Substrate specificity of the ileal and the hepatic Na^+/bile acid cotransporters of the rabbit. I. Transport studies with membrane vesicles and cell lines expressing the cloned transporters

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Abstract **The substrate specificity of the ileal and the hepatic Na^+/bile acid cotransporters was determined using brush border membrane vesicles and CHO cell lines permanently expressing the Na^+/bile acid cotransporters from rabbit ileum or rabbit liver. The hepatic transporter showed a remarkably broad specificity for interaction with cholephilic compounds in contrast to the ileal system. The anion transport inhibitor diisothiocyanostilbene disulfonate (DIDS) is a strong inhibitor of the hepatic Na^+/bile acid cotransporter, but does not show any affinity to its ileal counterpart. Inhibition studies and uptake measurements with about 40 different bile acid analogues differing in the number, position, and stereochemistry of the hydroxyl groups at the steroid nucleus resulted in clear structure–activity relationships for the ileal and hepatic bile acid transporters. The affinity to the ileal and hepatic Na^+/bile acid cotransport systems and the uptake rates by cell lines expressing those transporters as well as rabbit ileal brush border membrane vesicles is primarily determined by the substituents on the steroid nucleus. Two hydroxy groups at position 3, 7, or 12 are optimal whereas the presence of three hydroxy groups decreased affinity. Vicinal hydroxy groups at positions 6 and 7 or a shift of the 7-hydroxy group to the 6-position significantly decreased the affinity to the ileal transporter in contrast to the hepatic system. 6-Hydroxylated bile acid derivatives are preferred substrates of the hepatic Na^+/bile acid cotransporter. Surprisingly, the 3α-hydroxy group being present in all natural bile acids is not essential for high affinity interaction with the ileal and the hepatic bile acid transporter. The 3α-hydroxy group seems to be necessary for optimal transport of a bile acid across the hepatocyte canalicular membrane. A modification of bile acids at the 3-position therefore conserves the bile acid character thus determining the 3-position of bile acids as the ideal position for drug targeting strategies using bile acid transport pathways.**—Kramer, W., S. Stengelin, K-H. Baringhaus, A. Enhsen, H. Heuer, W. Becker, D. Corsiero, F. Girbig, R. Noll, and C. Weyland. **Substrate specificity of the ileal and the hepatic Na^+/bile acid cotransporters of the rabbit. I. Transport studies with membrane vesicles and cell lines expressing the cloned transporters.** ***J. Lipid Res.*** **1999.** 40: **1604–1617.**

Supplementary key words bile acids • Na^+/bile acid cotransporters • substrate specificity • molecular recognition • structure–activity relationships • cloned transporters • transport studies • ileum • liver

The ileal (IBAT) and the hepatic (LBAT) Na^+/bile acid cotransporters are integral membrane proteins belonging to a gene family of 7-transmembrane-domain transporters (1). The ileal transporter consists of 348 amino acids in human (2), hamster (3), rat (4), and mouse (5) and of 347 amino acids in the rabbit (6). The ileal transporter shows a high degree of identity and similarity among the different species, the rabbit transporter with 92% similarity and 87% identity being the closest relative to the human system. The hepatic Na^+/bile acid cotransporters have 349 amino acids in humans and 362 in the rat (7–9) showing 35–37% identity and 46–48% similarity to the ileal transporter. With the ileal system, the rabbit transporter has 82% homology and 87% similarity (9) most closely related to man. The ileal and the hepatic Na^+/bile acid cotransport systems work collaboratively to ensure efficacious biological recycling of bile acids during enterohepatic circulation (10, 11). The major purpose of the ileal system is to reabsorb and conserve bile acids for the body to maintain hepatic biliary secretion and to promote intestinal absorption. Bile acids recirculating with portal blood to the liver cause a feedback inhibition of cholesterol 7α-hydroxylase, the key enzyme for the degradation of cholesterol to bile acids (12) thereby influencing serum cholesterol

Abbreviations: BBMV, brush border membrane vesicles; BSP, bromosulfophthalein; CHO, Chinese hamster ovary cells; DIDS, 4,4′-diisothiocyanostilbene disulfonate; HPTLC, high performance thin-layer chromatography; IBAT, ileal bile acid transporter; ICG, indocyanine green; LBAT, liver bile acid transporter; 3β-NBD-TC, 2-(7α,12α-dihydroxy-3β-(4-nitrobenzo-2-oxa-1,3-diazolyl)-amino-5β-cholan-24-oylamino)-ethanesulfonic acid; LDL, low density lipoprotein; LTC-$3SO_4$, lithocholyltaurine-3-sulfate; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecylsulfate.

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levels. The liver plays a central role in the elimination of a wide variety of potentially toxic endogenous and exogenous amphipathic compounds from the body (13, 14). The different functions of the structurally related Na^+/bile acid cotransporters in the ileum and the liver is reflected in different specificities as reported so far. A multispecificity for the Na^+-dependent as well as for the Na^+-independent hepatic bile acid transport systems has been described for a variety of cholephilic compounds such as steroids and steroid conjugates, cyclic peptides (e.g., phalloidin, antamanide, and somatostatin analogues) and numerous drugs (e.g., BSP, ICG, bumetanide, and others) (15–20). None of these cholephilic substrates showed a significant interaction with the ileal Na^+/bile acid cotransport system in brush border membrane vesicles (BBMV) (21, 22). Only bile acid derivatives were known to interfere with ileal bile acid transport (21–24) and just recently non-bile acid-derived inhibitors of the ileal bile acid transporter have been reported (25).

Because interruption of the bile acid enterohepatic circulation results in increased hepatic low density lipoprotein (LDL) receptor expression and decreased serum cholesterol levels (12), specific nonabsorbable inhibitors of the ileal bile acid transport system represent a new class of potentially highly effective LDL cholesterol lowering agents (24, 26, 27). As a rational approach to the design of such inhibitors, we investigated the substrate specificity of the ileal and hepatic Na^+/bile acid cotransport systems for cholephilic compounds and bile acid analogues on a molecular level establishing clear structure activity relationships for interaction of bile acids with the ileal and the hepatic Na^+/bile acid cotransporters.

MATERIALS AND METHODS

Materials

Transport and photoaffinity-labeling studies were performed with 2-(7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oylamino)-ethanesulfonic acid (7,7-azo-TC; specific radioactivity 20 Ci/mmol) or 7,7-azo-3α,12α-dihydroxy-5β[3β-12β-^{3}H]cholan-24-oic acid (specific radioactivity 3.6 Ci/mmol) synthesized as described (16, 28, 29). The fluorescent bile acid analogue 2-(7α,12α-dihydroxy-3β-(4-nitrobenzo-2-oxa-1,3-diazolyl)-amino-5β-cholan-24-oylamino)-ethanesulfonic acid (3β-NBD-TC) for fluorescent bile acid uptake measurements was synthesized according to Schneider et al. (30). [G-^{3}H]cholyltaurine (specific radioactivity, 2.1 Ci/mmol), d-[U-^{14}C]glucose (specific radioactivity, 258.5 Ci/mmol) and l-[3-^{3}H]alanine (specific radioactivity, 84 Ci/mmol) were from DuPont-New England Nuclear (Dreieich, Germany). Marker proteins for the determination of molecular masses were from Sigma. Triton X-100, Serva Blue R-250, and other materials for electrophoresis were from Serva (Heidelberg, Germany). Cellulose nitrate filters for transport studies (ME 25; 0.45 μm; 25 mm diameter) were from Schleicher & Schüll (Dassel, Germany) and scintillators Quickzint 501 and 361 were from Zinsser Analytik GmbH (Frankfurt, Germany). Kit 3359 for the determination of the activity of the marker enzyme aminopeptidase N was from Merck. Protein was determined according to Bradford (31) using the Bio-Rad kit. Indocyanine green, estradiol, iopanoic acid, rifampicin, tetracyclin, digitoxigenin, novobiocin, 3-sulfolithocholyltaurine, reserpine, streptomycin sulfate, benzylpenicillin, cephalexin, chlorpromazine, 17β-estradiol-3-sulfate, and estrone-3-sulfate were purchased from Sigma (München, Germany), whereas strophantoside K was from Serva (Heidelberg, Germany) and bilirubin ditaurate was from Novabiochem Corporation (La Jolla, CA). Ofloxacin and cefixime were synthesized at Hoechst Marion Roussel. Cell culture medium was Minimal Essential Medium (MEM) with 1% (v/v) of MEM non-essential amino acids and 10% (v/v) fetal bovine serum. All three medium components and PBS were from GIBCO-BRL (Life Science Technologies GmbH, Eggenstein, Germany).

Synthesis of bile acid analogues

7,12-Dihydroxycholanoic acid, 3α,7α-dihydroxy-12-oxo-cholanoic acid, dehydrocholic acid and its taurine conjugate were purchased from Steraloids Inc. (Wilton, NH), whereas hyodeoxycholic acid, ursodeoxycholic acid and its taurine conjugates were from Sigma (München, Germany). 7α,12α-Dihydroxy-3α-diphenylmethoxy-cholanoic acid was synthesized as described (27). The following compounds were synthesized according to published procedures: allocholate (32), norcholate (33), α-muricholate (34), β-muricholate (34), ω-muricholate (34), hyocholate (34), ursocholate (35), and 7α,12α-dihydroxy-3-oxo-cholanoate (29, 36). The taurine conjugates of bile acids were synthesized using the mixed anhydride method (37). The purity of all compounds was analyzed by ^{1}H-NMR and mass spectrometry.

Synthesis of [^{3}H]taurine-conjugated bile acid analogues

One hundred μCi of [2,3-^{3}H]taurine (specific radioactivity 24.1 Ci/mmol) was evaporated in an Eppendorf tube to dryness and the residue was resolved in 20 μl of tetrahydrofuran and 20 μl n/100 sodium hydroxide solution. For the synthesis of [^{3}H]taurine bile acid analogues, 0.5 mg of the respective bile acid analogue was dissolved in 40 μl of freshly prepared dry tetrahydrofuran. After addition of 5 μl N-triethylamine, the tube was stored on ice and 5 μl of a 1% solution of chloroethylformate in tetrahydrofuran was added. After 30 min at 4°C the samples were centrifuged to precipitate triethylammonium chloride. Eight μl of the supernatant containing the bile acid mixed anhydride was added to the [^{3}H]taurine solution and left at 20°C for 12 h. After analysis of the reaction mixture by thin-layer chromatography in one of the solvent systems I–IV, the chromatograms were analyzed by radio-thin-layer chromatography using a Berthold radiochromatogram scanner (Berthold AG, Calw, Germany). Solvent systems were: I, chloroform–methanol 3:1 (v/v); II, n-butanol–water–acetic acid 5:3:2 (v/v/v); III, n-butanol–water–acetic acid 9:1:2 (v/v/v); and IV, n-butanol–water–acetic acid 10:1:1 (v/v/v).

Subsequently, the reaction mixture was put onto a 5 × 20 cm HPTLC thin-layer plate and developed in one of the above solvent systems. After detection of the radioactively labeled compounds by radio thin-layer chromatography, the respective bands were scratched off and the silica was poured into a one-way polycarbonate chromatography column (Pierce, Rockford, IL, Article No. 25 920). The radiolabeled bile acid analogues were eluted with 1500 μl of ethanol and, after rechromatography of an aliquot with the respective corresponding unlabeled compound in the above solvent systems, the ethanolic solutions containing the radioactively labeled bile acid were stored at 4°C. For biological experiments, the necessary amount of radioactively labeled bile acid was removed from the stock solution and evaporated to dryness. After dissolving in appropriate buffers with or without the addition of unlabeled compound, the solutions were immediately used for the respective studies.

Preparation of membrane vesicles and transport measurements

Brush border membrane vesicles from the ileum of male white New Zealand rabbits (4–5 kg) were prepared by the Mg^{2+}

precipitation method and characterized by transport measurements, immunological and enzymatic methods as described previously (38, 39). Plasma membrane subfractions from rat liver enriched with sinusoidal surfaces were prepared as described (40).

Uptake of radiolabeled substrates by brush border membrane vesicles was determined by the membrane-filtration method as described previously (21, 39). Transport reaction was initiated by adding 10 μl vesicle suspension (50 μg protein) to 90 μl incubation medium containing the radioactively labeled substrate at 30°C. The composition of the incubation medium for measurements in the presence of a Na^+ gradient was usually 10 mm Tris/HEPES, pH 7.4, 100 mm NaCl, 100 mm mannitol, and in the absence of Na^+ gradient 10 mm Tris/Hepes, pH 7.4, 100 mm KCl, 100 mm mannitol. For measurements of bile salt uptake, these media contained 50 μm (0.75 μCi) [^{3}H]cholyltaurine and the indicated concentrations of the respective inhibitors. Uptake studies with [^{3}H]taurine-conjugated bile acid analogues was performed accordingly using a concentration of 50 μm (0.2 μCi/determination). The transport reaction was terminated after 60 s by addition of 1 ml ice-cold 10 mm Tris/HEPES buffer (pH 7.4)/150 mm KCl and the radioactivity remaining on the filter was measured by standard liquid-scintillation techniques (38, 39). After correction for radioactivity from the medium bound to the filter in the absence of membrane vesicles and chemiluminescence, absolute solute uptake was calculated and expressed as nmol/mg protein. Uptake values were corrected for binding/transport in the absence of a Na^+ gradient thus representing Na^+-dependent bile acid uptake. All experiments were performed in triplicate, and uptake values are given as means ± SD.

Plasmids and cell lines

All cDNAs encoding bile acid transporters were cloned by reverse transcriptase polymerase chain reaction (RT-PCR). Cloning of IBAT and LBAT from humans was based on published sequences (2, 8). To obtain the hitherto unknown cDNAs for IBAT and LBAT from rabbit, partial cDNAs were first synthesized by the RT-PCR procedure using primers with homology to conserved regions, as determined by comparison of the published orthologous sequences from other species. Missing ends were then cloned by the RACE procedure. The two novel cDNA sequences from rabbit are deposited at the EMBL database (accession no. Z54357 and AJ 131361 for IBAT and LBAT, respectively). Parent plasmids to construct the eucaryotic expression vectors for the bile acid transporters were pcDNA1neo and pTracer-CMV from Invitrogen Corp. Both have a cytomegalovirus (CMV) promoter for constitutive expression of heterologous genes and a gene for expression of resistance against a selection compound (G418 in case of pcDNA1neo and zeocin in case of pTracer-CMV). Insertion of cDNA for human IBAT, human LBAT, and rabbit IBAT into pcDNA1neo led to the expression vectors pHIBAT8, pHLBAT5 and pKIBAT8, respectively, and insertion of cDNA for rabbit LBAT into pTracer-CMV led to the expression vector pKLBAT10. To create stable transgenic cell lines, expression vectors were introduced into Chinese hamster ovary (CHO) cells and transfectants were cultivated for 2 days in cell culture medium, followed by permanent cultivation in the same type of medium containing G 418 (400 μg/ml) or zeocin (250 μg/ml), respectively. Surviving cells were seeded at low density in microtiter plates and cultivated further. Aliquots of cells from wells with single colonies were then grown to near confluency and assayed for bile acid uptake activity. At room temperature, they were washed with PBS, exposed for 30 min to a 10 μm solution of the fluorescent bile acid 3β-NBD-TC in PBS, washed with PBS again, and examined with a fluorescence microscope. Clonal cell lines which stood out by showing bright intracellular fluorescence were selected for further use.

Bile acid transport assays

To measure uptake of radioactive bile acid derivatives, cells were seeded at equal density in 6-well plates and grown to near confluency. Cells of individual wells were then washed twice with PBS and treated at 22°C for the indicated time (1, 2, 4, 6, or 8 min) with 1 ml of a solution of the respective radioactive bile acid derivative at the indicated concentration in PBS. At the end, cells were washed five times with PBS and lysed by incubation at 22°C for 15 min with 1 ml of a solution of 0.1 m NaOH and 0.1% (w/v) SDS in water. Lysed material was triturated, mixed with 10 ml of a scintillation cocktail, and associated radioactivity was measured by liquid scintillation counting. During washing five times with PBS, adsorbed monomeric bile acids were removed and the radioactivity remaining with the cells represents the Na^+-dependent uptake of bile acids into the cell interior as we have shown previously by the lack of uptake in the absence of Na^+ (41). Uptake is expressed as pmol bile acid/mg cell protein. Inhibition of uptake of radioactive cholyltaurine by various compounds was measured according to a similar protocol using 96-well plates. Cells were seeded at 30,000 cells per well and grown to near confluency. After two washes with PBS, they were preincubated at 22°C for 30 min with the inhibitor of choice in PBS. Radioactive cholyltaurine was then added to a final concentration of 10 μm and incubation continued for an hour. At the end, cells were washed five times with PBS and remaining radioactivity was measured by liquid scintillation counting. All data were determined in triplicate and results were used to calculate mean values.

Liver perfusion experiments and in situ ileal perfusion

Rats starved for 18 h were anesthetized with urethane (25%, 5 ml/kg i.m.) and the common bile duct was cannulated. After an initial bile collection period of 30 min, 500 μl of a solution of the respective compounds (concentration usually 50 μm) dissolved in 10 mm of Tris/HEPES buffer (pH 7.4) 300 mm mannitol, ethanol content <5% was injected as bolus into a peripheral mesenteric vein and bile was collected after 2, 4, 6, 8, 10, 15, 20 min and subsequently in 10-min steps until 120 min after the initial injection. In situ ileal perfusion with radiolabeled bile acid analogues was performed as described elsewhere (23, 27, 42).

Photoaffinity labeling and SDS PAGE

For photaffinity labeling, 25 μl of rabbit ileal brush border membrane vesicles (150 μg of protein equilibrated with 10 mm Tris/HEPES buffer (pH 7.4)/300 mm mannitol or rat liver sinusoidal membranes (40) was photolabeled in 10 mm Tris/HEPES buffer (pH 7.4)/100 mm NaCl/100 mm mannitol with the radiolabeled photoreactive 7,7-azo-derivatives of bile acids and the putative nonradioactively labeled inhibitors as described (21–23, 38, 39, 42).

After washing the membranes, SDS-PAGE was carried out in vertical slab gels (20 × 17 × 0.15 cm) using an electrophoresis System LE 2/4 (LKB Pharmacia Biotechnologie, Freiburg, Germany) as described (39, 43). After staining with Serva Blue R 250, the gels were scanned with a densitometer CD 50 (DESAGA, Heidelberg, Germany) and the individual lanes were cut into slices of 2 mm thickness. Each slice was solubilized with 250 μl of tissue solubilizer Biolute S overnight and after addition of 4 ml of scinitillator Quickzint 501 the samples were counted for radioactivity. Alternatively, fluorography was used for detection of radioactively labeled proteins (43, 44).

RESULTS

Interaction of cholephilic substrates with the Na^+/bile acid cotransport system in rabbit ileal brush border membrane vesicles

Investigations in vivo as well as in vitro have demonstrated inhibition of Na^+-dependent hepatic bile acid transport by a great number of cholephilic organic substances of high structural diversity such as steroids, steroid conjugates, organic anions, drugs or (cyclic) peptides (45–47) showing competitive or noncompetitive interaction with cholyltaurine uptake depending on the particular compound. To identify and compare the substrate specificity of the ileal and hepatic Na^+/bile acid cotransport systems with respect to non-bile acid analogues, we have investigated the effect of 28 different hepatobiliary transported organic compounds and drugs on cholyltaurine transport in rabbit ileal brush border membrane vesicles and cell lines transfected with the cDNAs of ileal as well as hepatic Na^+/bile acid cotransporter proteins. **Table 1** summarizes the behavior of these cholephilic compounds on Na^+-dependent [^{3}H]cholyltaurine uptake by rabbit ileal brush border membrane vesicles. The anion transport inhibitor DIDS strongly inhibiting hepatic bile acid transport (48, 49) had no influence on cholyltaurine transport in ileal brush border membrane vesicles (21, 22). None of the antibiotics tested (the β-lactam antibiotics benzylpenicillin, cephalexin and cefixime, the gyrase inhibitor ofloxacin, rifampicin, tetracycline, streptomycin or novobiocin) showed a significant inhibition of the rabbit ileal Na^+/bile acid cotransport system in brush border membrane vesicles. Furthermore, none of the neutral steroids investigated (β-estradiol, digitoxigenine or strophantoside K, bromosulfophthalein, chlorpromazine, reserpine or the LDL-receptor inducer HOE 402) (50) showed any effect on [^{3}H]cholyltaurine transport. Hints for a slight inhibition of [^{3}H]cholyltaurine uptake by rabbit ileal brush border membrane vesicles were observed for indocyanine green, bilirubin ditaurate, iopanoic acid, lithocholyltaurine-3-sulfate, and estron-3-sulfate whereas 17β-estradiol-3-sulfate had no effect.

TABLE 1. Inhibition constants of cholephilic compounds for inhibition of [^{3}H]cholyltaurine uptake by rabbit ileal brush border membrane vesicles

	Ileal BBMV		
Compound	IC_{25}	IC_{50}	IC_{75}
Cholephilic anions			
Bromosulfophthalein		no inhibition	
Indocyanine green	50	200	˘
Bilirubin ditaurate	210	˘	˘
Iopanoic acid	125	>300	˘
DIDS		no inhibition	
Steroids			
Lithocholyltaurine-3-sulfate	120	300	˘
Estron-3-sulfate	300	˘	˘

The Na^+-dependent uptake of 50 μm [^{3}H]cholyltaurine into brush border membrane vesicles (50 μm) was measured in the presence of the indicated concentrations of compounds followed by determination of IC_x values. The IC_x values are the mean of 2–4 independent experiments with 3 individual uptake measurements for each concentration of the respective inhibitor; ˘, no effect.

Interaction of cholephilic substrates with rabbit ileal and hepatic Na^+/bile acid cotransporters expressed in CHO cells

In order to compare the substrate specificity of the ileal and hepatic Na^+/bile acid cotransport systems, we have cloned the rabbit ileal and hepatic Na^+/bile acid cotransport systems and expressed them permanently in CHO cells (6, 9). The effect of cholephiles on both transport systems was investigated by transport measurements at concentrations of 10 μm [^{3}H]cholyltaurine and 10^{-9} to 10^{-3} m for inhibitors followed by determination of IC_{50} values. No compound was found that inhibited only the ileal bile acid transporter without affecting the hepatic transport system. Out of the 28 compounds tested only 9 (the antibiotics streptomycin, cephalexin, benzylpenicillin, ofloxacine, rifampicin, tetracyclin, the steroids β-estradiol and α-aldosterone and the LDL-receptor inducer HOE 402) did not inhibit [^{3}H]cholyltaurine uptake by rabbit LBAT-transfected CHO cells, whereas 21 of the cholephilic compounds tested had no effect on the rabbit IBAT activity. Among the compounds that inhibited both transport systems, the affinity and inhibitory potency of these compounds were greater for the liver bile acid transporter in each case (**Fig. 1**). Only 7 compounds (lithocholyltaurine-3-sulfate, bromosulfophthalein, indocyanine green, iopanoic acid, strophantoside K, corticosterone, and chlorpromazine) inhibited the ileal transporter in CHO/pKIBAT 8 cells. Their inhibitory potency on [^{3}H]cholyltaurine was significantly greater compared to rabbit ileal brush border membrane vesicles resulting in IC_{50} values of 9.15 μm for lithocholyltaurine-3-sulfate, 5.6 μm for indocyanine green, 52 μm for bromosulfophthalein, 59 μm for iopanoic acid, 200 μm for chlorpromazine, and 350 μm for strophantoside K. The reason for these significant differences in the inhibitory potency between rabbit ileal brush border membrane vesicles and CHO cells transfected with the rabbit Na^+/bile acid cotransporter is probably caused by a hindered diffusion of these compounds across the unstirred water layer of the glycocalix in brush border membrane vesicles leading to a significant lesser inhibition compared to the transfected CHO cells lacking such a glycocalix.

The anionic steroids estrone-3-sulfate, 17β-estradiol-3-sulfate or lithocholyltaurine-3-sulfate are strong inhibitors of the hepatic Na^+/bile acid cotransporter, but only the bile acid derivative lithocholyltaurine-3-sulfate was also a strong inhibitor of the ileal system with an IC_{50} value of 9.15 μm compared to 0.81 μm for the hepatic system. Out of the 12 steroids investigated, only strophantoside K and corticosterone showed a weak inhibition of the ileal system with IC_{50} values of 350–400 μm. In contrast, with the exception of β-estradiol and α-aldosterone, all steroid hormones were able to inhibit the hepatic Na^+/bile acid cotransporter. The polar compounds estrone-3-sulfate, 17β-estradiol-3-sulfate, strophantoside K, and corticosterone showed moderate inhibition with IC_{50} values of 29–70 μm, whereas the other steroids (digitoxigenine, 6α-methylprednisolone, 5α-pregnane,

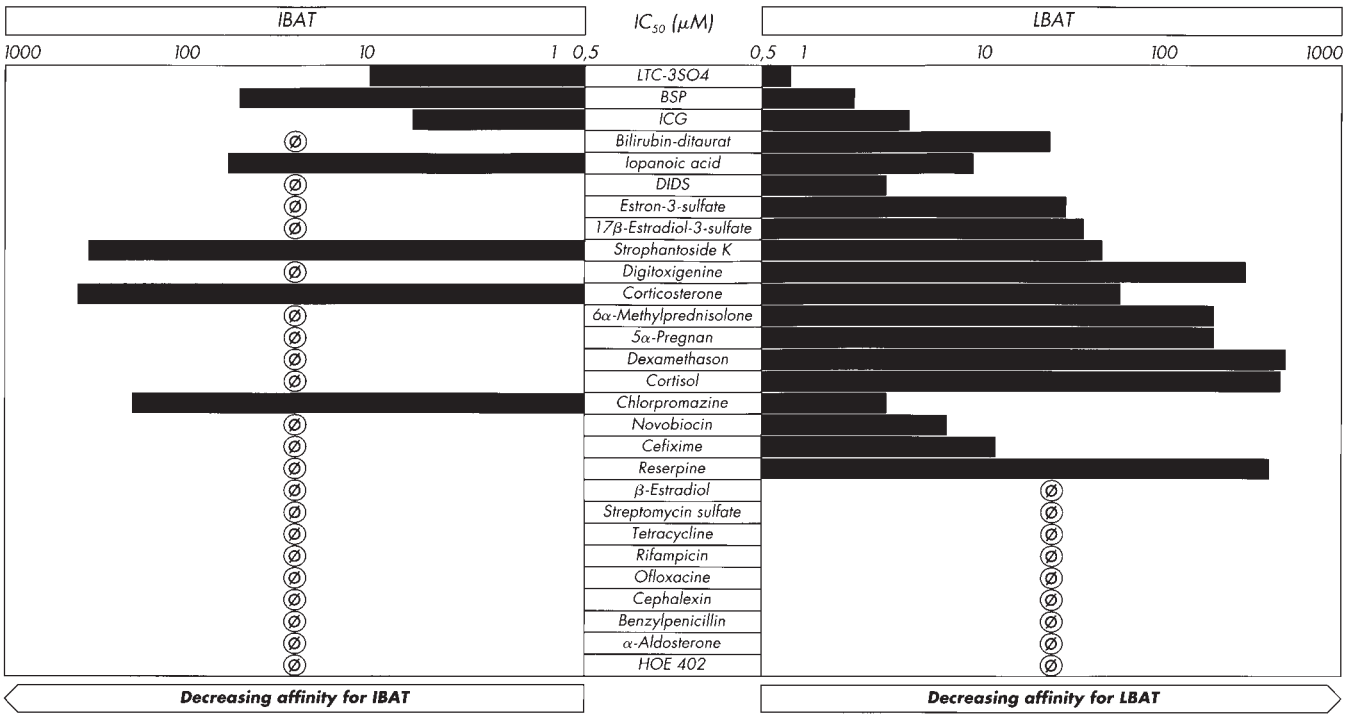


Fig. 1. Inhibition of [^{3}H]cholyltaurine uptake by cholephilic substrates into CHO cells expressing the rabbit ileal (IBAT) and rabbit liver (LBAT) Na^+/bile acid cotransporter. The uptake of 10 μm [^{3}H]cholyltaurine by CHO cells expressing the rabbit ileal (pKIBAT 8 cells) or rabbit liver Na^+/bile acid cotransporter (pKLBAT10 cells) was measured after incubation of cells with 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, or 10^{-3} m of the respective compounds for 30 min at 22°C followed by addition of 10 μm [^{3}H]cholyltaurine for 60 min. Uptake rates were plotted against inhibitor concentration and the IC_{50} values for inhibition of [^{3}H]cholyltaurine were calculated.

dexamethasone, and cortisol) had IC_{50} values of 200–400 μm. Of special importance is the effect of the anion transport inhibitor DIDS. DIDS strongly inhibited the Na^+/bile acid cotransporter from rabbit liver with an IC_{50} value of 3.1 μm but did not show any inhibition of the ileal Na^+/bile acid cotransport system. This difference is remarkable, especially as the anion transport inhibitor DIDS has been used for the identification of the liver bile acid transport system (48, 49) leading to the labeling of identical polypeptides as with reactive bile acid analogues (48, 49, 51). Photoaffinity labeling studies with photolabile bile acid analogues using rabbit ileal brush border membrane vesicles and sinusoidal membranes isolated from rat liver were in accordance with the transport inhibition studies. DIDS did not show any effect on the labeling pattern of rabbit ileal brush border membrane vesicles, whereas bromosulfophthalein and indocyanine green were able to suppress labeling of the 93 kDa bile acid binding polypeptide (21). The labeling of the bile acid binding polypeptides of M_r 48 kDa and 54 kDa was strongly suppressed upon photoaffinity labeling with the 7,7-azo-3α,12α-dihydroxy-5β[3β-^{3}H]cholan-24-oic acid or its taurine conjugate by DIDS, bromosulfophthalein, indocyanine green or the radioimaging agent iopodate in accordance with the transport studies (**Fig. 2**).

These extensive investigations demonstrate on a molecular level that the hepatic Na^+/bile acid cotransport system exerts a much broader specificity for the recognition of amphipathic compounds of diverse structure than its ileal counterpart. While the liver and ileal Na^+/bile acid cotransport systems share considerable sequence and predicted structural identity, these investigations demonstrate that the hepatic Na^+/bile acid cotransporter exhibits a much broader substrate specificity in accordance with preliminary earlier investigations (52, 53). Cyclosporin A was shown to inhibit the human ileal (52) as well as the rat hepatic transporter (53). Furthermore, testosterone, progesterone, and bumetamide were shown to inhibit the rat hepatic Na^+/bile acid cotransporter (53). It was thus of major importance to identify those structural elements that conferred the specific affinity for substrates and inhibitors in the ileal or hepatic transport systems, respectively. Consequently, we investigated the interaction of a large series of structurally different bile acid analogues with the ileal and hepatic Na^+/bile acid cotransporters.

Effect of bile acid analogues on [^{3}H]cholyltaurine transport in rabbit ileal brush border membrane vesicles

To identify molecular structure–activity relationships of bile acid derivatives for the ileal Na^+/bile salt cotransport system, we have investigated the effect of the bile acid analogues summarized in **Table 2** on Na^+-dependent [^{3}H]cholyltaurine uptake by brush border membrane vesicles isolated from rabbit ileum. The bile acid analogues led to a concentration-dependent inhibition of [^{3}H]cholyltaurine uptake by ileal brush border membrane vesicles, their inhibitory potency depending on the structure of the respective bile acid analogue. The concentrations necessary to achieve a 25% (IC_{25}), 50% (IC_{50}) or 75% (IC_{75}) inhibi-

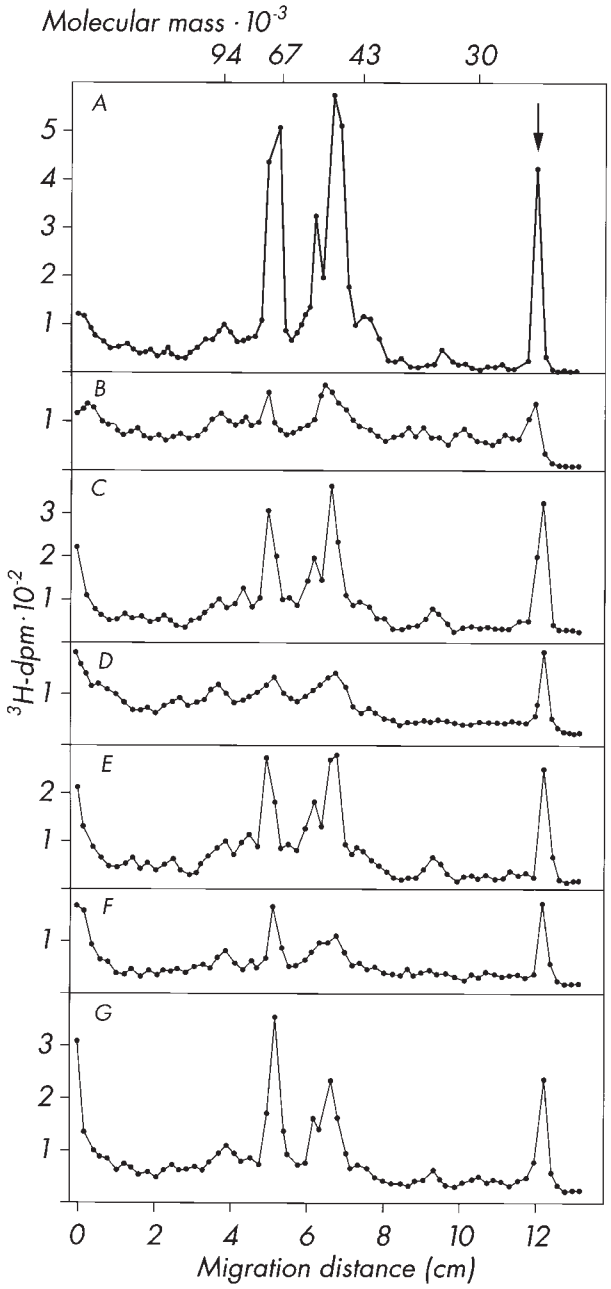


Fig. 2. Influence of cholephilic compounds on photoaffinity labeling of rat liver sinusoidal membranes by photolabile bile acids. Sinusoidal membranes prepared from rat liver (0.5 mg of protein) were incubated at 30°C in the dark for 10 min with 13.94 μm (10 μCi) 7,7-azo-3α,12α-dihydroxy-5β[3β,12β-^{3}H]cholan-24-oic acid in the absence (A) or presence of 400 μm of bromosulfophthalein (B), indocyanine green (C), DIDS (D), rifampicin (E), iopodate (F), or silybin (G) followed by ultraviolet irradiation at 350 nm for 10 min. After SDS-PAGE the distribution of radioactivity was determined by slicing of the gels in 2-mm pieces, hydrolysis of proteins with Biolute S, and liquid scintillation counting. The arrow indicates the gel front.

tion of initial Na^+-dependent uptake of 50 μm [^{3}H]cholyltaurine by these bile acid analogues are summarized in **Table 3**. Compared to the inhibitory potency of the reference compounds cholyltaurine and chenodeoxycholyltaurine, the different bile acid analogues could be grouped as follows with respect to their inhibitory potency:

higher affinity than TCDC: TLC, LC, 7,12-dihydroxycholanoate;
affinity similar to TCDC: TDC, DC;
affinity between TCDC and TC: $3N_3C$, $3N_3TC$, CDC, GDC, ˘$_2$C;
affinity similar to TC: UDC, allo-C, ˘$_2$TC;
weaker affinity than TC: TUDC, 12-ethinyl C, 12-ethinyl TC, 12KC, 12KTC, NorC, NorTC, αMC, αMTC, β-MC, β-MTC, ω-MTC, HDC, HDTC, HC, HTC, UC, UTC, UDTC, GC, 3KC, 3KTC;
no affinity: DHC, TDHC, ω-MC.

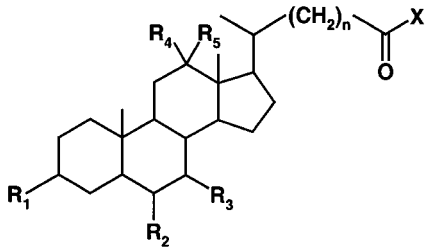
From these transport studies the following structure activity relationships can be developed.

Influence of the 3α-hydroxy group. The 3α-hydroxy group is generally considered to be important as it is present in all natural bile acids. However, the above results demonstrate that the 3α-hydroxy group is not essential for high affinity interaction with the ileal bile acid transport system in rabbit brush border membrane vesicles. In fact, removal of the 3α-hydroxy group increased the affinity of the bile acid analogues for the ileal transporter by a factor of 3–4. Substitution by an azido-function or modification by a hydrophobic diphenylmethyl moiety increased affinity whereas oxidation to a 3-carbonyl function decreased affinity. Epimerization at the 5 position to allocholate did not have a significant effect on the affinity to the ileal bile acid transporter (Table 3, panel I).

Influence of side chain conjugation. The inhibitory potency of the taurine conjugates was similar or slightly higher compared to the corresponding C-24-oic acids with the exception of two compounds, ursodeoxycholate and 3-O-diphenylmethylcholate (˘$_2$C), where taurine conjugation decreased IC_x values by a factor of 2 (Table 3, panel II). Therefore, with the exception of these compounds, the potency for inhibition of [^{3}H]cholyltaurine uptake is determined primarily by the substituents on the steroid nucleus. This finding is a very important step for determining the structure–activity relationships for molecular recognition and transport. Substitution of taurine by glycine decreased the affinity as did shortening of the side chain. The importance of an intact 4-methylbutanoyl side chain at carbon C-24 of the steroid nucleus for molecular recognition by the ileal Na^+/bile acid cotransporter is in accordance with our previous findings with HMG-CoA reductase inhibitor–bile acid hybrids where structural elements of a bile acid were combined with those of HMG-CoA reductase inhibitors to achieve liver-selective drugs (54, 55).

Influence of hydroxyl groups. The 12α-hydroxy group had a significant importance for molecular recognition of a bile acid molecule by the ileal bile acid transporter. Oxidation of the 12α-hydroxy function to a 12-carbonyl function or addition of an ethinyl group strongly decreased affinity whereas removal of the 12α-hydroxy group increased affinity (Table 3, panel III). Epimerization of the 7α-hydroxy group to the 7β-position in ursocholate decreased affinity by a factor of 3–4. Even more influential on the affinity was the role of the 7-position in the case of the dihydroxy bile acid chenodeoxycholate. Epimerization to ursodeoxy-

TABLE 2. Structure of bile acid analogues



Compound	Abbreviation	R_1	R_2	R_3	R_4	R_5	N	X
Cholate	C	α-OH	H	α-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Norcholate	NorC	α-OH	H	α-OH	α-OH	H	1	O^-, $NH(CH_2)_2$-SO_3^-
Ursocholate	UC	α-OH	H	β-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Hyocholate	HC	α-OH	α-OH	α-OH	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
α-Muricholate	α-MC	α-OH	β-OH	α-OH	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
β-Muricholate	β-MC	α-OH	β-OH	β-OH	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
ω-Muricholate	ω-MC	α-OH	α-OH	β-OH	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
12-Ethinylcholate	12-Ethinyl-C	α-OH	H	α-OH	ξ-OH	ξ-Ethinyl	2	O^-, $NH(CH_2)_2$-SO_3^-
12-Oxocholate	12-KC	α-OH	H	α-OH	O =	˘	2	O^-, $NH(CH_2)_2$-SO_3^-
Chenodeoxycholate	CDC	α-OH	H	α-OH	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Ursodeoxycholate	UDC	α-OH	H	β-OH	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Hyodeoxycholate	HDC	α-OH	α-OH	H	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Deoxycholate	DC	α-OH	H	H	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
7,12-Dihydroxy-cholanoate	7,12-DC	H	H	α-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
3-Oxocholate	3KC	O=	H	α-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
3β-Azidocholate	3-N_3-C	β-N_3	H	α-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
3-Diphenylmethyl-cholate	˘$_2$-C	α-$(C_6H_5)_2$CHO	H	α-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Lithocholate	LC	α-OH	H	H	H	H	2	O^-, $NH(CH_2)_2$-SO_3^-
Dehydrocholate	DHC	O=	H	O=	O=	˘	2	O^-, $NH(CH_2)_2$-SO_3^-
Glycocholate	GC	α-OH	H	α-OH	α-OH	H	2	NH-CH_2-COO^-
Glycochenodeoxy-cholate	GCDC	α-OH	H	α-OH	H	H	2	NH-CH_2-COO^-
Glycodeoxycholate	GDC	α-OH	H	H	α-OH	H	2	NH-CH_2-COO^-
Allocholate	AC	α-OH	H	α-OH	α-OH	H	2	O^-, $NH(CH_2)_2$-SO_3^-

cholate decreased the affinity about 7-fold whereas switching of the hydroxy group from the 7α- to the 6α-position in hyodeoxycholate reduced the affinity by a factor of 3–4. In contrast, removal of the 7α-hydroxy group to yield lithocholate increased the affinity to the ileal bile acid transporter. The strongest influence on the affinity of a bile acid to the ileal bile acid transport system was the introduction of an additional hydroxyl group into the 6-position of chenodeoxycholate; hyocholic acid and its taurine conjugate showed a 13-fold lesser affinity compared to chenodeoxycholate. As with hyocholic acid, the muricholic acids had a very low affinity to the ileal bile acid system with the ranking α > β > ω-muricholate for both the C-24 unconjugated acids and their taurine conjugates. It is evident that one hydroxy group either in position 7α or 6α mediates affinity to the ileal bile acid transport system whereas the concomitant presence of two hydroxyl groups in the 6- and 7-position strongly weakens the affinity to the ileal bile acid transport system.

Effect of bile acid analogues on [^{3}H]cholyltaurine uptake by CHO cells transfected with the cDNAs of the rabbit ileal and hepatic Na^+/bile acid cotransporters

In order to gain insight into the molecular differences in bile acid substrate recognition by the bile acid transporters, we used CHO cells stably transfected with the rabbit ileal or liver Na^+/bile acid cotransporter to measure the inhibition of [^{3}H]cholyltaurine uptake by the same series of bile acid analogues. **Table 4** shows that most of the bile acid analogues exhibited a similar ranking in their IC_{50} values for the rabbit ileal Na^+/bile acid cotransporter in transfected CHO cells and the ileal brush border membranes. Clear differences were found in the IC_{50} values for inhibition of [^{3}H]cholyltaurine uptake between the ileal and hepatic Na^+/bile acid cotransporter of the rabbit. Generally, the IC_{50} values for most compounds were at lower concentrations for the hepatic transport system, indicating a relatively higher affinity of these bile acids to the hepatic transporter. A comparable affinity to both transport systems was found for the most abundant physiological bile acids cholyltaurine, deoxycholyltaurine, chenodeoxycholyltaurine, and cholylglycine as well as for norcholyltaurine and 12-ethinylcholyltaurine. Bile acids carrying hydroxyl groups simultaneously at positions 6 and 7 (hyocholyltaurine, α-, β- or ω-muricholyltaurine) showed only a weak interaction with the ileal system but showed a remarkable high affinity to the hepatic transporter. Dehydrocholyltaurine and ω-muricholyltaurine had IC_{50} values of 900 and 800 μm for the ileal transporter, respectively, indicating a very weak affinity. In contrast, IC_{50} values of 2.25 and 7 μm indicate a high affinity to the hepatic Na^+/bile acid cotransporter with a 400- and 72-fold preference, respectively, for the hepatic transporter compared to the ileal system (measured as ratio of the IC_{50} values for the ileal and hepatic transporter).

TABLE 3. Inhibition constants of bile acid analogues for inhibition of [^{3}H]cholyltaurine uptake by rabbit ileal brush border membrane vesicles

I. Modification at the 3-position

Bile Acid	IC_{25} A	IC_{25} T	IC_{50} A	IC_{50} T	IC_{75} A	IC_{75} T
C	25	20	67	51	147	109
3-KC	35	21	151	72	>300	166
3-N_3C	23	16	47	40	94	91
˘ $_2$C	15	26	29	53	44	55
7,12-DC	8	n.d.	17	n.d.	25	n.d.

II. Effect of side chain conjugation

Bile Acid	IC_{25} A	IC_{25} T	IC_{25} G	IC_{50} A	IC_{50} T	IC_{50} G	IC_{75} A	IC_{75} T	IC_{75} G
C	25	20	70	67	51	121	147	109	210
AlloC	10.7	20	n.d.	21.5	67.1	n.d.	200	151	n.d.
NorC	122	57	n.d.	244	135	n.d.	>300	>300	n.d.
CDC	14	10	14	31	21	31	61	44	61
DC	10	10	28	19	20	41	38	43	64
UDC	24	78	n.d.	74	141	n.d.	135	>200	n.d.
LC	8	6	n.d.	15	13	n.d.	23	19	n.d.

III. Modification at hydroxyl groups

Bile Acid	IC_{25} A	IC_{25} T	IC_{50} A	IC_{50} T	IC_{75} A	IC_{75} T
C	25	20	67	51	147	109
CDC	14	10	31	21	61	44
DC	10	10	19	20	38	43
UDC	24	78	74	141	135	>200
HDC	49	29	122	71	˘	˘
HC	175	132	˘	˘	˘	˘
UC	80	67	178	190	˘	˘
12-KC	104	66	231	196	˘	˘
α-MC	73	77	154	173	˘	˘
β-MC	108	85	229	196	˘	˘
ω-MC	˘	145	˘	˘	˘	˘
12-Ethinyl-C	64	52	162	139	˘	˘
DHC	˘	˘	˘	˘	˘	˘

The Na^+-dependent uptake of 50 μm [^{3}H]cholyltaurine into rabbit ileal brush border membrane vesicles was measured as described in Materials and Methods followed by determination of IC_x values (the IC_x value indicates that concentration of compound necessary to achieve x% of inhbition of [^{3}H]cholyltaurine uptake); A, unconjugated bile acid; T, taurine conjugate of bile acid; G, glycine conjugate of bile acid.

TABLE 4. IC_{50} values of taurine-conjugated bile acid analogues for inhibition of [^{3}H]cholyltaurine uptake by rabbit ileal brush border membrane vesicles pKIBAT8 and pKLBAT10 cells

Taurine Conjugate of	IC_{50} Value pKIBAT8 cells	IC_{50} Values pKLBAT10 cells	Ratio of IC_{50} Values pKIBAT8 / pKLBAT10
C	25	36.5	0.69
NorC	39	47.5	0.82
UC	77	51	1.5
HC	175	9	19.4
α-MC	68	7	9.7
β-MC	79	21	3.76
ω-MC	800	11	72.72
12-Ethinyl-C	65	75	0.86
12-KC	150	107	1.40
CDC	4.45	2.0	2.23
UDC	17	3.0	5.66
HDC	10.5	4.15	2.53
DC	4.60	4.5	1.02
3KC	33	5.6	5.89
3-N_3C	13.75	5.5	2.5
˘ $_2$C	18.5	2.0	9.25
DHC	900	2.25	400
GC	31	30	1.03
Allo-C	150	26	5.77

The Na^+-dependent uptake of [^{3}H]cholyltaurine into rabbit ileal brush border membrane vesicles (50 μm) or CHO cells (10 μm) transfected with the cDNA of the rabbit ileal (pKIBAT8) or rabbit hepatic (pKLBAT10) cells was measured followed by determination of IC_{50} values. The ratio of IC_{50} values

$$\frac{\text{pKIBAT8}}{\text{pKLBAT10}}$$

is a measure for a preference to the hepatic transporter.

Uptake of [^{3}H]taurine-labeled bile acid conjugates by rabbit ileal brush border membrane vesicles

In order to evaluate whether the different affinities of the bile acid derivatives to the ileal or hepatic transport system are also reflected in a parallel ranking for the transport rates, we have measured the uptake of the [^{3}H]taurine-labeled conjugates of the bile acids shown in Table 2 by rabbit ileal brush border membrane vesicles. **Figure 3A** shows the uptake of selected [^{3}H]taurine-conjugated bile acid derivatives by rabbit ileal brush border membrane vesicles at a bile acid concentration of 50 μm. An in depth analysis of all bile acid analogues tested (**Table 5**) revealed that the dihydroxy bile acids chenodeoxycholyltaurine, deoxycholyltaurine, and (3β-azido-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonate showed higher uptake rates compared to cholyltaurine. Cholate, cholylglycine, norcholyltaurine, ursodeoxycholyltaurine, allocholyltaurine, and (7α,12α-dihydroxy-3-oxo-5β-cholan-24-oyl)- 2-aminethanesulfonate had initial uptake rates ranging from 52 to 82% compared to cholyltaurine. Oxidation of the 12α-hydroxy group in cholyltaurine to a 12-keto function or introduction of an additional 12-ethinyl group reduced uptake to 20–37%. All 6-hydroxylated bile acids showed low uptake rates with the ranking for uptake β-muricholyltaurine > α-muricholyltaurine > ursocholyltaurine > hyodeoxycholyltaurine > hyocholyltaurine > ω-muricholyltaurine. Dehydrocholyltaurine showed around 10–14% of initial uptake rate compared to cholyltaurine. 7α,12α-Dihydroxycholanoic acid and its taurine conjugate are unnatural bile acids having no functional group at the 3-position of the steroid nucleus. The unconjugated derivative strongly inhibited [^{3}H]cholyltaurine uptake in brush border membrane vesicles with an IC_{50} value of 17 μm similar to deoxycholate. In order to determine whether bile acids without any functional group at position 3 are transported by the ileal Na^+/bile acid cotransporter, we measured uptake of [^{3}H]7α, 12α-dihydroxycholanoyltaurine. As 7α,12α-dihydroxycholanoyltaurine was not available in sufficient amounts, we measured uptake at a substrate concentration of 0.2 μm as well as the uptake of the other bile acids at this concentration (Fig. 3B). Under these conditions, [^{3}H]7α,12α-dihydroxycholanoyltaurine showed a 3.3-fold higher uptake rate compared to cholyltaurine, even higher

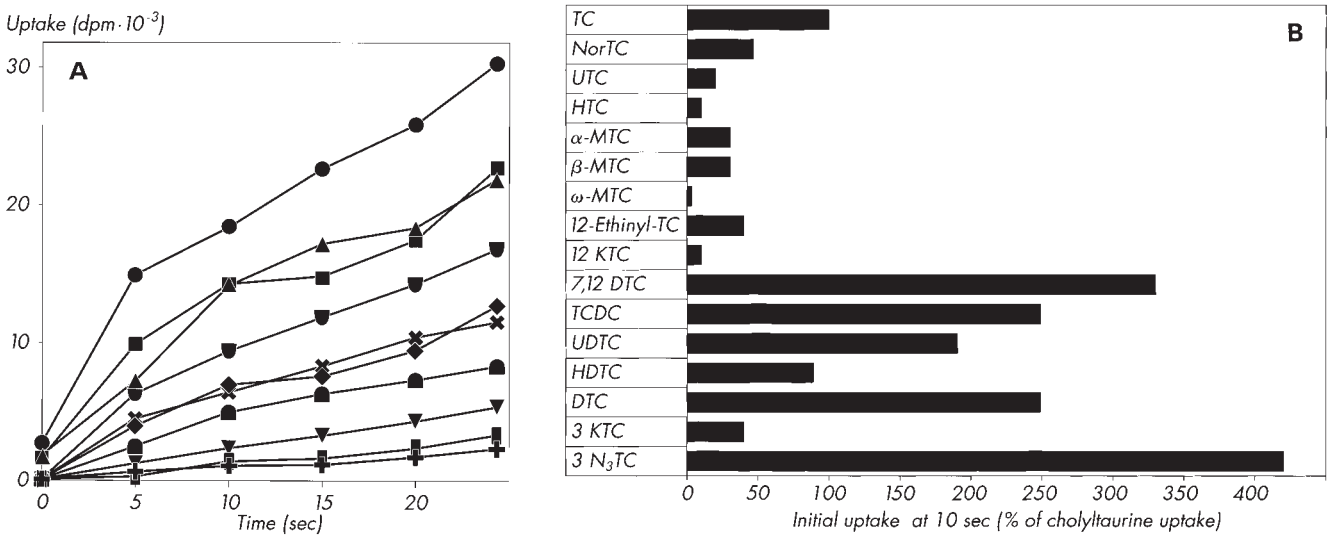


Fig. 3. Uptake of [^{3}H]taurine-conjugated bile acid analogues by rabbit ileal brush border membrane vesicles. Rabbit ileal brush border membrane vesicles (50 μg of protein) preloaded with 10 mm Tris/HCl buffer (pH 7.4)/300 mm mannitol were incubated at 30°C with radiolabeled bile acid analogues in 10 mm Tris/HCl buffer, 100 mm NaCl, 100 mm mannitol (pH 7.4) and uptake was measured for the indicated periods of time. A: Uptake at a concentration of 50 μm, ●-●: +3β-azido-7α,12α-dihydroxycholanoyltaurine; ■-■: + chenodeoxycholyltaurine; ▲-▲: + deoxycholyltaurine; ▾-▾: + cholyltaurine; ◆-◆: + ursodeoxycholyltaurine; x-x: + cholylglycine; ◓-◓: + norcholyltaurine; ▼-▼: + hyodeoxycholyltaurine; ▮-▮: + hyocholyltaurine; ✚-✚: + ω-muricholyltaurine. B: Uptake at a concentration of 0.2 μm. Uptake is shown as uptake compared to cholyltaurine after 10 sec of incubation.

than chenodeoxy- and deoxycholyltaurine with a 2.5-fold higher uptake rate. The best substrate at a concentration of 0.2 μm was 3β-azido-7α,12α-dihydroxycholanoyltaurine with a 4-fold higher uptake rate compared to cholyltaurine. The other bile acids showed the same ranking for uptake as with a substrate concentration of 50 μm.

TABLE 5. Uptake rates of [^{3}H]taurine-conjugated bile acid analogues by rabbit ileal brush border membrane vesicles, pKIBAT8 and pKLBAT10 cells

Bile Acid Derivative	BBMV	pKIBAT8 Cells	pKLBAT10 Cells
TC	100	100	100
C	n.d.	66.5 ± 0.70	83 ± 5.66
GC	70 ± 4.24	84 ± 2.83	73 ± 1.41
NorTC	55 ± 2.83	67 ± 12.56	84 ± 14.14
AlloTC	82	209 ± 14.14	113.5 ± 13.43
HTC	11.79 ± 5.94	17.5 ± 6.36	95.3 ± 19.6
UTC	30.3 ± 3.2	27 ± 5.94	52 ± 25.04
α-MTC	35.5 ± 12.03	46 ± 10.53	53 ± 16.37
β-MTC	41.46 ± 10.9	61.6 ± 13.05	77.5 ± 7.78
ω-MTC	8.33 ± 4.93	11.6 ± 3.51	61.6 ± 16.74
12-KTC	19.5 ± 0.71	33.75 ± 10.81	65 ± 22.22
12-Ethinyl-TC	37.5 ± 3.53	53 ± 18.73	61.5 ± 20.5
3-N_3C	120.6 ± 39.51	114 ± 44.44	180 ± 77
3-KTC	52	123 ± 2.83	101.5 ± 16.22
ˇ$_2$TC	n.d.	42.5 ± 0.70	145 ± 31
TCDC	143.7 ± 27.1	134.5 ± 37.41	143 ± 1.41
TDC	146.6 ± 24.8	130 ± 19.79	138 ± 9.89
UDTC	74.06 ± 14.3	100 ± 18.35	83 ± 0
HDTC	27.99 ± 8.67	52 ± 10.82	107.6 ± 21.96
DHTC	14.37 ± 11.1	3 ± 0	40.5 ± 7.78

The Na^+-dependent uptake of [^{3}H]cholyltaurine into rabbit ileal brush border membrane vesicles (50 μm) or CHO cells (10 μm) transfected with the cDNA of the rabbit ileal (pKIBAT8) or rabbit hepatic (pKLBAT10) Na^+/bile acid cotransporter was measured (1 min for brush border membrane vesicles, 8 min for CHO cells). Uptake rates are expressed as % uptake compared to [^{3}H]cholyltaurine. The values are the mean of ± SD of 2–4 independent measurements.

Uptake of [^{3}H]taurine conjugated bile acid analogues by CHO cells transfected with the cDNAs of the rabbit ileal and hepatic Na^+/bile acid cotransporter

Transport measurements with CHO cells transfected with the cDNA of the rabbit ileal Na^+/bile acid cotransporter revealed a similar ranking for uptake as with rabbit ileal brush border membrane vesicles. The dihydroxy bile acids chenodeoxycholyltaurine, deoxycholyltaurine, 3β-azido-7α,12α-dihydroxycholanoyl-taurine, 7α,12α-dihydroxy-3-oxo-cholanoyltaurine, and allocholyltaurine showed the highest uptake rates whereas the taurine conjugates of hyo-, hyodeoxy-, urso-, or the muricholic derivatives, 3α,7α-dihydroxy-12-oxo-cholanoyltaurine and dehydrocholyltaurine showed low uptake rates (Table 5, **Fig. 4**). CHO cells transfected with the cDNA of the rabbit liver transporter revealed striking differences to the ileal transporter. In general, the relative uptake rates for cholyltaurine were higher than with the ileal transporter. The relative uptake rates for cholylglycine, ursodeoxycholyltaurine, deoxycholyltaurine, and chenodeoxycholyltaurine were comparable for both transporters (Table 5). 6-Hydroxylated bile acids were handled quite differently by the ileal and the hepatic Na^+/bile acid transporters. Hyodeoxycholyltaurine, hyocholyltaurine, and ω-muricholyltaurine all contain an α-oriented hydroxyl group at the 6-position of the steroid nucleus. Whereas the shift of the 7α-hydroxy group of chenodeoxycholyltaurine to the 6-position to obtain hyodeoxycholyltaurine decreased the IC_{50} values for inhibition of [^{3}H]cholyltaurine by a factor of 2.5 and the initial uptake rate for uptake by pKIBAT 8 cells by a factor of 2, the hepatic system tolerated this structural change well with a minimal decrease of the relative uptake rate from 143% to 108% compared to cholyltaurine. As

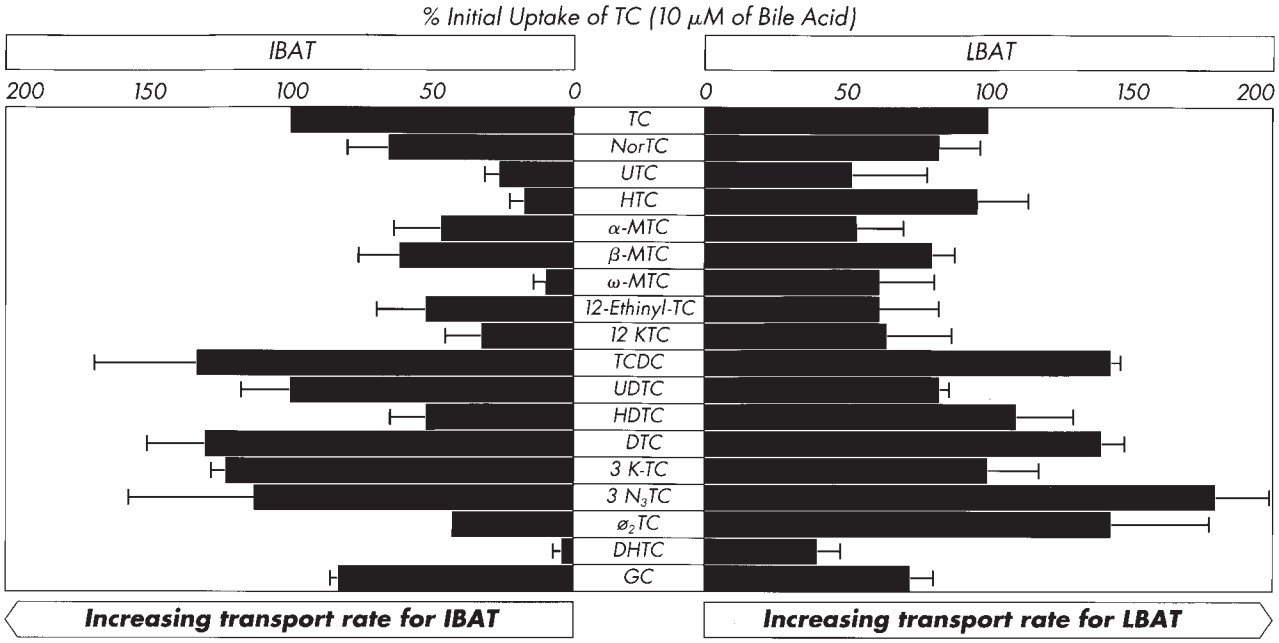


Fig. 4. Uptake of [^{3}H]taurine-conjugated bile acid analogues by recombinant CHO cells transfected with the rabbit ileal (pKIBAT8) or rabbit liver (pKLBAT5) Na^+/bile acid cotransporter. Recombinant CHO cells were incubated at 22°C with 10 μm of radiolabeled bile acid analogues and uptake was measured after 1, 2, 4, 6, and 8 min of incubation as described under Experimental Procedures.

with the ileal bile acid transport system, the presence of a 3α-hydroxyl group at the steroid nucleus is not essential for transport by the hepatic Na^+/bile acid cotransporter as is evident from the high uptake rate for 3β-azido-7α, 12α-dihydroxycholanoyltaurine (180%), the 3-diphenylmethyl derivative of cholyltaurine (145%), or 7α,12α-dihydroxy-3-oxo-cholanoyltaurine (101%). For both transport systems a slight decrease of uptake was observed after

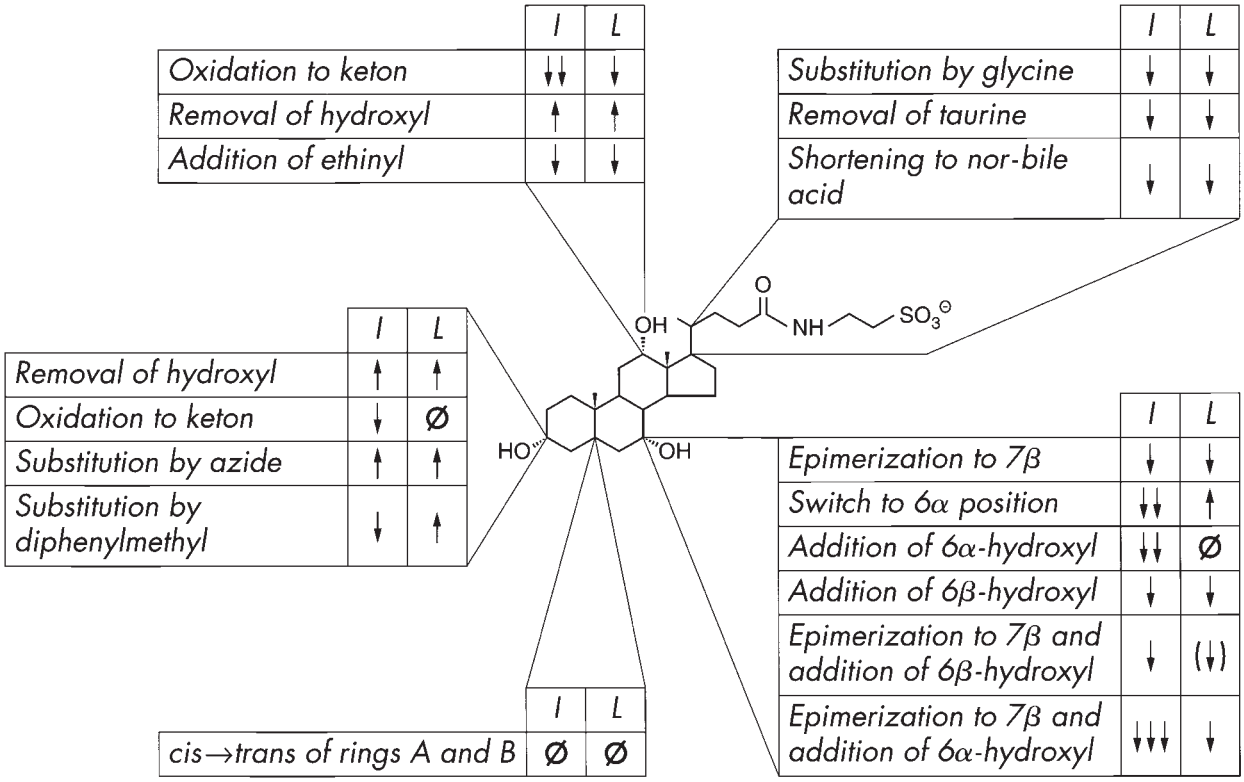


Fig. 5. Structure–activity relationships of bile acids for molecular recognition by Na^+/bile acid cotransporters from rabbit ileum (I) and rabbit liver (L).

shortening of the bile acid side chain by one methylene group to yield norcholyltaurine. This indicates a similar molecular recognition of the bile acid side chain by the ileal and the hepatic transporters. Oxidation of the 12α-hydroxy group of cholyltaurine led to a 3- to 5-fold decrease of uptake rate by the ileal system but only a 35% reduction of uptake by the hepatic transporter, indicating a lesser importance of the 12α-hydroxy group for molecular recognition of a bile acid molecule by the hepatic system. Whereas dehydrocholyltaurine was not significantly transported by the ileal system, this analogue was a moderate substrate for the hepatic Na^+/bile acid cotransporter and exhibited 40% relative uptake compared to cholyltaurine. Taken together, the structure–activity relationships for the bile acid derivatives with the ileal and hepatic Na^+/bile acid cotransporters are summarized in **Fig. 5** and are consistent with findings for the cholephilic substrates. Overall, the hepatic Na^+/bile acid cotransporter exhibited a significantly broader substrate specificity than the related ileal system. The transport and inhibition studies clearly demonstrated that the 3α-hydroxy group is neither necessary for molecular recognition nor for transport by the active Na^+-dependent bile acid transporters from ileum or liver. We therefore investigated the behavior of these bile acid derivatives using an in vivo ileal perfusion model.

In vivo ileal perfusion with [^{3}H]taurine-labeled bile acid derivatives

Ileal absorption is the rate-limiting step for the transport of natural bile acids from the intestinal lumen into bile; the appearance of bile acids in bile therefore reflects the ileal absorption rate. In a further series of experiments we investigated the structure–activity relationships of bile acids in the enterohepatic circulation in vivo. For this we used a closed-loop ileum perfusion model, where the radiolabeled bile acids (25 μm) were instilled into a small ileal segment which was perfused with a flow rate of 0.25 ml/min. After cannulation of the common bile duct, bile was fractionated over a period of 90 min. For practical reasons, to minimize the necessary amounts of compounds, we used the rat for the in vivo perfusion model. By transport experiments with transfected CHO cells we could show that the ileal Na^+/bile acid cotransporter from humans, rabbit, and rat have a very similar substrate specificity. Therefore, the in vivo perfusion experiments performed in the rat have a high predictability for the rabbit and human ileal transporter. An alternate model where the ileal segment is perfused with a constant concentration of bile acids could not be used owing to the high amount of compounds necessary for this model. As in the closed-loop model, the radiolabeled bile acids in the ileal lumen remain in contact with the absorbing mucosal surface during the entire experiment; the recovery of bile acids in bile after definite periods does not necessarily reflect differences caused by different ileal absorption rates as would be the case with the open perfusion model. The recoveries in bile after 90 min of collection, therefore, did not correlate with the transport rates for the ileal or hepatic Na^+/bile acid transporters. For most of the bile acid analogues investigated, recovery rates between 64 and 98% were found. Only the taurine conjugate of 3α-(diphenylmethyl)cholate had a significant lower recovery of only 26–44% after 90 min. Bile acids carrying a 3α-hydroxy group showed their maximum of biliary secretion between 12 and 16 min. A clearly delayed secretion maximum was observed for all bile acid analogues lacking a 3α-hydroxy group with the exception of 7α,12α-dihydroxy-3-oxo-cholanoyltaurine which showed a behavior similar to cholyltaurine. Most of the bile acid analogues carrying a 3α-hydroxy group were secreted unchanged into bile without any significant metabolism. Radio thin-layer analysis of the secreted biles revealed that ursodeoxycholyltaurine partially converted to chenodeoxycholyltaurine whereas the 3-oxo-derivative of cholyltaurine was completely reduced to cholyltaurine during liver passage explaining its secretion profile comparable to cholyltaurine. Secretion maxima were observed after 40 min for 3β-azido-7α,12α-dihydroxy-cholanoyltaurine, or after 60 min for 7α,12α-dihydroxy-cholanoyltaurine, 3α-diphenylmethylcholyl-taurine and dehydrocholyltaurine (**Fig. 6**) compared to 12 min for chenodeoxycholyltaurine and 16 min for cholylglycine. As the first two compounds showed high transport rates for both, the ileal as well as the hepatic Na^+/bile acid cotransporter, their in vivo pharmacokinetic behavior with a delayed secretion into bile may be caused either by a low transport rate from the ileocyte into blood or by a delayed hepatobiliary secretion across the hepatocyte canalicular membrane. In vivo liver perfusion experiments demonstrated a delayed hepatobiliary secretion of bile acid analogues lacking a free 3α-hydroxy group, suggesting that the efficacious secretion of a bile acid across the canalicular membrane depends on the presence of the 3α-hydroxy group.

DISCUSSION

The investigations performed in this study demonstrate that the hepatic Na^+/bile acid cotransport system exhibits a much broader substrate specificity compared to its ileal counterpart. Whereas only bromosulfophthalein and indocyanine green showed a significant inhibition of cholyltaurine transport by the rabbit ileal Na^+/bile acid cotransporter, the majority of the compounds tested were able to interact with the hepatic system. Among the steroids investigated, all except β-estradiol and α-aldosterone were inhibitors of the hepatic Na^+/bile acid cotransporter whereas only corticosterone and strophantoside K exerted a minimal effect on the ileal system. The organic anion transport inhibitor DIDS or bilirubin ditaurate were strong inhibitors of the hepatic transporter without any affinity to the ileal system. Among the antibiotics investigated, only novobiocin and the dianionic cephalosporin cefixime inhibited the hepatic transporter whereas the other antibiotics including penicillin, cephalosporins, tetracyclin, rifampicin, and gyrase inhibitors inhibited neither the ileal nor the hepatic bile acid transport systems. The interaction of various steroid hormones with hepatic bile acid transport may be of medical importance in patients with impaired liver function.

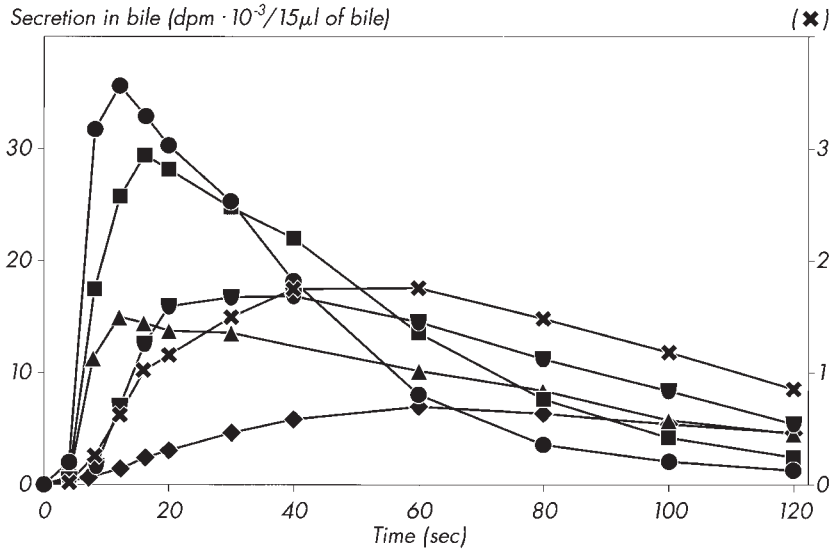


Fig. 6. Biliary secretion profiles of bile acid derivatives after in situ ileal perfusion. Ileal perfusion was performed in a closed loop model in the rat after instillation of 2 ml of a solution containing 137 mM NaCl, 0.9 mM $CaCl_2$, 0.51 mM $MgCl_2$, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 147 mM KH_2PO_4 (pH 7.4) containing the indicated concentrations of [^{3}H]taurine-labeled bile acid derivatives. Bile was collected after cannulation of the common bile duct and determination of the radioactivity in bile by liquid scintillation counting; ●–●: 25 µm chenodeoxycholyltaurine; ■–■: 25 µm cholylglycine; ◆–◆: 25 µm 3α-diphenylmethyl-7α,12α-dihydroxycholanoyltaurine; ▼–▼: 25 µm 3β-azido-7α,12α-dihydroxycholanoyltaurine; ▲–▲ : 25 µm dehydrocholyltaurine; ✖–✖: 1 µm 7α,12α-dihydroxycholanoyltaurine.

The investigations with the bile acid analogues led to clear and definite structure–activity relationships of a bile acid with the Na^+/bile acid cotransport systems from the ileum and the liver (Fig. 5).

Whereas the uptake rates of the major bile acids cholyltaurine, chenodeoxycholyltaurine, deoxycholyltaurine, and cholylglycine were comparable for the ileal and the hepatic transport systems, strong differences in the molecular recognition were found for bile acids carrying a hydroxy group at position 6. The affinities and transport rates of 6-hydroxylated bile acids (hyo-, hyodeoxy-, and muricholic acids) were low for the ileal systems, whereas these 6-hydroxylated hydrophilic bile acids are preferred substrates for the hepatic transport system.

Both the ileal and the hepatic Na^+/bile acid cotransporters recognize and transport bile acid derivatives lacking a hydroxy group at position 3 of the steroid nucleus; 3β-azido-7α,12α-dihydroxycholanoyltaurine and 7α,12α-dihydroxy-cholanoyltaurine show higher uptake rates by both transporters comparable to the physiological dihydroxy bile acids chenodeoxy- and deoxycholyltaurine whereas 3α-diphenylmethoxy-7α,12α-dihydroxycholanoyltaurine is a preferred substrate for the hepatic transporter.

Dehydrocholanoyltaurine, a non micelle-forming bile acid analogue lacking any hydroxy group, shows a negligible affinity and transport rate by the ileal transporter but it has a 40% relative uptake rate compared to cholyltaurine, a moderate substrate for the hepatic Na^+/bile acid cotransporter.

The different affinities of bile derivatives to the ileal and hepatic Na^+/bile acid cotransporters reflect the different physiological functions for the ileum and the liver. The ileal transporter conserves the majority of natural primary bile acids for the organism whereas the liver excretes metabolites and waste products. The observation that both the ileal as well as the hepatic Na^+/bile acid cotransport systems recognize and transport bile acid derivatives lacking a 3α-hydroxy group clearly demonstrates that the 3α-hydroxy group being present in all naturally occurring bile acids is not necessary for molecular recognition by the ileal and the hepatic active Na^+/bile acid cotransport systems. This raises the question concerning the physiological function of the 3α-hydroxy group in natural bile acids. In vivo ileum and liver perfusion studies demonstrated that bile acid analogues lacking a free 3α-hydroxy group showed a delayed secretion pattern into bile compared to 3α-hydroxylated bile acid derivatives, suggesting that a free 3α-hydroxy group is necessary for optimal transport of a bile acid across the hepatocyte canalicular membrane. Transport studies with cell lines transfected with the putative canalicular bile acid transporters using the different radiolabeled bile acid analogues described above are necessary to evaluate the molecular function of the 3α-hydroxy group of a bile acid for transport in the enterohepatic circulation.

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