \times 1.85) \quad (Eq.\ 1)$$

Determination of binding of CT to liposomes

The binding of CT to the liposomal membrane was determined as described (21). In Ultra-Clear™ centrifugation tubes (Beckman Instruments, Inc., Palo Alto, CA), 2 ml of a liposomal suspension containing 2 mM liposomal PC and 50 μM [^{3}H]CT was centrifuged at 125,000 *g* for 2 h. Free CT was determined in the clear supernatant, and bound CT was calculated by subtracting the amount of free CT from the total amount of CT found with the concentrated liposomes.

Isolation and primary culture of rat hepatocytes

Hepatocytes from rat liver were prepared by a two-step procedure of the collagenase perfusion method (22, 23) using a standard medium containing 118 mM NaCl, 4.74 mM KCl, 1.2 mM $MgCl_2$, 0.59 mM KH_2PO_4, 0.59 mM Na_2HPO_4, 10 mM HEPES, 1.25 mM $CaCl_2$, and 5.5 mM D-glucose, which was saturated with carbogen (95% O_2/5% CO_2) and adjusted to pH 7.4. Cell viability before cultivation was ≥90%, as determined by trypan blue exclusion.

Hepatocyte cultures used for binding and uptake studies were prepared on tissue culture six-well plates; the wells (~35 mm) were precoated with collagen type I (Biocoat®; Becton Dickinson Labware, Bedford, UK). Cell cultures used for confocal laser microscopic studies were cultivated on collagen-coated glass cover slips with a diameter of 30 mm. To replace the medium used for cell preparation by culture medium I, a modified Williams E medium without phenolsulfonphthalein and containing 10% fetal calf serum, 1 μM dexamethasone, 25 U/l bovine insulin, 200 U/l penicillin, and 0.2 g/l streptomycin was used. The cells were collected by centrifugation at 35 *g* for 2 min and resuspended in medium I. The centrifugation was repeated, and the number of hepatocytes in medium I was adjusted to 1.2–1.5 × 10^5 cells per milliliter. Two milliliters of the hepatocyte suspension was transferred into each well, and the cells were allowed to attach to the collagen layer in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. After 4 h, medium I was replaced by medium II, a phenolsulfonephthalein-free Williams E medium supplemented with 1 μM dexamethasone, removing unattached and damaged cells. Dexamethasone was used to stimulate bile salt transport activity in primary hepatocyte culture (24). After 24 h of cultivation, the confluence of the hepatocyte monolayers was controlled by light microscopy and the viability of the cells was determined. Only cultures with confluence ≥90% and viability ≥95% were used for the experiments.

Binding of liposomes to primary rat hepatocytes

Binding of liposomes to primary cultured hepatocytes was studied in the same standard medium that was used for the preparation of freshly isolated hepatocytes. To compensate for the missing amount of D-glucose and $CaCl_2$ in the liposomal stock solutions, the appropriate amount of a solution containing 125 mM $CaCl_2$ and 550 mM D-glucose was added. The medium used to wash the culture wells and the liposomal suspensions prepared for incubation were kept at 37°C. After 24 h in culture, the cells were washed three times with 2 ml of medium. After removing the medium as completely as possible, 800 μl of the respective liposomal suspension was added and the cells were incubated for 5 min at 37°C with gentle shaking at 100 rotations per minute in a water bath. Subsequently, the liposomal suspension was removed completely and the cells were cautiously washed three times with 2 ml of medium. The cells and the attached liposomes were dissolved in 5% (w/v) SDS and 1% (v/v) Triton X-100 in 0.2 N NaOH (1 ml/well) by gentle mixing and avoiding foaming. Radioactivity and protein content in the wells were determined as described.

The effect of CT on the attachment of liposomes to the cultured hepatocytes was analyzed under exactly the same experimental conditions, except that CT at different concentrations was present in both the liposomal suspensions used for the incubation of the cells and the medium used for the washing procedure. In some experiments, cells were kept for 10 min in standard medium containing 100 μM CT before adding liposomal suspensions containing 2 mM liposomal PC and 100 μM CT.

Inhibition of CT uptake into cultured primary rat hepatocytes by liposomes

Uptake of CT into cultured primary hepatocytes was compared in the absence and presence of liposomal suspensions corresponding to 2 mM PC. Uptake was measured from a 50 μM CT solution containing 10 kBq [^{3}H]CT/ml. The culture plates were kept at 37°C in a water bath at 100 rpm. Uptake was started by the addition of 800 μl of the corresponding [^{3}H]CT-containing solutions and stopped after 10, 20, 30, 40, 50, or 60 s by quickly removing the supernatants and quickly washing the cells three times with 1.25 ml of ice-cold medium. The cells were then processed as described above.

All uptake experiments were performed in duplicate. Initial uptake rates were calculated by linear regression. For each individual experiment, the uptake rate of the liposome-free solution was determined and the inhibition of the uptake of CT by the different kinds of liposomes was calculated by dividing the measured uptake rates in the presence of liposomes by the uptake rate of CT in the absence of liposomes.

Confocal laser scanning microscopy

Fluorescence images were recorded with a confocal laser scanning microscope (LSM 510 UV; Zeiss, Jena, Germany) equipped with a galvanometer-driven scanning stage for fast focusing in the z direction (HRZ-200; Zeiss). The scanning speed was set to a pixel time of 2.24 μs. The usual pixel size, depending on the optical magnification, was ~0.2–0.4 μm in the x and y directions. The z-focus step size was usually set to values between 0.05 and 0.15 μm. The confocal pinhole was set to achieve a full-width, half-maximum z resolution between 0.5 and 1 μm. Ph_2-DiOC was excited at 488 nm with an argon laser. Fluorescence emission signals >505 nm were collected. At the given time points after incubation with fluorescence-labeled liposomes, images were acquired sequentially, whereas the differential interference contrast images were taken simultaneously with 488 nm excitation and a water-immersion 40× objective (C-APO 40/1.2 water; Zeiss).

Model calculations

A liposome with a diameter of 122 nm has a surface of 4.9 × 10^{-14} m^2. As a membrane is ~4.5 nm thick, the surface of the inner membrane leaflet is 4.2 × 10^{-14} m^2. Assuming the average space of a lipid molecule to be 44 × 10^{-20} m^2, as determined for a 6:4 mixture of DPPC/CH (25), each liposome is made up of ~2.1 × 10^5 lipid molecules and has a mass of ~1.4 × 10^8 Da. Thus, each liposome consists of ~1.7 × 10^{-19} mol of PC, or 1 nmol of liposomal PC is equivalent to 5.9 × 10^9 liposomes. As 1 g

of hepatocyte protein equals 5×10^5 cells, 1 nmol liposomal PC/mg protein equals ~12,000 liposomes/cell. As 1 mol% of BSLs are disodium 3-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy)-5β-cholan-24-oyl-2′-aminoethansulfonate (DSPE-3-LCT), the surface area of each DSPE-3-LCT is 4.3×10^{-17} m^2 or 43 nm^2. As 78.5% of a surface may be covered by circles, each DSPE-LCT is surrounded by a circle of 33.8 nm^2, having a radius of ~3.3 nm. Thus, the average distance between two DSPE-3-LCT molecules on the liposomal surface is ~6.6 nm. With this distance between two molecules, the corresponding concentration of bile salts on the liposomal surface can be calculated. As spheres in close packing cover a maximum of ~74% of total volume, each molecule in solution is surrounded by a total volume of $\sim 2.0 \times 10^{-25}$ m^3 or 2.0×10^{-22} liters. Thus, 5×10^{21} molecules, having a distance of 6.6 nm between each other, are found in a volume of 1 liter, corresponding to a concentration of bile salts on the liposomal surface of ~8 mM.

Analysis of organic compounds

Melting points (MPs) were determined with a hot-stage apparatus (Büchi, Flawil, Switzerland) and are uncorrected. ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded on a Bruker AM 400 spectrometer (Bruker GmbH, Karlsruhe, Germany) with tetramethylsilane as an internal standard. Chemical shifts are reported as ppm (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Mass spectra were determined with a TSQ 7000 ESI (Finnigan, Sunnyvale, CA). The compounds were ionized by electron spray ionization at a temperature of 200–250°C and a voltage of 4–5 kV. Nitrogen at a pressure of 30 pounds per square inch was used as inert gas. Bile salt derivatives were visualized after TLC on silica-coated 60 F_{254} plates (E. Merck, Darmstadt, Germany) by spraying with concentrated H_2SO_4 containing 1% (w/v) vanillin followed by heating at 100°C for 5 min (26), and phospholipids were visualized by spraying with a solution of 25 N H_2SO_4 containing molybden salts (27).

Syntheses

Preparative column chromatography was carried out under hydrostatic pressure on 20 × 5 cm columns of silica gel 60, 230–400 mesh (ICN Biomedicals, Meckenheim, Germany), if not stated otherwise. Solvent systems (SSs) for chromatographic separations were as follows: SS 1, cyclohexane-ethyl acetate (30:1, v/v); SS 2, cyclohexane-ethyl acetate (5:1, v/v); SS 3, cyclohexane-ethyl acetate-acetic acid (80:20:1, v/v/v); SS 4, cyclohexane-ethyl acetate-acetic acid (70:30:1, v/v/v); SS 5, cyclohexane-ethyl acetate-acetic acid (5:15:1, v/v/v); SS 6, chloroform-methanol (3:1, v/v); SS 7, chloroform-methanol (2:1, v/v); SS 8, chloroform-methanol-water (10:10:0.1, v/v/v); SS 9, chloroform-methanol-water (9:3:0.1, v/v/v); SS 10, chloroform-methanol-water (8:2:0.1, v/v/v). If chloroform-methanol mixtures were used as solvents for chromatography, the isolated products were separated from eluted silica gel by adsorption chromatography on a 30 × 3 cm column of Serdolit® PAD-1 (Serva Feinbiochemica, Heidelberg, Germany) using ~50 g of Serdolit® PAD-1 per gram of product. The silica gel-containing product was suspended in warm water (~250 ml/g), and the suspension was passed through the column at ~1 ml/min. The column was washed twice with the same volume of water, then the product was eluted with methanol.

3α-Hydroxy-5β-cholan-24-oic acid methyl ester (1). For esterification, 14 g of 3α-lithocholic acid was dissolved in 150 ml of dried methanol and 70 μl of concentrated HCl was added. After heating the reaction mixture for 4 h under reflux, the solvent and the HCl were removed under reduced pressure. The resulting crude product was purified by recrystallization in methanol-water yielding ~13.6 g (34.8 mmol, 94% yield) of compound **(1)**. MP, 115°C; TLC: relative mobility (R_f) = 0.18 (SS 3), R_f = 0.66 (SS 5); ^{1}H-NMR ($CDCl_3$): δ 0.68 (s, 3H, C$\underline{H}_3$-18); 0.91 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 0.92 (s, 3H, C$\underline{H}_3$-19); 3.64 (m b, C$\underline{H}$-3); 3.69 (s, 3H, OC$\underline{H}_3$); ^{13}C-NMR ($CDCl_3$): δ 12.1 (C-18); 18.3 (C-21); 51.5 (O$\underline{C}H_3$); 71.9 (C-3); 174.9 (C-24); mass spectrum (MS): *m/z* 373 $(M\text{-}H_2O+H)^+$, 413 $(M+Na)^+$.

3α-(2-Propenoxy)-5β-cholan-24-oic acid methyl ester (2). Under an atmosphere of argon, 5 g (12.8 mmol) of compound **(1)** was dissolved in 15 ml of dry *N,N*-dimethylformamide and 6.5 ml of *N,N*-diisopropylethylamine was added. Under refluxing, 15 ml (173 mmol) of allyl bromide was added dropwise over a period of 8 h. After removing excess allyl bromide and amine by distillation at normal pressure, the solvent was removed under reduced pressure. The crude product was purified by chromatography using SS 1. The yield of pure product was 4.2 g (9.8 mmol, 77% yield). MP, 74°C; TLC: R_f = 0.19 (SS 1); R_f = 0.48 (SS 2); ^{1}H-NMR ($CDCl_3$): δ 0.63 (s, 3H, C$\underline{H}_3$-18); 0.91 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 0.92 (s, 3H, C$\underline{H}_3$-19); 3.29 (m, 1H, C$\underline{H}$-3); 3.68 (s, 3H, OC$\underline{H}_3$); 4.02 (d, J = 6 Hz, 2H, -CHC$\underline{H}_2$O); 5.18 (d, $^3J_{cis}$ = 10 Hz, 1H, -CHC$\underline{H}_2$); 5.28 (d, $^3J_{trans}$ = 16 Hz, 1H, -CHC$\underline{H}_2$); 5.95 (m, 1H, H_2CC$\underline{H}$C$\underline{H}_2$-); ^{13}C-NMR ($CDCl_3$): δ 12.1 (C-18); 18.3 (C-21); 51.5 (-OC$\underline{H}_3$); 69.0 (=CH$\underline{C}H_2$O-); 78.7 (C-3); 116.4(-CH$\underline{C}H_2$); 135 (H_2C$\underline{C}$HCH$_2$-); 174.7 (C-24); MS: *m/z* 453 $(M+Na)^+$, 373 $(M\text{-}CH_2CHCH_2O)^+$.

3α-(2-Oxoethoxy)-5β-cholan-24-oic acid methyl ester (3). A sample of 3.5 g of compound **(2)** (8.1 mmol) was dissolved in 50 ml of dioxane, and 56 mg of OsO_4 (0.21 mmol) in 10 ml of water was added. Then, 4.5 g (21 mmol) of $NaIO_4$ was added in small portions at room temperature (RT) over a period of 45 min. After stirring the reaction mixture at RT for 10 h, the precipitated salt was removed by filtration. The solvent was removed under reduced pressure, and the residue was purified by chromatography using SS 2. The yield of pure compound **(3)** was ~2.75 g (6.3 mmol, 78% yield). MP, 111°C; TLC: R_f = 0.22 (SS 2), R_f = 0.74 (SS 5); ^{1}H-NMR ($CDCl_3$): δ 0.67 (s, 3H, C$\underline{H}_3$-18); 0.93 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 0.95 (s, 3H, C$\underline{H}_3$-19); 3.36 (m, 1H, C$\underline{H}$-3); 3.70 (s, 3H, -OC$\underline{H}_3$); 4.17 (s, 2H, OHCC$\underline{H}_2$O-); 9.75 (s, 1H, OC$\underline{H}$-); ^{13}C-NMR ($CDCl_3$): δ 12.1 (C-18); 18.3 (C-21); 51.6 (-O$\underline{C}H_3$); 73.9 (OHC$\underline{C}H_2$O-); 80.6 (C-3); 174.7 (C-24); 201.6 (O$\underline{C}$H-); MS: *m/z* 455 $(M+Na)^+$, 373 $(M\text{-}OCHCH_2O)^+$.

3α-(2-Hydroxyethoxy)-5β-cholan-24-oic acid methyl ester (4). In 40 ml of a mixture of tetrahydrofuran and water (4:1, v/v), 3.5 g (8.0 mmol) of compound **(3)** was dissolved and 250 mg of $NaBH_4$ was added. The reaction mixture was stirred at RT for 14 h. After destroying the excess borohydride with 0.1 N HCl, the organic compounds were extracted with ether. The crude product was purified by chromatography in SS 4. The yield of pure product was 2.1 g (4.8 mmol, 62% yield). MP, 116°C; TLC: R_f = 0.25 (SS 4), R_f = 0.60 (SS 5); ^{1}H-NMR ($CDCl_3$): δ 0.65 (s, 3H, C$\underline{H}_3$-18); 0.91 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 0.92 (s, 3H, C$\underline{H}_3$-19); 3.31 (m, 1H, C$\underline{H}$-3); 3.60 (t, J = 6 Hz, 2H, -CH$_2$C$\underline{H}_2$O-); 3.69 (s, 3H, -OC$\underline{H}_3$); 3.71 (t, J = 6 Hz, 2H, HOC$\underline{H}_2$-); ^{13}C-NMR ($CDCl_3$): δ 12.1 (C-18); 18.3 (C-21); 51.5 (-O$\underline{C}H_3$); 62.2 (HO$\underline{C}H_2$-); 69.0 (-CH$_2$$\underline{C}H_2$O-); 79.7 (C-3); 174.8 (C-24); MS: *m/z* 457 $(M+Na)^+$, 373 $(M\text{-}HOCH_2CH_2O)^+$.

3α-(2-Hydroxyethoxy)-5β-cholan-24-oic acid (5). A mixture of 1 g (2.3 mmol) of compound **(4)** and 260 mg (4.6 mmol) of KOH in 20 ml of methanol and 5 ml of water was stirred under reflux for 4 h. After acidification of the reaction mixture with 250 ml of 0.1 N HCl, the product was extracted with ether and purified by chromatography using SS 4. The yield of pure compound **(5)** was 0.89 g (2.1 mmol, 92% yield). MP, 131–133°C; TLC: R_f = 0.13 (SS 4), R_f = 0.61 (SS 5); ^{1}H-NMR (d_6-DMSO): δ 0.67 (s, 3H, C$\underline{H}_3$-18); 0.95 (s, 3H, C$\underline{H}_3$-19); 0.95 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 3.31 (m, 1H, C$\underline{H}$-3); 3.62 (t, J = 6 Hz, 2H, -CH$_2$C$\underline{H}_2$O-); 3.72 (t, J = 6 Hz, 2 H, HOC$\underline{H}_2$-); ^{13}C-NMR (d_6-DMSO): δ 11.8 (C-18); 18.1 (C-21); 62.8 (HO$\underline{C}H_2$-); 70.3 (-CH$_2$C$\underline{H}_2$O-); 78.5 (C-3); 174.7 (C-24); MS: *m/z* 443 $(M+Na)^+$, 359 $(M\text{-}HOCH_2CH_2O)^+$.

3β-(2-Hydroxyethoxy)-5β-cholan-24-oic acid (6). Under an atmosphere of argon, 5 g (13.3 mmol) of lithocholic acid was dissolved in 10 ml of dried and freshly distilled pyridine. After cooling the solution to 0°C, 1.3 ml (16 mmol) of methansulfonylchloride was added dropwise over 30 min. The reaction mixture was allowed to warm to RT and stirred for 2 h. Then, 20 ml of dried glycol was added and the mixture was stirred at 100°C for another 2 h. After cooling to RT, the mixture was poured into 500 ml of 0.1 N HCl. The organic compounds were extracted with ether. The crude product was purified by chromatography using SS 4, to give 1.2 g (2.9 mmol, 22%) of compound **(6)**. MP, 135–137°C; TLC: R_f = 0.14 (SS 4), R_f = 0.63 (SS 5); ^{1}H-NMR (d_6-DMSO): δ 0.62 (s, 3H, C$\underline{H}_3$-18); 0.89 (s, 3H, C$\underline{H}_3$-19); 0.89 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 3.35 (t, J = 5 Hz, 2H, -C$\underline{H}_2$O-); 3.48 (t, J = 5 Hz, 2H, HOC$\underline{H}_2$-); 3.56 (m, 1H, C$\underline{H}$-3); 4.46 (m, 1H, $\underline{H}$OCH$_2$-); 11.93 (m, 1H, -COO$\underline{H}$); ^{13}C-NMR (d_6-DMSO): δ = 11.8 (C-18); 18.1 (C-21); 60.6 (HO$\underline{C}$H$_2$-); 69.0 (-CH$_2\underline{C}$H$_2$O-); 73.6 (C-3); 174.7 (C-24); MS: *m/z* 443 $(M+Na)^+$, 359 $(M\text{-}HOCH_2CH_2O)^+$.

3α- and 3β-(2-acetyloxyethoxy)-5β-cholan-24-oic acids [(7) and (8)]. To a solution of 1 g (2.4 mmol) of compound **(5)** or **(6)** in 20 ml of dried and freshly distilled pyridine, 4 ml of acetic acid anhydride was added, and the reaction mixture was stirred at RT for 1 h. Excess acetic acid anhydride was hydrolyzed by adding 10 ml of water. After removing the solvent as azeotrope with toluene under reduced pressure, the residue was dissolved in SS 3 and purified by chromatography. Compound **(7)** yielded 780 mg (1.7 mmol, 71% yield). MP, 107°C; TLC: R_f = 0.21 (SS 3), R_f = 0.70 (SS 5); ^{1}H-NMR (d_6-DMSO): δ 0.62 (s, 3H, CH3-18); 0.89 (d, J = 7 Hz, 3H, CH3-21); 0.90 (s, 3H, CH3-19); 2.02 (s, 3H, H$_3$CCOO-); 3.26 (m, 1H, CH-3); 3.59 (t, J = 6 Hz, 2H, -CH$_2$O-); 4.08 (t, J = 6 Hz, 2H, AcOCH$_2$-); ^{13}C-NMR (d_6-DMSO): δ 11.8 (C-18); 18.1 (C-21); 20.3 (H$_3\underline{C}$CO-); 63.6 (-COO$\underline{C}$H$_2$-); 65.0 (-CH$_2\underline{C}$H$_2$O-); 78.4 (C-3); 170.2 (H$_3$CCO-); 174.7 (C-24); MS: *m/z* 485 $(M+Na)^+$, 463 $(M+H)^+$, 359 $(M\text{-}H3CCOOCH2CH2O)^+$. Compound **(8)** yielded 980 mg (2.1 mmol, 88% yield). MP, 109°C; TLC: R_f = 0.18 (SS 3), R_f = 0.71 (SS 5); ^{1}H-NMR (d_6-DMSO): δ 0.62 (s, 3H, C$\underline{H}_3$-18); 0.90 (s, 3H, C$\underline{H}_3$-19); 0.90 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 2.01 (s, 3H, $\underline{H}_3$CCOO-); 3.51 (t, J = 5 Hz, 2H, -C$\underline{H}_2$O-); 3.58 (m, 1H, C$\underline{H}$-3); 4.10 (t, J = 5 Hz, 2H, HOC$\underline{H}_2$-); 11.96 (m, 1H, -COO$\underline{H}$); ^{13}C-NMR (d_6-DMSO): δ 11.8 (C-18); 18.1 (C-21); 20.3 (H$_3\underline{C}$CO-); 63.7 (-COO$\underline{C}$H$_2$-); 65.1 (-CH$_2\underline{C}$H$_2$O-); 73.8 (C-3); 170.2 (H$_3\underline{C}$CO-); 174.7 (C-24); MS: *m/z* 485 $(M+Na)^+$, 463 $(M+H)^+$, 359 $(M\text{-}H_3CCOOCH2CH2O)^+$.

Sodium (3α- and 3β-(2-hydroxyethoxy)-5β-cholan-24-oyl)-2′-aminoethanesulfonate [(9) and (10)]. Under an atmosphere of argon, 660 mg (1.4 mmol) of compound **(7)** or **(8)**, 430 mg (2.1 mmol) of DCCI, and 240 mg (2.1 mmol) of *N*-hydroxysuccinimide were dissolved in 10 ml of dry dioxane, and the mixtures were stirred at RT for 12 h. The precipitated *N,N*-dicyclohexylurea was removed by filtration, and a solution of 263 mg (2.1 mmol) of taurine and 177 mg (2.1 mmol) of sodium hydrogen carbonate in 5 ml of water was added to the filtrates. After stirring at RT for 16 h, the reaction mixture was poured into 500 ml of 0.1 N NaOH and the organic compounds were extracted with ether. The corresponding bile acid derivative was extracted out of the aqueous phase by adsorption with Serdolit® PAD-1. After washing, the product was eluted with methanol. The methanol was evaporated under reduced pressure, and the residue was heated for 2 h in 500 ml of 1 N NaOH under reflux to remove the protective acetyl group. After the reaction mixture had been cooled to RT, adsorption chromatography was repeated. Finally, the crude product was purified by chromatography using SS 6. Compound **(9)** yielded 550 mg (1.0 mmol, 72% yield). MP, 223°C; TLC: R_f = 0.33 (SS 6), R_f = 0.71 (SS 8); ^{1}H-NMR (CD_3OD): δ 0.70 (s, 3H, C$\underline{H}_3$-18); 0.94 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 0.96 (s, 3H, C$\underline{H}_3$-19); 2.97 (t, J = 7 Hz, 2H, -C$\underline{H}_2$SO$_3$-), 3.32 (m, 1H, C$\underline{H}$-3); 3.54–3.68 (m b, 6H); ^{13}C-NMR (CD_3OD): δ 12.6 (C-18); 18.9 (C-21); 33.1 (-NH$\underline{C}$H$_2$-); 51.6 (-C$\underline{H}_2$SO$_3$-); 62.6 (HO$\underline{C}$H$_2$-); 70.4 (-CH$_2\underline{C}$H$_2$O-); 80.9 (C-3); 176.7 (C-24); MS: *m/z* 572 $(M+Na)^+$, 550 $(M+H)^+$. Compound **(10)** yielded 610 mg (1.1 mmol, 79% yield). MP: 214°C; R_f = 0.35 (SS 6), R_f = 0.76 (SS 8); ^{1}H-NMR (CD_3OD): δ 0.69 (s, 3H, C$\underline{H}_3$-18); 0.98 (s, 3H, C$\underline{H}_3$-19); 0.98 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 2.96 (t, J = 7 Hz, 2H, -C$\underline{H}_2$SO$_3$-); 3.47 (t, J = 5 Hz, 2H, -C$\underline{H}_2$O-); 3.60 (m, 1H, C$\underline{H}$-3); 3.61–3.71 (m, 4H); ^{13}C-NMR (CD_3OD): δ 12.6 (C-18); 18.9 (C-21); 33.1 (-NH$\underline{C}$H$_2$-); 51.5 (-C$\underline{H}_2$SO$_3$-); 62.7 (HO$\underline{C}$H$_2$-); 70.3 (-CH$_2\underline{C}$H$_2$O-); 76.3 (C-3); 176.5 (C-24); MS: *m/z* 572 $(M+Na)^+$, 550 $(M+H)^+$.

Sodium (3α- and 3β-(2-succinyloxyethoxy)-5β-cholan-24-oyl)-2′-aminoethanesulfonate [(11) and (12)]. To a solution of 200 mg (0.38 mmol) of compound **(9)** or **(10)** in 5 ml of dry pyridine, 120 mg (1.2 mmol) of succinic anhydride was added under an argon atmosphere. The reaction mixture was stirred at RT for 12 h, then 2 ml of water was added. Water and pyridine were removed under reduced pressure, and the residue was chromatographed on silica gel with SS 7. Compound **(11)** yielded 170 mg (0.27 mmol, 71% yield). MP, 178°C; TLC: R_f = 0.22 (SS 7), R_f = 0.51 (SS 8); ^{1}H-NMR (CD_3OD): δ 0.69 (s, 3H, C$\underline{H}_3$-18); 0.95 (s, 3H, C$\underline{H}_3$-19); 0.96 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 2.61 (m b, 4H, -OCC$\underline{H}_2\underline{C}H_2$CO-), 2.96 (t, J = 7 Hz, 2H, -C$\underline{H}_2$SO$_3$-); 3.35 (m, 1H, C$\underline{H}$-3); 3.61 (t, J = 7 Hz, 2H, -HNC$\underline{H}_2$-); 3.69 (t, 2H, J = 6 Hz, -C$\underline{H}_2$OCH-); 4.19 (t, 2H, J = 6 Hz, -COOC$\underline{H}_2$-); ^{13}C-NMR (CD_3OD): δ 12.5 (C-18); 18.9 (C-21); 30.3 (-OOC$\underline{C}$H$_2$-); 33.1 (-NH$\underline{C}$H$_2$-); 51.5 (-C$\underline{H}_2$SO$_3$-); 65.3 (-COO$\underline{C}$H$_2$-); 66.9 (-C$\underline{H}_2$OCH-); 81.0 (C-3); 174.3 (HOO$\underline{C}$- and -$\underline{C}$OOCH$_2$-); 176.5 (C-24); MS: *m/z* 672 $(M+Na)^+$, 650 $(M+H)^+$. Compound **(12)** yielded 190 mg (0.30 mmol, 79% yield). MP, 176°C; TLC: R_f = 0.33 (SS 7), R_f = 0.49 (SS 8); ^{1}H-NMR (CD_3OD): δ 0.69 (s, 3H, C$\underline{H}_3$-18); 0.96 (s, 3H, C$\underline{H}_3$-19); 0.96 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 2.62 (m b, 4H, -OCC$\underline{H}_2\underline{C}H_2$CO-), 2.96 (t, J = 7 Hz, 2H, -C$\underline{H}_2$SO$_3$-); 3.44 (t, 2H, J = 6 Hz, -C$\underline{H}_2$OCH-); 3.60 (m, 3H); 4.21 (t, 2H, J = 6 Hz, -COOC$\underline{H}_2$-); ^{13}C-NMR (CD_3OD): δ 12.5 (C-18); 18.9 (C-21); 30.3 (-OOC$\underline{C}$H$_2$-); 33.1 (-NH$\underline{C}$H$_2$-); 51.6 (-C$\underline{H}_2$SO$_3$-); 65.2 (-COO$\underline{C}$H$_2$-); 66.9 (-C$\underline{H}_2$OCH-); 76.3 (C-3); 174.4 (HOO$\underline{C}$- and -$\underline{C}$OOCH$_2$-); 176.5 (C-24); MS: *m/z* 672 $(M+Na)^+$, 650 $(M+H)^+$.

DSPE-3α-LCT and DSPE-3β-LCT. A solution of 250 mg (0.39 mmol) of compound **(11)** or **(12)**, 116 mg (0.58 mmol) of DCCI, and 65 mg (0.58 mmol) of *N*-hydroxysuccinimide in 3 ml of anhydrous *N,N*-dimethylformamide was stirred at RT for 16 h under an atmosphere of argon. The reaction mixtures were cooled to 4°C and filtered. The precipitate was washed with 2 ml of ice-cold *N,N*-dimethylformamide. The clear filtrate was then poured into 50 ml of cold ether and centrifuged (5 min at 2,000 *g*). The ether was decanted and the precipitate was dissolved in a mixture of 10 ml of $CHCl_3$, 5 ml of methanol, and 200 μl of water. To this solution, 300 mg (0.4 mmol) of 1,2-*O*-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and 400 μl of 1 N NaOH were added. The mixture was stirred at RT for 2 h, then the solvent was removed under reduced pressure and the crude product was chromatographed with SS 9. DSPE-3α-LCT yielded 170 mg (0.12 mmol, 31% yield). MP, 211°C; TLC: R_f = 0.08 (SS 9), R_f = 0.05 (SS 10); ^{1}H-NMR: ($CDCl_3$-CD_3OD-D_2O 2:1:0.1 (v/v/v)) δ 0.69 (s, 3H, C$\underline{H}_3$-18); 0.90 (t, J = 8 Hz, 6H, 2 × $\underline{H}_3$CCH$_2$-); 0.95 (s, 3H, C$\underline{H}_3$-19); 0.95 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 2.32 (m, 4H, 2 × -CH$_2\underline{C}$H$_2\underline{C}$H$_2$OCO-); 2.56 (t, J = 7 Hz, 2H, -COC$\underline{H}_2\underline{C}H_2$COO-); 2.69 (t, J = 7 Hz, 2H, -NHCOC$\underline{H}_2$CH$_2$CO-); 3.01 (t, J = 7 Hz, 2H, -C$\underline{H}_2$SO$_3$-); 3.35 (m, 1H, C$\underline{H}$-3); 3.44 (m, 2H, -OCH$_2\underline{C}$H$_2$NH-); 3.61 (t, J = 7 Hz, 2H, -HNC$\underline{H}_2$CH$_2$SO$_3$Na); 3.70 (t, 2H, J = 6 Hz, -C$\underline{H}_2$OCH-); 3.81–4.18 (m b, 6H); 4.21 (t, 2H, J = 6 Hz, -COOC$\underline{H}_2$-); 5.23 (m, -OCH$_2\underline{H}$COHCH$_2$O-); ^{13}C-NMR ($CDCl_3$-CD_3OD-D_2O 2:1:0.1 (v/v/v)): δ 12.3 (C-18); 14.5 (stearoyl-CH$_3$); 18.5 (C-21); 25.2 (stearoyl-C-3′) 30.0 (stearoyl-CH$_2$); 30.8 (succinyl-C$\underline{H}_2$); 33.4 (taurine-NH$\underline{C}$H$_2$-); 50.6 (-C$\underline{H}_2$SO$_3$-); 63.1 (glyceryl-C-3′); 64.0 (glyc-

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eryl-C-1′); 64.7 (-COO$\underline{C}H_2CH_2$-O); 66.0 (-$\underline{C}H_2$OCH-); 71.2 (glyceryl-C-2′); 80.2 (C-3); 173.9 (stearoyl-$\underline{C}$O); 174.0 (succinyl-$\underline{C}$O); 174.4 (C-24); MS: *m/z* 1,424 $(M+Na)^+$, 1,402 $(M+H)^+$, 1,380 $(M\text{-}Na^+2H)^+$, 1,378 $(M\text{-}Na)^-$, 677 (M-2Na) (2-). DSPE-3β-LCT yielded 220 mg (0.16 mmol, 41% yield). MP, 206°C; TLC: R_f = 0.08 (SS 9), R_f = 0.03 (SS 10); ^{1}H-NMR ($CDCl_3$-CD_3OD-D_2O 2:1:0.1 (v/v/v)): δ 0.68 (s, 3H, C$\underline{H}_3$-18); 0.92 (t, J = 8, 6H, 2 × $\underline{H}_3CCH_2$-); 0.96 (d, J = 7 Hz, 3H, C$\underline{H}_3$-21); 0.97 (s, 3H, C$\underline{H}_3$-19); 2.35 (m, 4H, 2 × -$CH_2CH_2C\underline{H}_2$OCO-); 2.56 (t, J = 7, 2H, -COC$H_2C\underline{H}_2$COO-); 2.68 (t, J = 7 Hz, 2H, -NHCOC$H_2C\underline{H}_2$CO-); 3.01 (t, J = 7 Hz, 2H, -C$\underline{H}_2$-SO_3-); 3.44 (m, 2H, -OC$H_2C\underline{H}_2$NH-); 3.58 (t, J = 7 Hz, 2H, -HNC$\underline{H}_2CH_2SO_3$Na); 3.62 (t, 2H, J = 6 Hz, -C$\underline{H}_2$OCH-); 3.67 (m, 1H, C$\underline{H}$-3); 3.92–4.21 (m b, 6H); 4.21 (t, 2H, J = 6 Hz, -COOC$\underline{H}_2$-); 5.27 (m, -OC$H_2\underline{H}$COHCH_2O-); ^{13}C-NMR ($CDCl_3$-CD_3OD-D_2O 2:1:0.1 (v/v/v)): δ 12.5 (C-18); 14.5 (stearoyl-CH_3); 18.7 (C-21); 25.5 (stearoyl-C-3′) 30.8 (succinyl-$\underline{C}H_2$); 30.0; 30.2 30.3 (stearoyl-CH_2); 33.9 (taurine-NH$\underline{C}H_2$-); 50.8 (-$\underline{C}H_2SO_3$-); 63.4 (glyceryl-C-3′); 64.2 (glyceryl-C-1′); 64.9 (-COO$\underline{C}H_2CH_2$-O); 66.0 (-$\underline{C}H_2$OCH-); 71.2 (glyceryl-C-2′); 75.8 (C-3); 174.1 (stearoyl-$\underline{C}$O); 174.4 (succinyl-$\underline{C}$O); 176.0 (C-24); MS: *m/z* 1,424 $(M+Na)^+$, 1,402 $(M+H)^+$, 1,380 $(M\text{-}Na^+2H)^+$, 1,378 $(M\text{-}Na)^-$, 677 (M-2Na) (2-).

RESULTS

Syntheses

To determine whether bile salts may serve as ligands to direct liposomes to hepatocytes, a bile salt had to be linked to the liposomal membrane. Therefore, a bile salt-phospholipid conjugate was synthesized and characterized. In a first step, the secondary 3-hydroxyl group of lithocholic acid was converted in a stereoselective manner to the 3α- or 3β-glycol ether derivative (**Fig. 1**). With slight modifications, this synthesis followed the synthesis for the corresponding cholic acid derivatives (28). The modified bile salts were then conjugated with taurine (**Fig. 2**). Activating the carboxyl group with *N*-hydroxysuccinimide gave higher yields under milder conditions than the methods used previously (29). A spacer between the lipid anchor and the homing device is necessary for efficient binding (30), and succinate was chosen as a physiological linker. The succinyl-bile salt derivatives were linked to DSPE via the *N*-hydroxysuccinimide method. The low solubility of the two amphipathic molecules required N,N-dimethylformamide as a solvent. Subsequent to the formation of the glycol ether, all reactions were performed under mild conditions. Thereby, the fixed stereochemistry at the 3 position of the bile salt was preserved, as confirmed by ^{1}H- and ^{13}C-NMR.

Liposomes

To reduce nonspecific coulomb interactions of the liposomes with the hepatocytes, the liposomes used had a negative net charge. The negative net charge of the liposomal membrane introduces a repulsive force against the negative charge of the taurine-conjugated side chain of the bile salt, presumably leading to ligands that protrude as far as possible from the liposomal membrane. COLs were prepared with 10 mol% DSPG as the negatively charged phospholipid, whereas BSLs were prepared with 8 mol% DSPG and 1 mol% DSPE-3-LCT. As DSPE-3-LCT carries two charges, COLs and BSLs had the same net charge.

The prepared BSLs had a mean diameter of 122 ± 14 nm, and COLs had a mean diameter of 118 ± 6 nm. The polydispersity of the liposomes was 0.050 ± 0.035 for BSLs and 0.073 ± 0.026 for COLs. Diameter and size distribution were as expected for the extrusion method used. The prepared BSLs and COLs were stable over a period of several days (as measured by changes in diameter). The zeta potential of BSLs was 31.2 ± 3.6 mV, and that of COLs was 35.3 ± 2.7 mV. The difference was slightly significant (P = 0.06). The amount of encapsulated lipid (lamellarity) was measured according to a modified method described by McIntyre and Sleight (20). Instead of using detergent to destroy the liposomes, cholate was used to increase the permeability of the liposomal bilayer while the liposomal structure was maintained (21). Adding cholate to a final concentration of 2 mM allows the dithionite anion to reach the interior of the liposomes and to reduce the NBD lipids inside the liposomes. At this concentration, no quenching of fluorescence by the addition of cholate was observed, and the amount of light scattering by the liposomes (baseline in **Fig. 3**) was not altered. This allows the continuous measurement of lamellarity. A typical measurement is shown in Fig. 3. Approximately 6 ± 2% of total lipid was encapsulated within the COLs, and ~2 ± 2% was encapsulated within the BSLs (n = 4). Thus, the BSLs as well as the COLs were almost unilamellar.

Binding of CT to liposomes

For bile salt uptake studies, CT at a concentration of 50 μM was used as a reference. Therefore, the binding of 50 μM CT to the liposomes was measured. The amount of bound CT was determined by ultracentrifugation under equilibrium conditions as described (31). Binding of CT to the liposomes was very low. Approximately 0.4 ± 0.2 or 0.2 ± 0.2 mmol CT/mol liposomal PC was bound to BSLs or COLs (n = 3). The difference between BSLs and COLs was almost significant (P = 0.1). Between DSPE-3α-LCT-bearing and DSPE-3β-LCT-bearing liposomes, no difference in binding of CT was observed. Bile salts tend to interact with each other. Depending on the concentration, they form dimers, multimers, or micelles (32), and probably CT interacts weakly with bile salts protruding from the liposomal membrane.

Inhibition of bile salt uptake

To examine whether bile salts covalently linked to the surface of a liposome are able to interact with the bile salt carriers of the hepatocytes, the inhibition of the uptake of CT in the presence of liposomes was examined (**Fig. 4**). Liposomes and CT were added simultaneously to the cells. The uptake rates were measured under initial rate conditions within the first 60 s. The concentration of free CT was reduced from 50 to 49.6 μM for COLs and from 50 to 49.2 μM for BSLs as a result of binding of CT to the liposomes (see above). To consider this effect on uptake rates, the kinetic constants published by Schramm et al. (10) were used. Binding of CT to the liposomes reduces uptake rates of CT by ~0.2% (COLs) or 0.4% (BSLs) in the experimental setting applied. The measured uptake rates in the absence of liposomes were re-

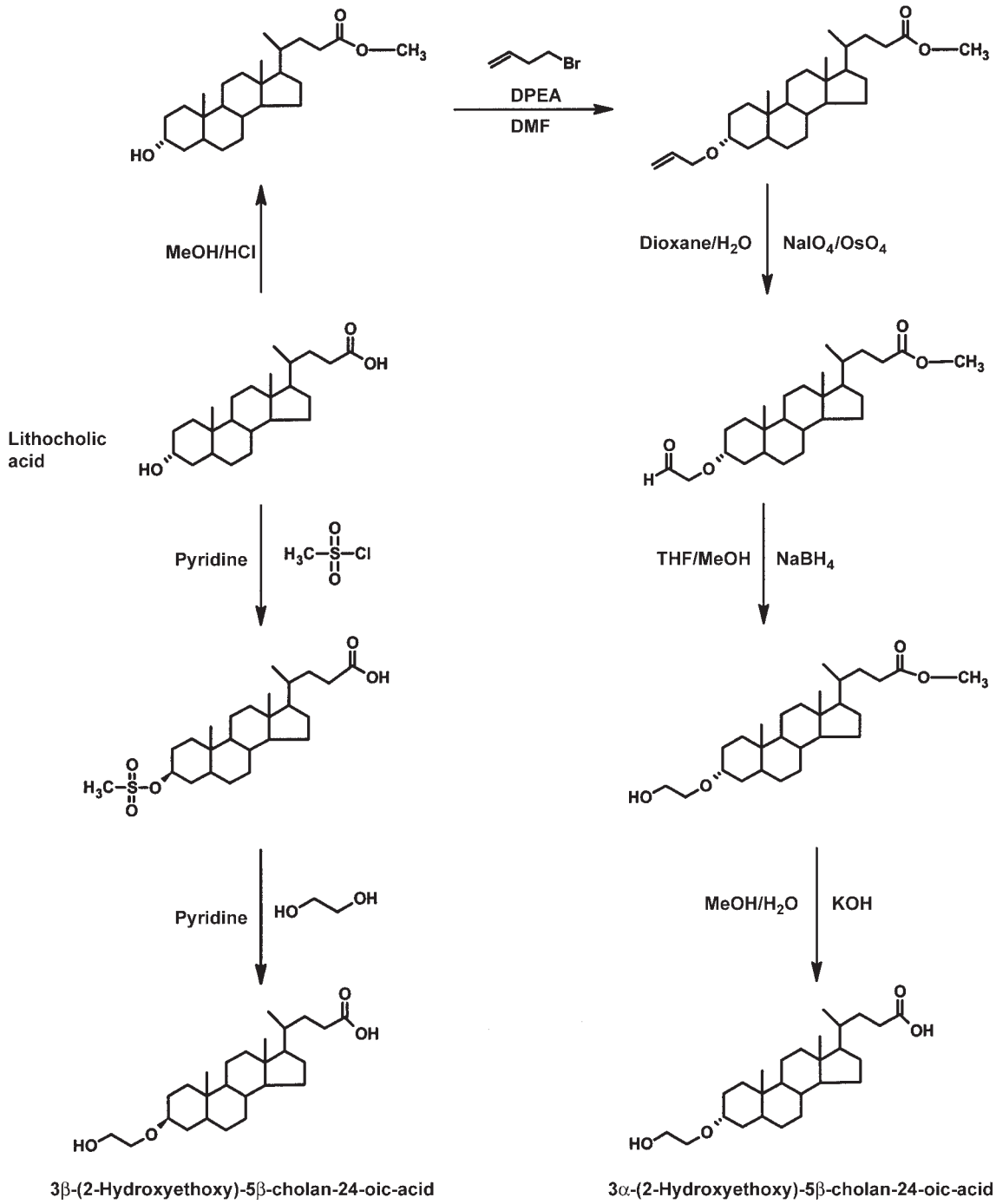

Fig. 1. Synthesis of 3α- and 3β-(2-hydroxyethoxy)-5β-cholan-24-oic acid. DMF, N,N-dimethylformamide; DPEA, N,N-diisopropylethylamine.

duced by 0.2% or 0.4%, respectively, before the calculation of relative uptake rates in the presence of liposomes.

Uptake was linear within the first 60 s. The uptake rates in the presence of different liposomes determined in five independent experiments, normalized to the respective rates in the absence of liposomes, are shown in Fig. 4. In the presence of COLs, the uptake rate of CT was virtually unaffected. In contrast to COLs, the uptake of CT was significantly reduced in the presence of BSLs ($P = 0.017$ for DSPE-3α-LCT and $P << 0.01$ for DSPE-3β-LCT). Bile salts covalently attached to the surface of liposomes are able to interact with the bile salt carrier proteins on the surface of hepatocytes. The presence of DSPE-3β-LCT-bearing liposomes leads to a greater inhibition of CT uptake than the presence of DSPE-3α-LCT-bearing liposomes ($P = 0.03$). Thus, the interaction of BSLs with hepatocytes is dependent on the stereochemical orientation in the 3 position of the bile salt moiety linked to the liposomal surface.

Binding of liposomes to the surface of hepatocytes

To determine whether BSLs bind to the surface of hepatocytes, confocal laser scanning microscopy (LSM) was per-

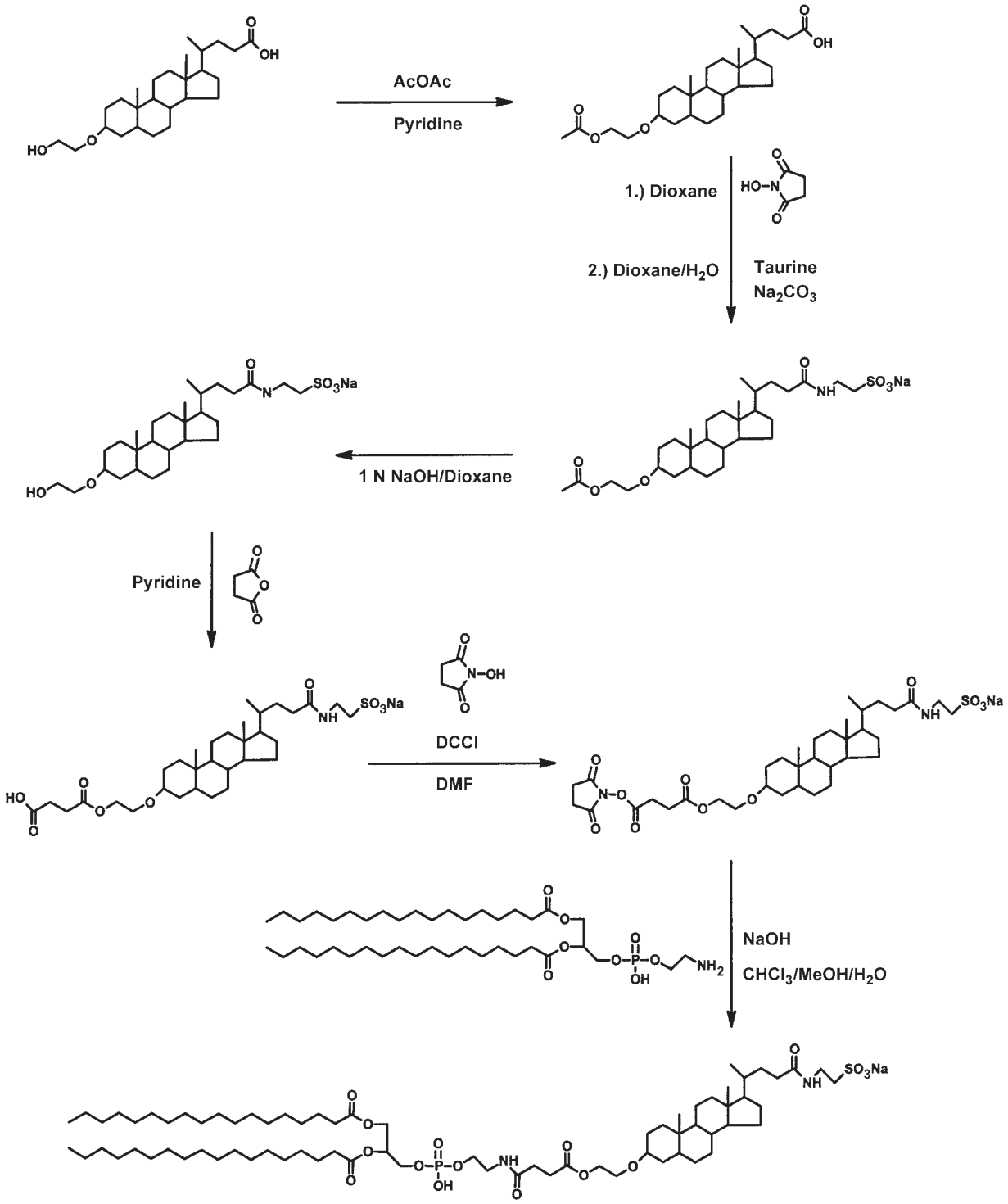

Fig. 2. Synthesis of disodium 3α-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy)-5β-cholan-24-oyl-2′-aminoethansulfonate (DSPE-3α-LCT) and disodium 3β-(2-(1,2-*O*-distearoyl-*sn*-glycero-3-phospho-2′-ethanolamidosuccinyloxy)ethoxy-5β-cholan-24-oyl-2′-aminoethansulfonate (DSPE-3β-LCT).

formed. Liposomes were labeled with the fluorescent dye Ph_2-DiOC18. Ph_2-DiOC18 is a very hydrophobic membrane marker that is not exchanged between liposomes and the cytoplasmic membrane during the experiments (10–15 min). Fluorescent liposomes were added to hepatocytes, and after 5 min the liposomes were removed. The cells were washed with buffer and examined by confocal LSM. Typical images are shown in **Fig. 5**. Fig. 5A, B show COLs, and Fig. 5C, D show DSPE-3β-LCT-bearing liposomes. Fig. 5A, C show differential interference contrast images combined with fluorescence images along the plane of the cover slips the cells were grown on (x-y plane). The

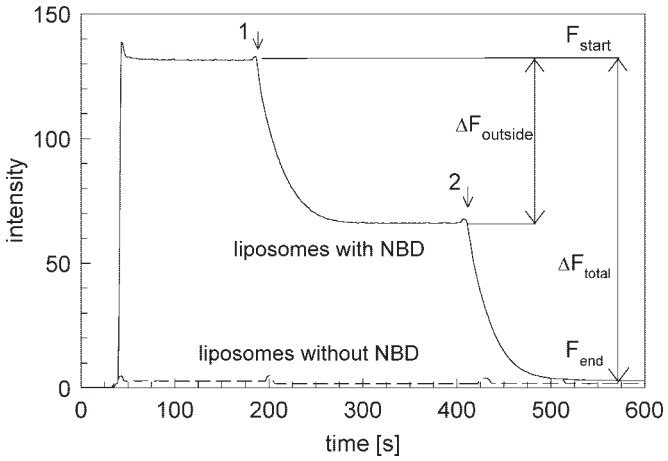

Fig. 3. Measurement of encapsulated lipid. The graph shows a typical measurement of DSPE-3α-LCT-bearing liposomes. DSPE-3α-LCT-bearing liposomes, DSPE-3β-LCT-bearing liposomes, and control liposomes (COLs) showed the same general behavior. After the addition of sodium-dithionite (1), the fluorescent lipid in the outer membrane layer was reduced and the respective fluorescence disappeared ($\Delta F_{outside}$). After the addition of cholate to the liposomes (2), the liposomal membrane became leaky and the dithionite was able to reduce the fluorescent lipid inside the liposomes (ΔF_{total}). NBD, 4-nitrobenzo-2-oxa-1,3-diazol.

confocal plane in the images focuses on the surface of the cover slip. Fig. 5B, D show cross-sections (x-z plane) of the cells along the white lines shown in Fig. 5A, C.

COLs attach only in a very sporadic manner to the hepatocytes. By contrast, BSLs attach to a much higher extent more or less all over the surface of the hepatocytes. Thus, bile salts conjugated to a lipid in the membrane of a liposome are able to anchor the liposome on the surface of a hepatocyte. The binding sites seem to be more or less evenly distributed on the surface of the hepatocytes.

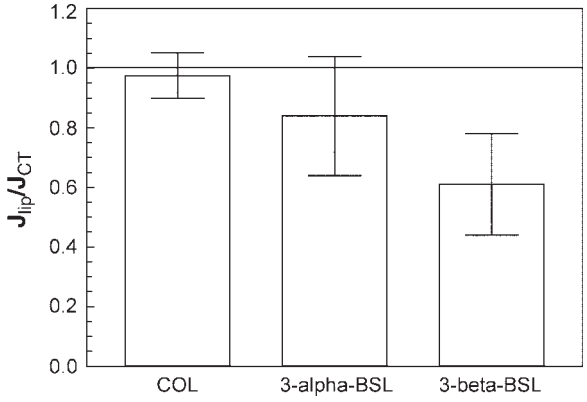

Fig. 4. Inhibition of cholyltaurine (CT) uptake by liposomes. The uptake of ^{3}H-labeled CT was measured in the absence and the presence of liposomes in a concentration of 2 mM liposomal phosphatidylcholine (PC). The concentration of CT was 50 μM. Liposomes and CT were added simultaneously to the cells. The graph shows the uptake in the presence of liposomes (J_{lip}) related to the uptake in the absence of liposomes (J_{CT}). J_{CT} was 0.5 ± 0.2 nmol/min/mg protein. The means and SD of five individual experiments are shown. BSL, bile salt-bearing liposome.

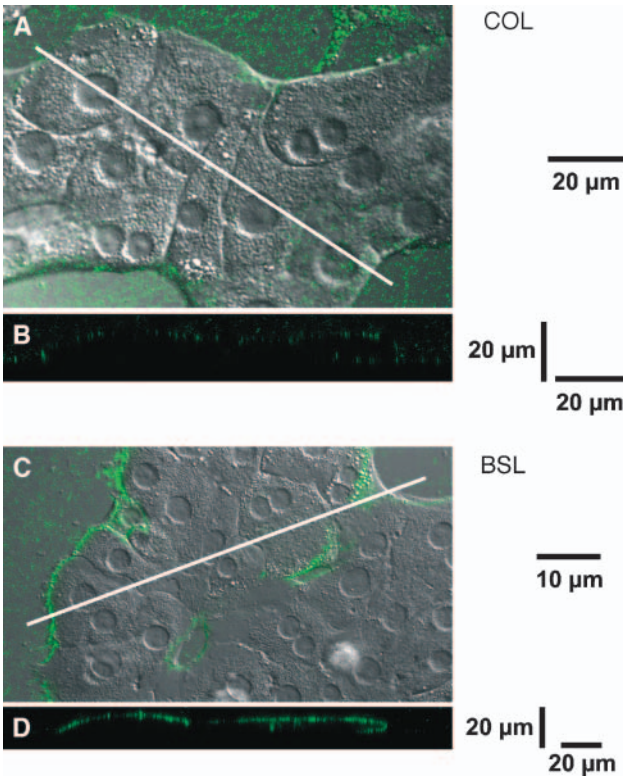

Fig. 5. Binding of liposomes to the surface of hepatocytes. Liposomes in a concentration of 2 mM liposomal PC were added to hepatocytes cultured for 24 h. After 5 min, the cells were carefully washed and subjected to laser scanning microscopy. A, C: Interference contrast images along the x-y plane, focusing on the surface of the cover slips on which the cells were grown. B, D: x-z cross-sections along the white lines indicated in A, C, respectively. Liposomes without DSPE-LCT (COLs) are shown in A, B, whereas DSPE-3β-LCT-bearing liposomes (BSLs) are shown in C, D.

To quantify the amount of bound liposomes, liposomes were labeled with [^{3}H]cholesterylhexadecylether, a non-exchangeable membrane marker (33). Liposomes were added to the cells for 5 min. After washing (three times), the amount of radioactivity remaining associated with the cells was quantified. The amount of liposomes associated with the cells was calculated as nanomoles of liposomal PC per milligram of cellular protein. As described in Materials and Methods, a rough estimate was made to calculate the number of liposomes bound per cell. Hepatocytes were cultured on six-well plates covered with collagen. Because there is some significant binding of liposomes to collagen not covered with cells (Fig. 5), only cultures with a confluence >90% were used. The binding of liposomes to the collagen was quantified under identical conditions, and the amount of liposomes associated with the cells was corrected for nonspecific binding to 10% of the surface area of a culture well.

In **Fig. 6**, means ± SD are shown for 10–12 individual experiments. In all experiments, more BSLs than COLs remained associated with the cells. The difference in binding was highly significant ($P < 0.01$). Again, DSPE-3β-LCT-bearing liposomes showed a stronger interaction with the hepatocytes than DSPE-3α-LCT-bearing liposomes ($P < 0.01$).

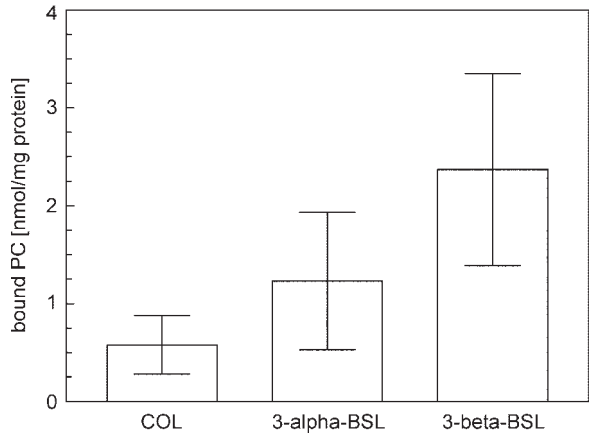

Fig. 6. Binding of liposomes to hepatocytes. ^{3}H-labeled liposomes in a concentration of 2 mM liposomal PC were added for 5 min to primary rat hepatocytes kept in culture for 24 h. Cells were washed, and the amount of associated radioactivity was quantified as described. In a rough estimation, 1 nmol liposomal PC/mg protein = ~12,000 liposomes/cell (see model calculations for details). The means and SD of 10–12 individual cell culture experiments are shown.

After three washing steps, there were virtually no liposomes left in the cell culture medium. Therefore, a fast equilibration between bound and free liposomes is ruled out, and the binding of BSLs has a quasi-irreversible character. To test this observation, the binding of DSPE-3β-LCT-bearing liposomes was observed during different washing steps (**Fig. 7**). By the first two washing steps, there was a significant reduction in liposomes remaining with the cells, COLs as well as BSLs. Because the supernatant cannot be removed completely during each washing step, these liposomes were most likely unbound liposomes remaining in the supernatant. Bile salt-mediated binding was calculated by subtracting COLs from BSLs remaining in the culture well (error bars indicate the sum of SD for COLs and SD for BSLs). As shown in Fig. 7, there was no significant reduction in bile salt-mediated binding by washing. Although the concentration of free liposomes was reduced significantly by each washing step, bound BSLs stayed firmly associated with the cells. Thus, there is no simple and fast dissociation equilibrium between bound and unbound BSLs.

Anionic liposomes are bound by scavenger receptors, and binding is dependent on charge density and the anionic lipid present (34). To rule out the possibility that the observed binding of BSLs is mediated only by scavenger receptors, liposomes that readily bind to scavenger receptors (having 25 mol% DPPS) (35) were used as inhibitors for BSL binding. Even though binding of DPPS-bearing liposomes (PSLs) showed an even higher binding to hepatocytes than BSLs, PSLs showed only a modest inhibition of BSL binding when BSLs were incubated together with PSLs (**Fig. 8**). Inhibition of BSLs was ~22% when 2 mM liposomal PC was used for both BSLs and PSLs. Because PSLs consisted of 35 mol% PC, there were even more PSLs present than BSLs. Inhibition should be 50% or greater if BSL binding is mediated only by scavenger receptors. As expected, there is some interaction of the anionic BSLs with scavenger receptors, but BSLs bind to a major extent to some other cell surface proteins that might bind liposomes by other than charge-charge interactions.

Influence of CT on binding of BSLs

Free bile salts should compete with BSLs for binding to the carriers and thereby inhibit, at least in part, the bind-

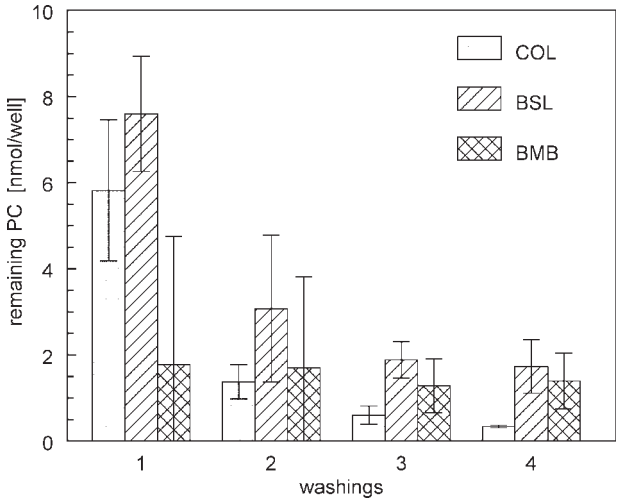

Fig. 7. Binding of liposomes during different washing steps. ^{3}H-labeled liposomes in a concentration of 2 mM liposomal PC were added for 5 min to primary rat hepatocytes cultured for 24 h on six-well plates (35 mm/well). The cells were washed different times with 2 ml of buffer per well. The amount of radioactivity remaining in the well was quantified as described. COLs and BSLs prepared with DSPE-3β-LCT were used. To calculate bile salt-mediated binding (BMB), the amount of COLs associated with the cells was subtracted from the amount of BSLs associated with the cells. The means and SD of 3 individual cell culture experiments are shown. SD for BMB was calculated by adding the SD for COL and BSL.

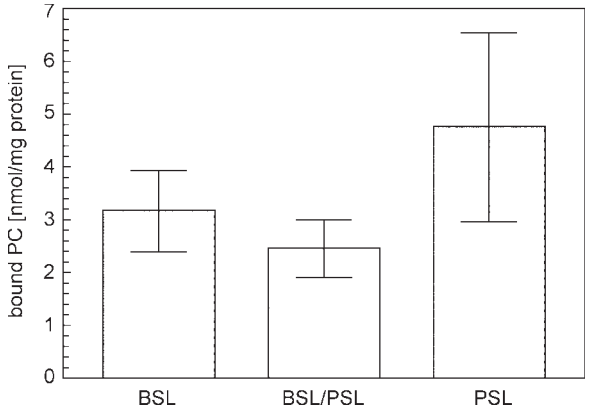

Fig. 8. Binding of anionic liposomes to hepatocytes. Liposomes in a concentration of 2 mM liposomal PC were added for 5 min to primary rat hepatocytes kept in culture for 24 h. Cells were washed, and the amount of associated radioactivity was quantified as described. The means and SD of 3 individual cell culture experiments are shown. BSL, liposomes prepared with 8 mol% 1,2-*O*-distearoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) and 1 mol% DSPE-3β-LCT; BSL/PSL, ^{3}H-labeled BSLs and unlabeled PSLs, each in a concentration of 2 mM liposomal PC, were added simultaneously to the cells; PSL, liposomes prepared with 25 mol% 1,2-*O*-dipalmitoyl-*sn*-glycero-3-phosphoserine.

ing of BSLs to the hepatocytes. To investigate the influence of CT on the binding of BSLs, binding of BSLs was quantified in the presence of different CT concentrations. The experiments were performed as before, CT and BSLs were added simultaneously, and the washing buffer contained the same concentration of CT as the liposomal solutions added. Because DSPE-3β-LCT-bearing liposomes bound to a greater extent to the hepatocytes than DSPE-3α-LCT-bearing liposomes, DSPE-3β-LCT-bearing liposomes were used as BSLs for the experiments.

CT up to a concentration of 500 μM had no significant effect on the binding of COLs. The effect of increasing CT concentrations on the binding of BSLs to hepatocytes is shown in **Fig. 9**. The bile salt-mediated binding was calculated as described before. Within each test series, the amount of bile salt-mediated binding in the presence of CT was divided by the amount of bile salt-mediated binding in the absence of CT.

The bile salt-mediated binding in the presence of CT did not follow a simple competitive characteristic. At low concentrations of CT (20 and 50 μM), there was some slight inhibition of BSL binding to the hepatocytes (P = 0.025 for 20 μM CT and P = 0.068 for 50 μM CT). Increasing concentrations of CT led to increased bile salt-mediated binding, and BSLs with concentrations > 300 μM CT showed even higher bile salt-mediated binding than BSLs in the absence of CT (P = 0.083 for 500 μM). Besides a slight inhibition of BSL binding by CT, there must be a second process leading to increased binding of BSLs at increasing concentrations of CT.

The transport of bile salts is regulated by a feed-forward mechanism (36, 37), and the presence of bile salts leads to an increasing amount of bile salt carrier in the hepatocyte membrane (38, 39). The activation of bile salt transport is transient and time-dependent. For primary hepatocytes cultivated for 24 h, the maximum activation of intracellular signal cascades by CT was observed 10 min after adding the bile salts to the culture dishes (37). Binding of BSLs in the presence of CT was observed within 5 min, so the activation of bile salt transport by the presence of CT had presumably not reached its peak. To determine whether there is CT-mediated activation of BSL binding, hepatocytes were treated for 10 min with medium containing 100 μM CT, then BSLs were added in the presence of 100 μM CT and binding was observed as before. The amounts of bound liposomes are shown in **Fig. 10**.

Again, there was no significant difference in binding of COLs, either in the presence of 100 μM CT or after pretreatment of the cells with 100 μM CT for 10 min. As described before, the total amount of BSL binding was not affected by the presence of 100 μM CT when bile salt was added simultaneously with the liposomes. After pretreatment of the cells with 100 μM CT for 10 min, a duplication in the amount of bound BSLs was observed. Thus, the presence of CT is able to promote the binding of BSLs to hepatocytes. When simultaneously adding liposomes and CT, the stimulation of bile salt transport activity and the inhibition of binding of BSLs by the presence of bile salts seem to neutralize each other. Within 5 min, the stimulation of bile salt transport activity has not reached its maximum. When the stimulation of bile salt transport activity has more time to proceed, the stimulation of BSL binding is much stronger than the weak competitive inhibition of BSL binding by the free bile salt.

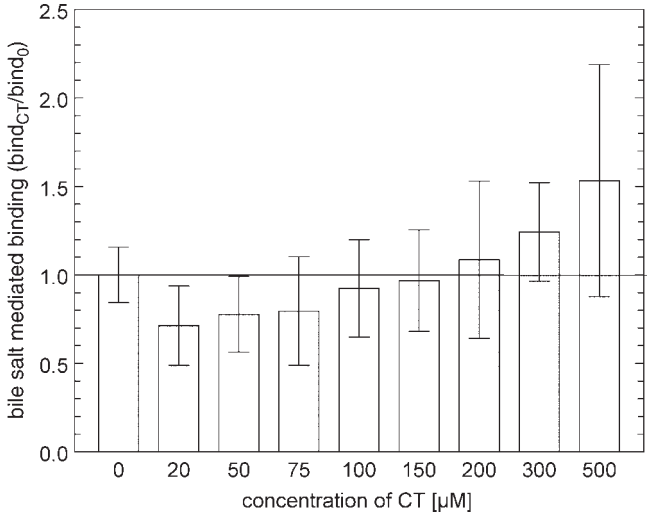

Fig. 9. Influence of CT on the association of BSLs with hepatocytes. Liposomal suspensions containing ^{3}H-labeled liposomes in a concentration of 2 mM liposomal PC and different concentrations of CT were added for 5 min to primary rat hepatocytes in culture for 24 h. Cells were washed, and the amount of associated radioactivity was quantified as described. The means and SD of 6 individual cell culture experiments are shown. BSLs prepared with DSPE-3β-LCT were used. To calculate bile salt-mediated binding, the amount of COLs associated with the cells was subtracted from the amount of BSLs associated with the cells.

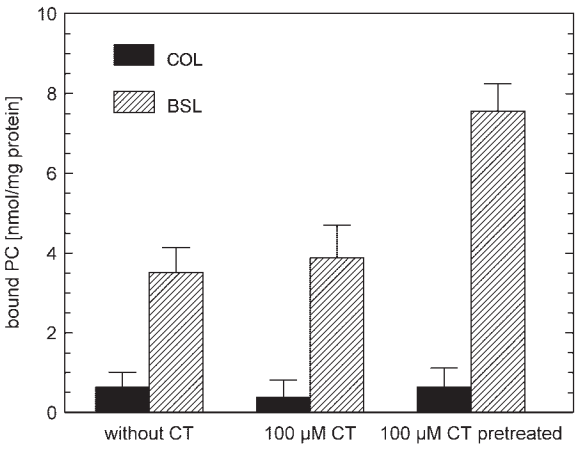

Fig. 10. Influence of CT on the association of liposomes with hepatocytes. ^{3}H-labeled liposomes in a concentration of 2 mM liposomal PC were added for 5 min to 24 h cultured primary hepatocytes. The liposomal suspensions contained either no CT or CT in a concentration of 100 μM. Some hepatocytes were pretreated for 10 min with medium containing 100 μM CT. This medium was removed followed by immediate addition of CT-containing liposomal suspensions. The amount of associated liposomes was measured as described. COLs without DSPE-3-LCT and BSLs prepared with DSPE-3β-LCT were used. The means and SD of 6 individual experiments are shown.

DISCUSSION

Synthesis

To determine whether bile salts may serve as ligands to direct liposomes to hepatocytes, a bile salt-lipid conjugate was synthesized. The synthesis path allows the stereoselective preparation of DSPE-3α-LCT and DSPE-3β-LCT in a convenient way with an acceptable yield. DSPE was chosen as the lipid component of the conjugate because a phospholipid as a natural building block of lipid bilayers should not alter the membrane properties of the liposomes. Lithocholic acid was chosen as the bile salt because it bears only one hydroxyl group at the C-24 position of the bile acid skeleton, which allows chemical modification without the necessity to protect further functional groups. Other bile salts having two or three hydroxyl groups, such as cholic acid or the therapeutically used ursodeoxycholic acid, may be useful as ligands as well. Higher amounts of these bile salts are less toxic than lithocholic acid, but synthesis with these bile salts is more complex. The aim of this study was a proof of principle, and the demonstration of other bile salts as potential ligands is the subject of further work. Taurine-conjugated bile salts were favored because they bear a permanent negative charge at physiological pH. This charge should prevent the insertion of the hydrophobic bile salt backbone into the liposomal membrane, leading to a buried anchor, and this charge should prevent the insertion of the hydrophobic bile salt backbone into cellular membranes, leading to nonspecific binding of the liposomes to cells.

Different from several other potential ligands for hepatocyte-specific targeting, DSPE-3-LCT may be synthesized at low cost. The lipid-coupled ligand allows the addition of desired amounts of ligand to the lipid mixture of which the liposomes are prepared, without any further elaborate coupling procedure. BSLs may be produced at much lower cost than other targeting systems, using antibodies, for example, as a targeting moiety. Because bile salts are physiological molecules of low molecular weight, it is very unlikely that BSLs trigger any bile salt-specific immune response.

Influence of stereochemistry

The interaction of BSLs with the carrier systems for CT is dependent on the stereochemical orientation of the bridging 3-O of the bile salt. The different degree of inhibition of CT uptake by COLs and BSLs bearing either the stereoisomer DSPE-3α-LCT or DSPE-3β-LCT indicates that inhibition of CT transport is bile salt-specific with interaction between liposomal DSPE-3-LCT and bile salt carrier proteins.

Conjugation of the fluorescent dye NBD to CT via the 3β position of the bile salt leads to a compound that readily interferes with CT, whereas the isomeric 3α compound does not (32). As shown by Kramer et al. (13), a 3β-conjugated drug-bile salt is secreted faster into bile than its respective 3α derivative. In accordance with these data for dissolved bile salt conjugates, liposomes bearing DSPE-3β-LCT interfere to a greater extent with CT transport than liposomes bearing DSPE-3α-LCT. Furthermore, higher amounts of DSPE-3β-LCT-bearing liposomes bind to hepatocytes than DSPE-3α-LCT-bearing liposomes. Thus, DSPE-3β-LCT is more suitable for hepatocyte-specific targeting than its 3α derivative. In general, conjugation via the 3β position seems to be favorable for using hepatocyte-specific bile salt-transporting systems for drug delivery.

Interaction of BSLs with bile salt carrier systems

DSPE-3β-LCT-bearing liposomes inhibited the uptake of 50 μM CT by ~40%. At a concentration of 2 mM liposomal PC, the overall concentration of liposomal bile salt in the outer membrane layer is ~20 μM. Thus, the observed inhibition is in the same range as expected for a simple competitive mechanism. The mechanism of inhibition is definitely more complex than a simple competitive inhibition, but the affinity of liposomal DSPE-3-LCT for bile salt carrier must be of the same order of magnitude as the affinity for CT.

CT is transported by different carrier systems, such as Na^+/cholyltaurine-cotransporting polypeptide (Ntcp) and different organic anion-transporting polypeptides (7). Because CT uptake is not inhibited completely in the presence of BSLs, at least under the experimental conditions used in the described experiments, it cannot be definitely determined whether these interactions occur with all or certain different carrier systems. Expression, activity, and kinetic parameters of the different carrier systems are not well defined for primary hepatocyte cultures. Other model systems than cultured hepatocytes might be useful to obtain further kinetic data on the interference of BSLs with bile salt transport.

Binding of BSLs to hepatocytes

Bile salts covalently attached to the surface of hepatocytes are able to anchor liposomes on the surface of hepatocytes. As revealed by confocal LSM, the BSLs are more or less evenly distributed on the surface of a single cell, and all vital hepatocytes bind BSLs. Thus, the potential binding sites for BSLs are distributed all over the cell surface. As expected, no endocytosis of bound liposomes was seen during the experiments (~10–15 min for binding, washing, and processing). Whether this is also true for longer time periods has to be established and will be discussed in detail in a subsequent publication.

Anionic liposomes are bound by scavenger receptors (34), such as scavenger receptor class B type I, which is found on the surface of hepatocytes. The binding is dependent on the lipid providing the anionic charges and the net charge of the liposomes. COLs had the same net charge and an even higher zeta potential as BSLs, and the binding of COLs and BSLs to scavenger receptors should be comparable, but anionic charges of DSPE-3-LCT protrude from the liposomal surface. So it is possible that binding of BSLs to scavenger receptors may be facilitated by DSPE-3-LCT. To rule out the possibility that the observed binding of BSLs to the hepatocytes is mediated by scavenger receptors only, highly anionic liposomes (PSLs) were used as inhibitors that readily bind to scavenger re-

ceptors. When given at the same concentration of liposomal PC, the inhibition of BSL binding by PSLs was significant, but much less than expected for a direct competition at the same receptor(s). Thus, BSLs and PSLs may compete for binding at some binding sites where highly anionic liposomes show a high affinity, but the binding of BSLs is mediated to a major extent by binding sites where BSLs show a much higher affinity than PSLs. Because the PSLs bound to a slightly higher extent to the hepatocytes than the BSLs did, these binding sites cannot be scavenger receptors. Furthermore, DSPE-3α-LCT- and DSPE-3β-LCT-carrying liposomes show no difference in charge distribution. Because there is a significant difference in the binding of these BSLs to hepatocytes, there must be at least some interactions that discriminate between the 3α and 3β positions of the bile salt, as is well known for bile salt carriers. These date indicate that there is some interaction of the anionic BSLs with receptors that might bind negatively charged liposomes, such as scavenger receptors, but BSLs bind to a major extent to some other cell surface proteins. Because there is substantial interference of BSLs with bile salt transport, these cell surface proteins are most likely bile salt carriers.

At least in terms of dissociation, the binding of liposomes seems to be a high-affinity process. On a molecular scale, a liposome is a huge particle with a lipid mass of $\sim 1.4 \times 10^8$ Da. It is very unlikely that a particle with a diameter of ~120 nm is bound by a single DSPE-3-LCT-carrier complex. More likely, the anchoring of a liposome occurs by several DSPE-3-LCT-carrier complexes. Assuming that the contact surface between the cell surface and a liposome is 5% of the liposomal surface, ~100 DSPE-3-LCT molecules are present at the contact surface. The total number of bile salt carriers on a hepatocyte surface is not known, but at least $\sim 3.3 \times 10^6$ Ntcps were measured on a single hepatocyte (40). Under the conditions applied, ~28,000 BSLs are bound to a single hepatocyte. Given the ratio of >100 bile salt carriers to a bound liposome, each BSL may interact with more than one bile salt carrier. A rough estimation gives an average distance of 6.6 nm between the DSPE-3-LCT molecules on the liposomal surface (see Materials and Methods), and cooperativity might be possible. Furthermore, DSPE-3-LCT is free to move in the liposomal membrane, and bile salt carriers are most likely mobile in the hepatocyte membrane, so both may accumulate at the contact site. In terms of concentration, an average distance of 6.6 nm between two molecules corresponds to a concentration of ~8 mM. Thus, once the first DSPE-3-LCT-carrier complex is formed, the formation of more complexes is very likely and the dissociation of a bound liposome becomes very unlikely.

Specific binding to the target cell still keeps the cellular membrane between the intracellular compartments and a therapeutic molecule. For diagnostic purposes such as cell-specific contrast agents, hepatocyte-specific binding may be sufficient, but for drug delivery, the membrane barrier has to be overcome. One approach is the use of lipophilic prodrugs (6), and the close contact of cellular and liposomal membranes, as revealed by LSM, may facilitate the diffusion of lipophilic drugs into the cell. Close contact of the membranes is a prerequisite for the fusion of membranes. The binding of liposomes by DSPE-3-LCT will bring the liposomes into close contact with the cellular surface. BSLs bound to the surface of the hepatocytes may be useful to study the fusion of liposomes with cellular membranes as a possible mechanism for drug delivery.

Influence of CT on binding of BSLs to hepatocytes

Bile salt-mediated binding is modulated by the presence of CT. At least two competing processes are observed. On the one hand, a minor inhibition, presumably by competition for binding sites, occurs at low concentrations of CT. On the other hand, higher concentrations of CT are able to reverse this inhibitory effect, probably by feed-forward stimulation of bile salt transport activity. Binding by several DSPE-3-LCT-carrier complexes may explain why the inhibition of binding in the presence of CT is very week. The formation of the first DSPE-3-LCT-carrier complex may be less likely in the presence of CT, but once the first complex has formed, the concentration of DSPE-3-LCT in the vicinity of the first complex is much higher than the concentration of free CT, and the formation of further DSPE-3-LCT-carrier complexes may not be inhibited by CT present in the medium.

The presence of bile salts leads to the activation of bile salt transport activity in vitro and in vivo (41). Activation of bile salt transport is mediated by the dephosphorylation of Ntcp (42) and by increasing the number of Ntcps on the hepatocyte surface by extravasation of stored carrier (38, 39). Both processes may stimulate the binding of liposomes, and at least the increase in the number of carriers on the hepatocyte surface may allow the anchoring of more liposomes. It is not known whether transport processes other than bile salt transport are stimulated by CT within the observed time scale, but stimulation of bile salt-mediated binding by CT gives strong evidence for the involvement of bile salt carriers in the binding of BSLs to hepatocytes.

BSLs bind to the surface of rat hepatocytes in primary culture and interact with the hepatocyte-specific carrier systems for bile salts. Thus, bile salt-lipid conjugates such as DSPE-3β-LCT target liposomes to hepatocytes and should be further analyzed for hepatocyte-specific drug delivery. More generally, not only substrates for cellular endocytosing receptors but also substrates for cellular carrier proteins should be suitable ligands for the cell-specific targeting of nanoscale particles such as liposomes.

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