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Review

Exploitation of Bile Acid Transport Systems in Prodrug Design

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Abstract: The enterohepatic circulation of bile acids is one of the most efficient recycling routes in the human body. It is a complex process involving numerous transport proteins, which serve to transport bile acids from the small intestine into portal circulation, from the portal circulation into the hepatocyte, from the hepatocyte into the bile, and from the gall bladder to the small intestine. The tremendous transport capacity and organ specificity of enterohepatic circulation combined with versatile derivatization possibilities, rigid steroidal backbone, enantiomeric purity, availability, and low cost have made bile acids attractive tools in designing pharmacological hybrid molecules and prodrugs with the view of improving intestinal absorption, increasing the metabolic stability of pharmaceuticals, specifically targeting drugs to organs involved in enterohepatic circulation, as well as sustaining therapeutically reasonable systemic concentrations of active agents. This article briefly describes bile acid transport proteins involved in enterohepatic circulation, summarizes the key factors affecting on the transport by these proteins, and reviews the use of bile acids and their derivatives in designing prodrugs capable of exploiting the bile acid transport system.

Keywords: Bile acids, Prodrugs, Active Transport, Carrier-Mediated Transport, Drug Targeting

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Introduction

Several potential drug molecules fail in the developmental phase, since they lack features that enable them to overcome barriers. These barriers can be economical, physical, chemical, or biological. The biological barriers, *e.g.* epithelial, endothelial, and/or elimination barriers, restrict access of the drug molecule to its site of action. A vast amount of strategies have been developed for efficient delivery of a drug molecule from the site of administration to its pharmacological target in the body. It can be incorporated into a delivery system, equipped with structural features necessary for interacting with the target and/or for crossing the barriers, as well as conjugated with a non-toxic and rapidly excreted promoiety, which once past the barrier will be cleaved from the parent drug or its active form (prodrug strategies). The traditional prodrug approach aims to mask undesirable drug properties, such as bad taste or odor, pain or irritation at injection sites, gastrointestinal irritability, poor solubility or stability, incomplete absorption, premature metabolism, or toxicity. Recently, prodrug design has shifted towards targeting specific enzymes or membrane transporters [1].

Bile acids, which are steroidal molecules derived from the catabolism of cholesterol and essential for the digestion and absorption of lipids and lipid-soluble vitamins [2], pass through several cellular membranes *via* both active and passive transport processes during enterohepatic circulation [3-8]. The classes of transporters specific for bile acids consist of Na^+-dependent transport proteins that mediate reabsorption from the intestine and biliary epithelium as well as uptake into hepatocytes from the portal circulation as well as an ATP-dependent transport protein that pumps bile acids into bile. In addition, Na^+-independent organic anion carriers with broad multi-substrate specificities including bile acids have been identified [2].

Due to the identification and cloning of different nutrient transporters in recent years, the active nutrient transport systems have become a target for prodrug design [1]. A successful example of a prodrug approach to target an intestinal transporter to enhance oral absorption is valacyclovir, an amino acid ester prodrug of acyclovir. The bioavailability of acyclovir *via* oral administration was shown to markedly enhance as a result of conjugation to valine, since valine-containing di- and tripeptides act as substrates for the small intestine peptide transporter [9, 10].

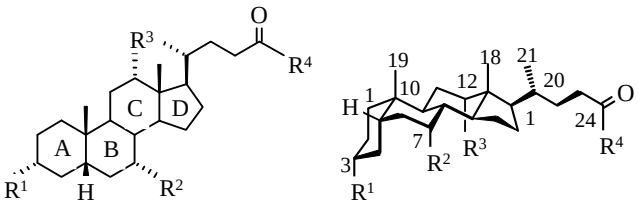
Bile acids consist of a convex hydrophobic upper side, a concave hydrophilic α-side, and a polar side chain, which makes them amphiphilic by nature [8]. The rigid steroidal backbone, chemically different hydroxyl groups, enantiomeric purity, availability, and low cost make bile acids ideal building blocks for various synthetic purposes [11-18]. Because of the specificity and high capacity of the ileal and hepatic transport systems for bile acids, attempts have been made to take advantage of these properties and the organotropism of bile acids for pharmaceutical applications [8, 19]. Additionally, the intestinal bile acid transport system is a key regulator of the cholesterol homeostasis in the human body. Specific inhibition of the bile acid transporters is thus another important medical application [20].

This overview is aimed at highlighting the chemistry, biochemistry, and physiological functions of bile acids, their recirculation in the body and proteins involved in this complex process as well as factors affecting on the transport activity from the membrane protein-targeted prodrug design point of view not forgetting the fascinating examples of bile acid-containing prodrug applications.

Bile Acids – Chemistry, Biochemistry, and Physiological Functions

The most abundant naturally occurring bile acids in higher vertebrates are derivatives of cholanic acid (or cholan-24-oic-acid), a cyclopentanoperhydrophenanthrene ring-containing steroid consisting of 24 carbon atoms (see Figure 1). The two isomeric groups of cholanic acids are 5β- and 5α- (or *allo*) cholanic acids, the former possessesing *cis*- and the latter *trans*-oriented plane of fusion of the A/B rings [21]. A common structural feature of all natural bile acids is the *cis* configuration at C-5 combined with a pentanoic acid side chain attached to ring D. Their differences are determined by the number, position, and stereochemistry of the hydroxyl groups [8]. The bile acid nucleus possesses a curved profile with the angular methyl groups at positions C_{18} and C_{19} on the convex hydrophobic β side and the hydroxyl groups on the concave hydrophilic α side. Consequently, bile acids are facially amphipathic [8,18]. Reactions of the bile acids might be expected at their various functional groups. The carboxylic acid group may be esterified, reduced, amidated, or subjected to salt formation with metal ions, alkaloids, or organic bases. The reactivity of the hydroxyl groups towards oxidation is $C_7 > C_{12} > C_3$ and $C_6 > C_3$ [21]. It, however, depends on the oxidative agent: oxidation of cholic acid with *N*-bromosuccinimide preferentially yields the 7-oxo-derivative, whereas Oppenauer-oxidation with aluminum *t*-butoxide of methyl cholate favors formation of the 3-oxo product. The order of acetylation, hydrolysis, *vis-á-vis* reduction, or hydrogenation is $C_3 > C_7 > C_{12}$ [21].

Figure 1. Structures of the most important bile acids and amino acid conjugates of cholic acid.

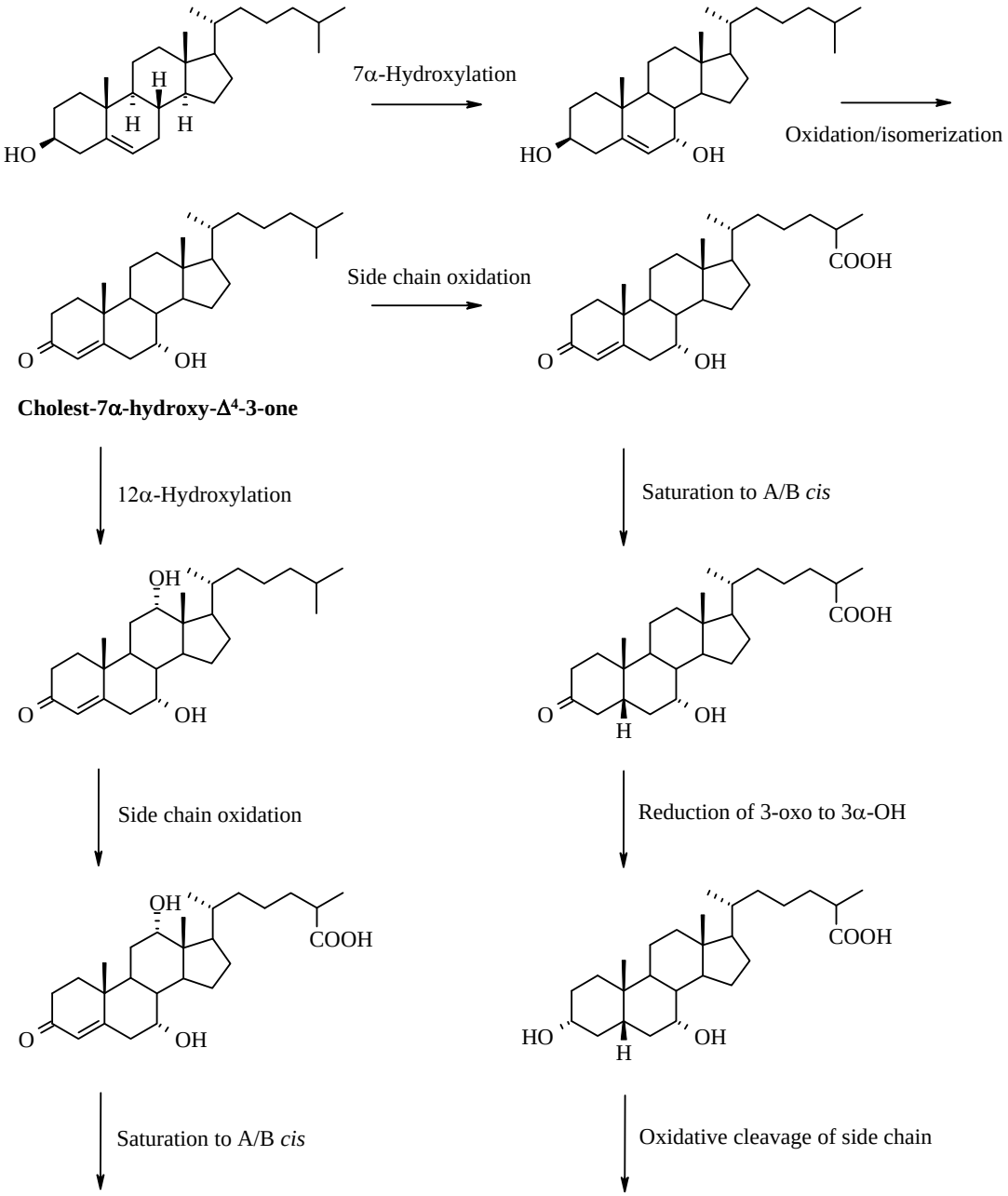


	R^1	R^2	R^3	R^4
cholanic acid	H	H	H	OH
cholic acid	OH	OH	OH	OH
chenodeoxycholic acid	OH	OH	H	OH
deoxycholic acid	OH	H	OH	OH
lithocholic acid	OH	H	H	OH
glycocholate	OH	OH	OH	$NHCH_2CO_2^-$
taurocholate	OH	OH	OH	$NHCH_2CH_2SO_3^-$

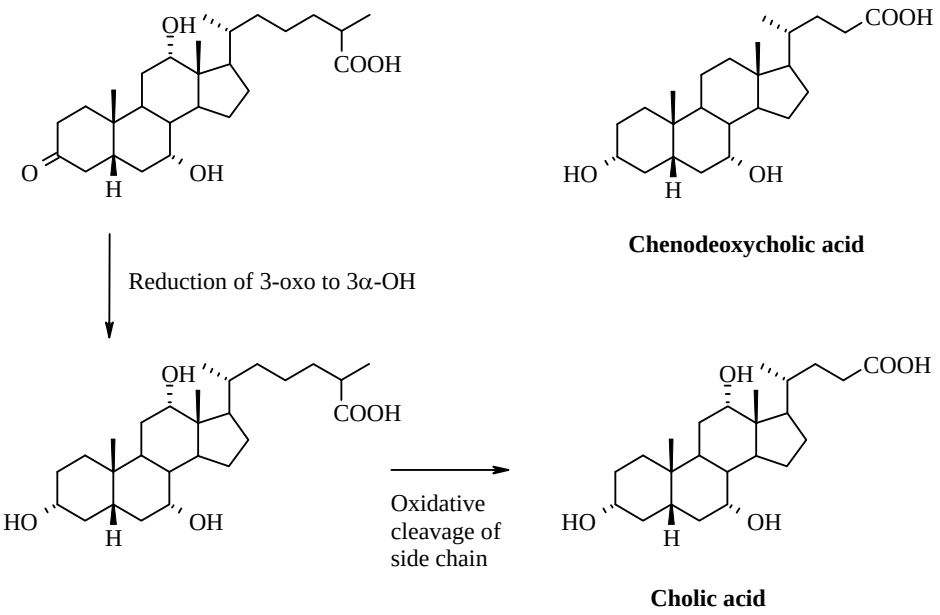
Bile acids form as end products of cholesterol catabolism in the liver [21]. The biosynthesis involves several steps on the steroid nucleus and the side chain. These steps may, in principle, occur in any order, which makes description and elucidation of the pathway complicated [18]. The classical (dominant) biosynthetic pathway (Scheme 1) begins with 7α-hydroxylation of cholesterol catalyzed by cholesterol 7α-hydroxylase [2]. This is believed to be the rate-limiting step of the biosynthesis of bile acids [22]. Oxidation and isomerization then produce a key intermediate, cholest-7α-hydroxy-Δ^4-3-one, the branching point for cholic (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic (3α,7α-dihydroxy-5β-cholan-24-oic acid) acid biosyntheses. 12α-Hydroxylation leads to formation of the trihydroxy derivative and stereoselective reduction affords the 5β-bile acid skeleton. The second key metabolic step in the biosynthesis of bile acids is the oxidation of the side

chain to a C_{27}-acid. This is believed to take place in mitochondria. Oxidative cleavage of the side chain to afford the C_{24}-acid then follows resulting in the primary bile acids [18]. The acidic pathway, on the other hand, starts with side chain hydroxylation at position C_{25}, C_{26}, or C_{27}.

Scheme 1. Biosynthesis of bile acids from cholesterol according to ref. [18].



Scheme 1. Cont.



The primary bile acids are conjugated at the terminal (C_{24}) carboxyl group with the amino acids glycine and taurine by bile acid CoA:amino acid *N*-acyltransferase [23]. Conjugation increases bile acid polarity and lowers passive transport leading to control of bile acid reabsorption by intestinal transporters. This ascertains high intraluminal bile acid concentration important for absorption of lipids. Additionally, conjugation prevents precipitation in the presence of high calcium ion concentration in the gall bladder thus improving bile acid solubility [24, 25]. In the colon the amides can be partially cleaved by hydrolases, followed by transformation to secondary bile acids by bacterial enzymes [2, 8]. Examples include deoxycholic (3α,12α-dihydroxy-5β-cholan-24-oic acid), hyodeoxycholic (3α,6α-dihydroxy-5β-cholan-24-oic acid) and lithocholic (3α-hydroxy-5β-cholan-24-oic acid) acids [21]. To a certain extent bile acids can be sulphated or glucuronidated leading to tertiary bile acids [8].

The biosynthesis of bile acids is under strict regulation [2, 26]. Decrease in the return of bile acids to the portal circulation increases the conversion of cholesterol to bile acids. In addition, the dietary intake of cholesterol affects the synthesis of bile acids [27-30]. The hepatic synthesis of bile acids is one of the predominant mechanisms for the excretion of excess cholesterol in the body [18]. In order to prevent the return of bile acids to portal circulation and thereby to enhance the biosynthesis of bile acids in order to treat hyperlipidemic conditions, bile acid transporter inhibitors have been developed [20, 31-40] in addition to polymeric anion exchange resins, such as cholestyramine and colestipol [41], which, for one, are capable of binding bile acids in the intestine. The gene responsible for expression of cholesterol 7α-hydroxylase (CYP7A1), the enzyme catalyzing the rate-limiting step in bile acid biosynthesis, is subject to both feedforward and feedback regulation [42]. Several nuclear receptors, such as the liver X receptor α (LXRα), the retinoid X receptor (RXR), and the farnesoid X nuclear receptor (FXR) have been identified to participate in the regulation. Bile acids act as endogenous ligands for FXR, which, through the transcription factor SHP, controls the expression of several bile acid transporters as well as the key enzyme in bile acid biosynthesis [18, 43-47]. Additionally, bile acids are able to activate mitogen-activated protein kinase pathways and are ligands for the G-protein-

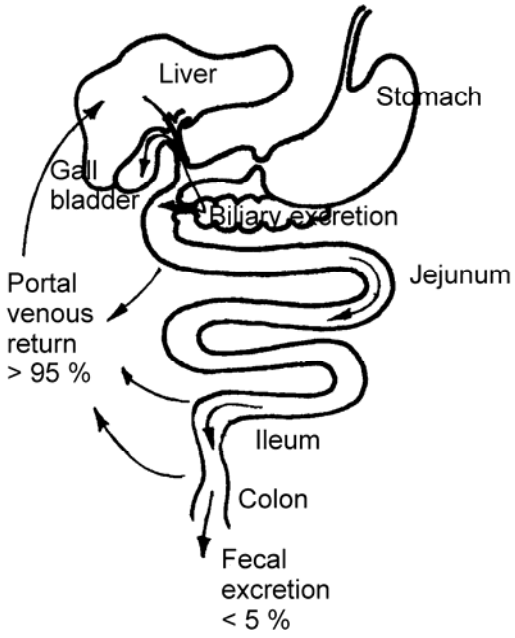
coupled receptor TGR5, making them signaling molecules with systemic endocrine functions. Besides regulating their own enterohepatic circulation, bile acids are able to regulate triglyceride, cholesterol, energy, and glucose homeostasis [47]. Since bile acids are capable of downregulating their own biosynthesis, investigations of the structural changes of bile acids on the regulation of bile acid biosynthesis have been performed [48, 49]. The obtained results indicate that the 6α-position plays a fundamental role in determining affinity and that the side chain of the bile acid is amenable to a variety of chemical modifications [49].

The essential function of bile acids in the human body is to emulsify dietary lipids and lipid soluble vitamins through the formation of mixed micelles. These micelles contain, in addition to bile salts, lecithin and glycerides, and are responsible for lipid/cholesterol solubilization in the small intestine. Their formation significantly increases the surface area of lipids, making them more readily available for lipase enzymes [18].

Enterohepatic Circulation

Bile, which consists of water (80 %) and dissolved components, for example bile acids, phospholipids, cholesterol, biliary pigments and proteins, is concentrated and stored in the gall bladder. After food intake, bile is secreted into the small intestine, where the bile acids perform their essential function in the digestive processes. The bile acids are then almost completely (> 95 %) reabsorbed by both active and passive mechanisms. Passive transport can occur down the length of the small intestine, whereas active transport is carried out mainly by the terminal ileum. After ileal uptake, the bile acids recirculate to the liver *via* the portal vein (see Figure 2). This process is termed enterohepatic circulation and it happens 6-15 times per day [8, 18]. The bile acid pool in humans is about 2.5-5 g, resulting in turnover of 12-18 g of bile acids each day. In spite of this repeated cycling, the loss of bile acids in feces is less than 0.5 g per day, reflecting the tremendous capacity and efficiency of the bile acid transport system [8, 25].

Figure 2. Enterohepatic circulation of bile acids [8].

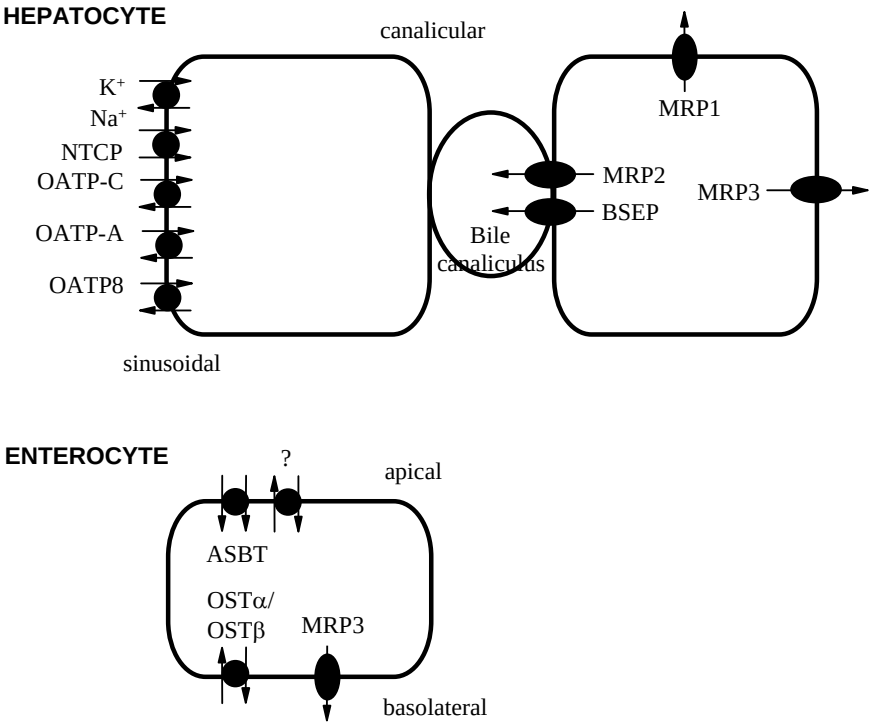


The enterohepatic recirculation of bile acids is a complex process involving numerous transport proteins. These include the sodium/taurocholate cotransporting polypeptide (NTCP), the organic anion transporting polypeptides OATP-C, OATP8, and OATP-A, the ATP-dependent bile salt export pump (BSEP), the apical sodium-dependent bile acid transporter (ASBT), the multidrug resistance protein MRP3 [2,25], and the heteromeric organic solute transporter (OST) OSTα-OSTβ [50, 51]. NTCP and ASBT are sodium-dependent members of the SLC10 family of solute carrier proteins. NTCP is expressed in hepatocytes and localized on their sinusoidal surface. It reabsorbs bile acids from the portal circulation. ASBT, for one, is expressed on the apical membrane of enterocytes in the terminal ileum and mediates the reabsorption of bile acids from the intestine. ASBT and NTCP serve to transport bile acids from the small intestine into portal circulation and from the portal circulation into the hepatocyte [2,25].

Bile Acid Transport Proteins

St-Pierre *et al.* [2] and Balakrishnan and Polli [25] have recently published excellent review articles describing bile acid carriers within the enterohepatic circulation. In this chapter the bile acid transport proteins, which might find use in prodrug applications, are highlighted. The sodium-dependent carrier proteins in the liver and small intestine are the most probable targets with that respect. Other bile salt transporters in hepatocytes and enterocytes will be briefly covered, in order to provide a more complete view of the relevant transport processes. A simplified scheme of bile salt carriers in human hepatocytes and enterocytes is represented in Figure 3.

Figure 3. A schematic representation of the bile salt carriers in human hepatocytes and enterocytes [2].



The entry of bile acids into hepatocytes at the sinusoidal surface takes place by two processes. The principal system is Na^+-dependent and is driven by the transmembrane Na^+-gradient, which is maintained by Na^+/K^+-ATPase [2]. The sodium/taurocholate cotransporting polypeptide, NTCP, is a 349 amino acid residue glycoprotein [52] exclusively expressed in the hepatocytes and localized at the sinusoidal membrane [53]. The coupled transport mediated by NTCP is electrogenic. The stoichiometry of Na^+:bile acid is identified of being 2:1 [54]. The second uptake system is Na^+-independent and involves several members of the organic anion transporting polypeptide (OATP) family. Three OATPs, which are expressed in the liver and accept bile salts as substrates, have been cloned. These include OATP-C, OATP8, and OATP-A; OATP-C being the most important one [2]. It transports taurocholate, but with slightly lower affinity than does NTCP. Since OATPs are multispecific organic anion carriers, substrates of OATP-C include other organic compounds as well [55,56]. It seems as OATP8 and OATP-A play negligible roles in the bile acid transport of humans [2].

At the canalicular surface of hepatocytes, conjugated bile salts are transported against a 1000-fold concentration gradient [2]. The protein involved in the efflux of bile salts into the canalicular space has been named as bile salt export pump (Bsep) [57]. Transport *in vitro* was shown to be saturable and ATP-dependent. In addition, high affinity for conjugated bile acids was detected [58,59]. The human BSEP has not yet been functionally expressed or characterized, but its gene locus is linked to progressive liver disease, PFIC2 (familiar intra-hepatic cholestasis type 2) [60]. Dianionic conjugated bile salts, formed for example by sulphation or glucuronidation at the C_3 position, are not substrates of Bsep [61], but rather of another class of proteins termed the multidrug resistance protein (MRP) family. MRP2 provides hepatocytes a mechanism for elimination of lithocholic acid metabolites by exporting sulphated tauro- or glycolithocholate [2, 62]. Lam *et al.* [63] have recently reported that P-glycoprotein (Mdr1) is capable of transporting bile acids across the canalicular membrane in *spgp* $^{-/-}$ knockout mice protecting them from bile acid-induced cholestasis. They believe that this finding might have potential medical implications for PFIC2 [63].

In addition to efflux on the canalicular surface, hepatocytes are able to remove bile acids through the sinusoidal surface as well [2]. Two members of the multidrug resistance protein family, MRP1 and MRP3, are expressed at the sinusoidal surface of normal hepatocytes, but at extremely low levels [64-66]. Similarly to MRP2, MRP1 and MRP3 are ATP-dependent pumps with a wide range of substrates including glucuronide and glutathione conjugates of endo- and exogenous compounds [67]. MRP1 is able to export dianionic bile salts [68] and MRP3 transports glycocholate, although at low affinity [69].

The apical sodium-dependent bile salt transporter (ASBT) consists of 348 amino acids [70] and is expressed at high levels in the terminal ileum, renal proximal tubules, and biliary epithelium [25, 71, 72]. Similarly to NTCP it is electrogenic, coupled with sodium in a 2:1 sodium-bile acid stoichiometry [73], and exhibits a seven transmembrane (7 TM) topology. The ligand binding site is localized near the C-terminus of the transport protein. Combination of photoaffinity labeling, enzymatic digestion, and epitope-specific antibodies further allowed identification of the bile acid attachment site (of position 7) to the C-terminal 56 to 67 amino acids [25, 74-76]. Recent studies by Banerjee, Ray, and colleagues [77, 78] have aimed at preparing bile acid-containing MTS agents capable of modifying the bile acid binding site of ASBT. Use of these conjugates provides information about the protein-ligand interactions of ASBT, and the prepared bile acid-MTS conjugates may find use in characterizing other

bile acid transporters, such as NTCP and BSEP as well [77, 78]. Kramer and co-workers [79] have widely studied the substrate specificity of ASBT and NTCP. They have demonstrated that the sodium/taurocholate cotransporting polypeptide exhibits a much broader substrate specificity compared to its ileal counterpart [79]. Bile salts are the sole substrates for ASBT, whereas NTCP can transport at least one other organic anion, the C_3OH-sulphate conjugate of oestrone. Both primary and secondary conjugated and unconjugated bile acids are substrates for ASBT [2, 72]. ASBT especially has been the target when designing drug delivery protocols, due to its unique substrate specificity and pivotal role in cholesterol homeostasis [80, 81].

The localization of a multispecific member of the rat Oatp family, Oatp3, is consistent with a Na^+-independent uptake of bile acids from intestinal brush border membranes [2, 82]. It accepts a range of amphipathic anions as its substrates [82], including bile acids [83]. Its role in bile acid uptake, however, has yet to be investigated [2]. OATP-A has been proposed as the human ortholog of Oatp3 [83]. However, it has not been detected in intestine, and its identity and importance in bile acid uptake in humans remain to be confirmed [2].

A peripheral protein, ileal lipid-binding protein (ILBP), specifically interacts with ASBT at the cytoplasmic face of the enterocyte. This suggests that it has a function in active ileal bile acid transport [84-86]. Moreover, binding of bile acids to ILBP seems to increase the affinity of ILBP for bile acids, indicating a regulatory role of ILBP on the ileal bile acid resorption system allowing a maximal adaptation of transport activity to changing substrate loads [87]. Kramer, Kurz, and colleagues [88,89] have investigated the bile acid binding site of ILBP by photoaffinity labeling, MALDI mass spectrometry, and NMR spectroscopy. By enzymatic fragmentation, gel electrophoresis, and MALDI-MS, the attachment sites of the C_3 and C_7 positions of cholyltaurine could be identified. The attachment site of the 3-position was localized to a sequence of three amino acids, and that of the 7-position to the C-terminal part as well as to the N-terminal part, suggesting more than one binding site for bile acids [88]. Combination of 2D- and 3D-homo and heteronuclear NMR techniques with GRID/CPCA analysis for investigating the structure of ILBP in its free form and in complex with its natural ligand cholyltaurine resulted in an observation according to which the tertiary structure of human ILBP is similar to other lipid-binding proteins, but there exist differences in binding of the ligand within the inner core [89]. The bile acid is deeply buried within the core, while its flexible side chain is located in the vicinity to the so-called fatty acid portal, which acts as an entry region to the inner core of the protein [88, 89].

The secretion of bile salts through the basolateral surface of enterocytes into circulation has been attributed to an anion-exchange protein [90]. Roles of t-Asbt (a truncated form of Asbt) in rat and MRP3 in humans have been suggested for pumping out bile acids at this site [2, 68, 90-93]. Only recently, a heteromeric organic solute transporter (OST) was identified as the major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia [50, 51]. The two subunits of this bile acid and sterol transporter are coexpressed in tissues that also express the sodium-dependent apical bile acid transporter ASBT. Both subunits, OSTα and OSTβ, are localized to the basolateral plasma membrane in these epithelial tissues. OSTα-OSTβ appears to mediate facilitating diffusion of bile acids and sterols from the cell. The transport has been found to be independent of Na^+, K^+, H^+, and Cl^- gradients as well as of intracellular ATP levels. Moreover, it occurs in both directions across the plasma membrane and is able to accept a variety of sterols as substrates [51].

Bile acids undergo cholehepatic recycling and are reabsorbed after filtration through glomerulus, which is why bile acid transporters are expressed in cholangiocytes and renal tubular cells. In addition, active carrier-mediated bile acid transportation takes place in the placenta [2]. It has even been observed that the placental barrier is able to protect the fetal compartment from a well-known cytostatic drug cisplatin, when the drug is coupled to a bile acid derivative, such as cholylglycinate [94].

Factors Affecting Bile Acid Transport

The first description of factors affecting on the intestinal bile acid transport was published by Lack and Weiner as early as the 1960's [95], as described, *e.g.*, in the review article by Balakrishnan and Polli [25]. Lack and Weiner used everted gut sacs of guinea pigs in their studies and observed that trihydroxy derivatives were better transported than dihydroxy ones. Additionally, they concluded that changes in conjugating moieties did not affect on the transport activity, unless the number of potential negative charges (normal singly-charged species were best transported) was altered. Further, the transport of a bile salt was observed to be depressed in the presence of another [95].

Later, a comprehensive study by Kramer and colleagues [79] on the substrate specificity of ASBT and NTCP using brush border membrane vesicles and CHO cell lines permanently expressing the sodium/bile acid cotransporters from rabbit ileum or rabbit liver with more than 20 different bile acid analogs in transport and inhibition studies and approximately 40 cholephilic compounds for inhibition studies demonstrated that the hepatic sodium/bile acid cotransporter system exhibited a much broader substrate specificity compared to its ileal counterpart. The affinity and uptake rates of the bile acid analogs were primarily determined by the substituents on the steroid nucleus. Two hydroxyl groups at positions C_3, C_7, or C_{12} were optimal, whereas the presence of a third one decreased the activity. The affinities and transport rates of bile acids possessing a hydroxyl group at position C_6 were low in ileal but preferred in hepatic systems. According to these studies, the 3α-hydroxyl group, which is present in all naturally occurring bile acids, was not necessary for molecular recognition by ASBT or NTCP. The authors suggested that it might, however, play an important role for optimal transport of a bile acid across the hepatocyte canalicular membrane. By using a training set of 17 inhibitors of the rabbit ileal sodium/bile acid cotransporter, Baringhaus, Kramer, *et al.* [96] were able to design a 3D QSAR pharmacophore model of ASBT. The model was characterized by five chemical features – one hydrogen bond donor, one hydrogen bond acceptor, and three hydrophobic features – and exactly explained the experimentally found structure-activity relationships described above. Based on their studies, the authors consequently suggested an updated model (compared to the one presented by Lack and co-workers) for the interaction of bile acids with the ileal sodium/bile acid cotransporter: the ε-amino group of a lysine residue interacts with the negatively-charged side chain of the bile acid representing the hydrogen bond acceptor. The α-hydroxyl groups at positions C_7 or C_{12} act as the hydrogen bond donors. Two of the three hydrophobic sites, for one, are mapped by the 5-membered D-ring of the steroid nucleus and the C_{21} methyl group of the side chain. A *cis*-configuration of rings A and B seems not essential for high affinity of a bile acid to the ileal transporter, in contrast to Lack and Weiner's suggestion. Tolle-Sander *et al.* [97] have recently proved that negative charge at C_{24} is not a prerequisite for the transport.

Balakrishnan and Polli [25] believe that the contradictory results obtained concerning the structure-transport activity investigations stem from a lack of a comprehensive study of bile acid transport using an appropriate method. For this reason their group has developed an ASBT-MDCK model [98] and used it for systematically elucidating ASBT-transport and inhibition profiles of native bile acids [99]. Based on their observations they concluded that glycine or taurine conjugation at C_{24} enhances the inhibitory potency of bile acids and that there is a reverse relationship between the number of steroidal hydroxyl groups and inhibitory potency. Further, 7α-OH seemed to be favored over 7β-OH, since chenodeoxycholate exhibited greater inhibitory potency than did ursodeoxycholate. The hydroxylation degree of the steroid skeleton had a significant effect on the transport activity. Fewer hydroxyl groups promoted transport activity toward ASBT [25, 99].

The transport of C_{24}-conjugated peptidyl bile acids has been studied by Swaan and his colleagues, who observed that the affinity towards the receptor decreased when an amino acid was replaced by a dipeptide. Further increase of the peptidyl chain did not markedly affect on the transport activity. The conjugates were translocated to the cells *via* passive diffusion as well, but with an increased rate compared to mannitol. Conjugation thus seems to aid the passive transport also, most probably by increasing the lipophilicity of the compound [100].

Anwer *et al.* [101] have investigated the influence of side chain charge of bile acids and their analogs on hepatic transport and biliary secretion. They reported that a fully positively charged bile acid derivative and two fully zwitterionic bile acid derivatives showed no appreciable uptake. Bile acid derivatives existing mostly in cationic form at pH 7.4, in neutral form, or in a divalent anion form had an increased uptake. This, however, was less than 20 % of that of cholyltaurine. The side chain charge seemed additionally to affect on the secretion rate into bile. Bile acids existing in mono- or dianionic form were well secreted, but the other derivatives had lower secretion rates. Based on their studies Anwer and colleagues concluded that although uncharged and anionic derivatives of cholic acid may be taken up by the liver at a moderate rate, only anionic derivatives were well secreted from within the liver cell into bile. A single negative charge appeared to be optimal for transport of a bile acid from sinusoidal blood into bile. These studies, however, were performed using the isolated perfused rat liver and anesthetized rats with bile fistula [101], and not with human transport systems.

The hydrophobicity of the bile salts has been observed to regulate the activity of the transport functionality. In the liver the activity of Mrp3 and Bsep and in the intestine the activity of ASBT has been observed to increase toward hydrophobic bile acids [102].

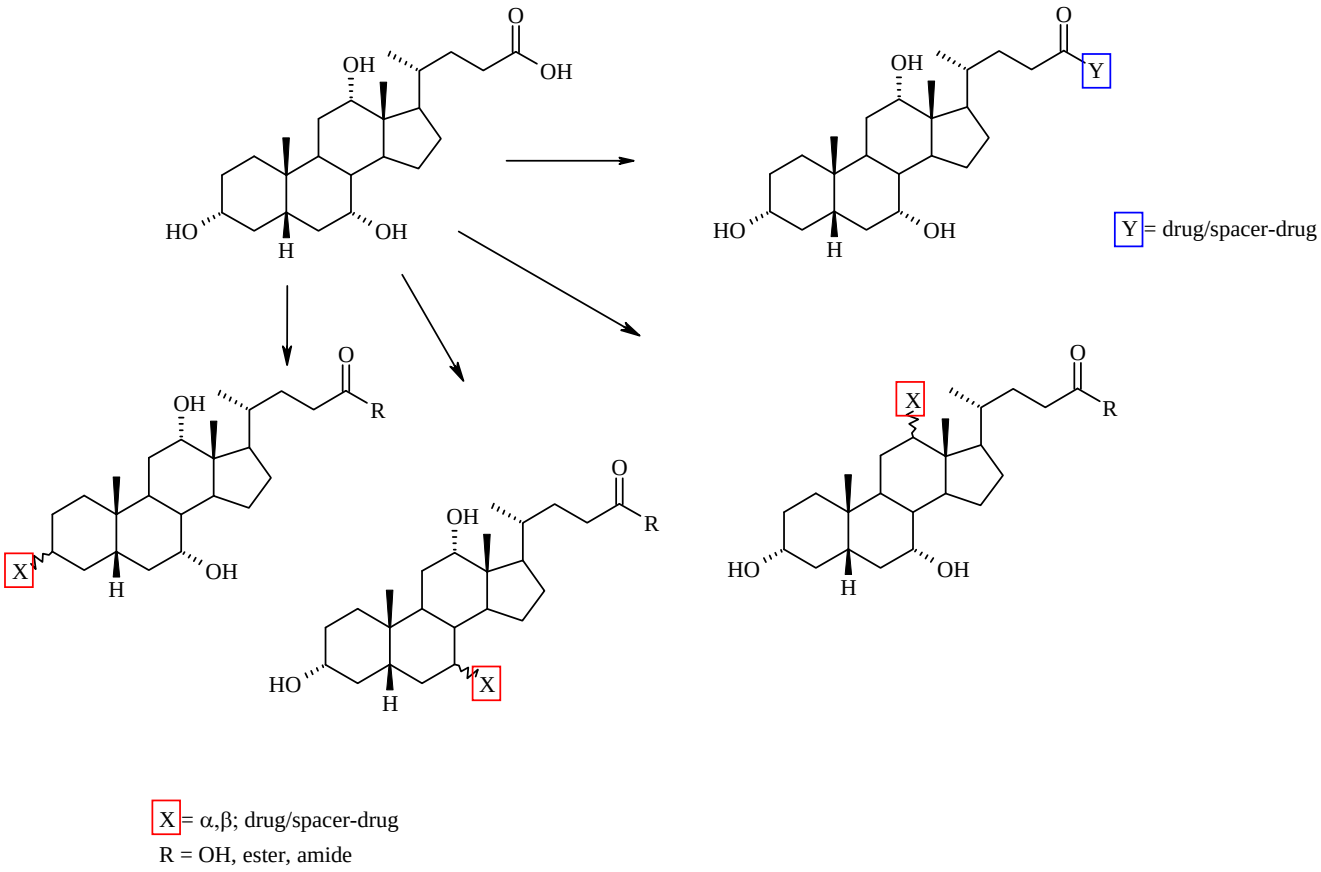
Bile Acid-Containing Prodrugs

The physiology of bile acid transport exclusively involving liver and small intestine overviewed above, should ideally suit for the use of bile acids as promoieties aimed at organ-targeted drug delivery and/or enhancement of absorption as well as increase of metabolic stability [8, 19]. The prodrug approach may involve coupling of a drug candidate to a natural substrate for a transporter or so-called "substrate mimicry", wherein the three-dimensional drug structure resembles that of a natural substrate [103]. Pharmacological applications of bile acids have utilized both of these techniques.

Because of the unique structure of bile acids, they are versatile building blocks for numerous synthetic applications, as mentioned above. Drug substances can be attached to different positions of

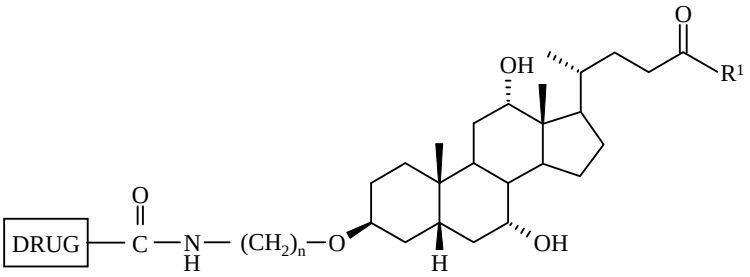
the steroidal skeleton or to the side chain *via* different chemical bonds, which can be further varied by linkers with different structure, length, stereochemistry, polarity, and/or functional group. Scheme 2 highlights the versatility of attaching drugs and/or spacers appropriate for coupling to drugs to bile acids. The carboxylic acid group of the bile acid molecule may be esterified, amidated, reduced, or subjected to anhydride bond formation. The steroidal hydroxyl groups, for one, are available for esterification, etherification, oxidation, or reduction. Further, they can be converted into amines [104].

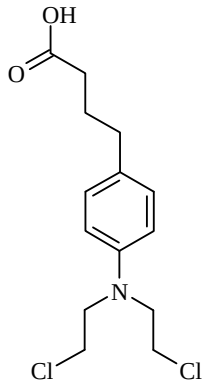
Scheme 2. Structures of potential bile acid derivatives. Drugs may be attached either directly or *via* spacer molecules at positions C_3, C_7, C_{12}, or C_{24} [104].



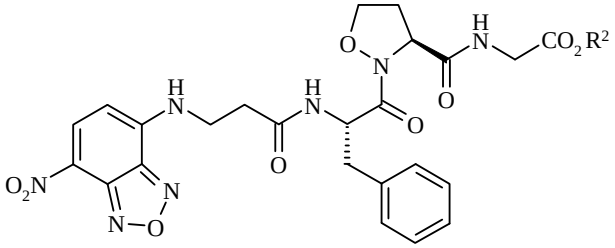
The research group of Kramer and Wess can undoubtedly be considered as a pioneer in developing bile acid-derived prodrugs. They have prepared bile acid-drug conjugates containing, for example, an alkylating cytostatic agent chlorambucil, collagen biosynthesis inhibiting oxaprolylpeptide, as well as cholesterol biosynthesis inhibitors HR780 and lovastatin (see Figure 4) [8, 19, 104-107]. The spacer attached at C_3-β position in these conjugates was commonly ω-aminoalkoxy residue [108, 109], except in the case of oxaproline peptide derivative, which was attached to 3β-(5-aminopentoxy)-7α,12α-dihydroxy-5β-cholan-24-oic acid.

Figure 4. Examples of bile acid-drug conjugates prepared by Kramer, Wess, *et al.* [19, 104-107].

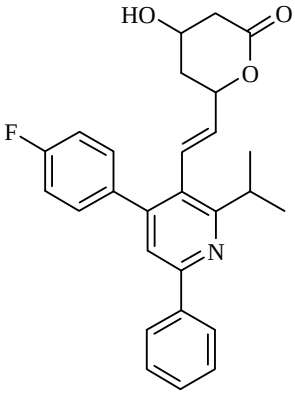




Chlorambucil



Oxaproline peptide



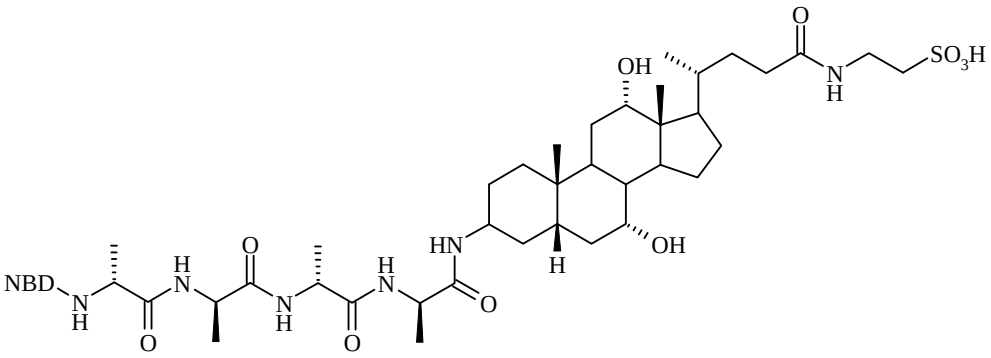
HR780

Bile acid chlorambucil conjugates strongly inhibited the hepatic taurocholate transport in freshly isolated rat hepatocytes. Specific interaction was also observed for intestinal brush border membrane vesicles. The chlorambucil conjugates were absorbed from the ileum and secreted by the liver, in contrast to the renal clearance of chlorambucil itself, as demonstrated by *in situ* perfusion experiments of rat liver and ileal segments. Moreover, the characteristic alkylating activity was retained in the intact bile acid-drug conjugates [8, 19, 104-106]. In a later study, it was demonstrated that human hepatocellular carcinomas expressed both Na^+-dependent and -independent basolateral bile acid transport proteins, which were capable of mediating the uptake of chlorambucil-taurocholate conjugate. The NTCP-mediated uptake of the conjugate was of high affinity, which further indicates the feasibility of bile acid-drug conjugates in liver-specific drug targeting [110].

Enzymatic hydrolysis and poor oral bioavailability are the major drawbacks, when considering the use of peptide drugs. Conjugation of peptides to bile acids is believed to increase their metabolic stability as well as to improve their absorption from the intestine. Kramer, Wess, and colleagues have evaluated the pharmacokinetic behavior of an NBD-oxaprolylpeptide, 4-nitrobenzo-2-oxa-1,3-diazol-β-Ala-Phe-5-Opr-Gly-OH, its *t*-butyl ester, and the related cholic acid conjugate, all of which are inhibitors of isolated prolyl-4-hydroxylase, a key enzyme in collagen biosynthesis [19, 104-107]. It was demonstrated that intestinal absorption of the bile acid-oxaprolyl peptide conjugate occurred *via* the specific ileal bile acid transport system and that the conjugate inhibited hepatic prolyl-4-hydroxylase as well as collagen biosynthesis in the liver. The results obtained clearly demonstrate that coupling to modified bile acids partially prevents the usual metabolism of peptides and allows their access to tissues served by bile acid transport systems [8, 19, 104-107].

Kramer, Wess, *et al.* further attached a series of small, linear model peptides to a modified bile acid resulting in peptidyl 3β-(ω-aminoalkoxy)-7α,12α-dihydroxy-5β-cholan-24-oic acids or their methyl esters. The peptidyl chains consisted mainly of D-alanine residues, due to the inert methyl side chain of this particular amino acid. The affinity towards the bile acid transport system in the intestine was observed to increase, when the length of the peptide chain was decreased [107]. In yet another study, L-alanine and two biooligomers, a tetrapeptide L-$(Ala)_4$ and a 15mer oligodeoxynucleotide (ODN) were covalently attached *via* linker molecules to the C_3 position of bile acids. The conjugate with L-Ala attached to C_3 position of taurocholate mimicked the hepatic transport of natural bile acids. Also the tetrapeptide conjugate (see Figure 5) showed significantly improved hepatic and intestinal cell uptake and rendered the peptide conjugate resistant to peptidases. The hepatocellular uptake or biliary elimination of the ODN, on the other hand, was not markedly improved by conjugation to a bile acid [111].

Figure 5. Structure of the C_3-conjugated NBD-L-$(Ala)_4$ derivative of taurocholate [111].

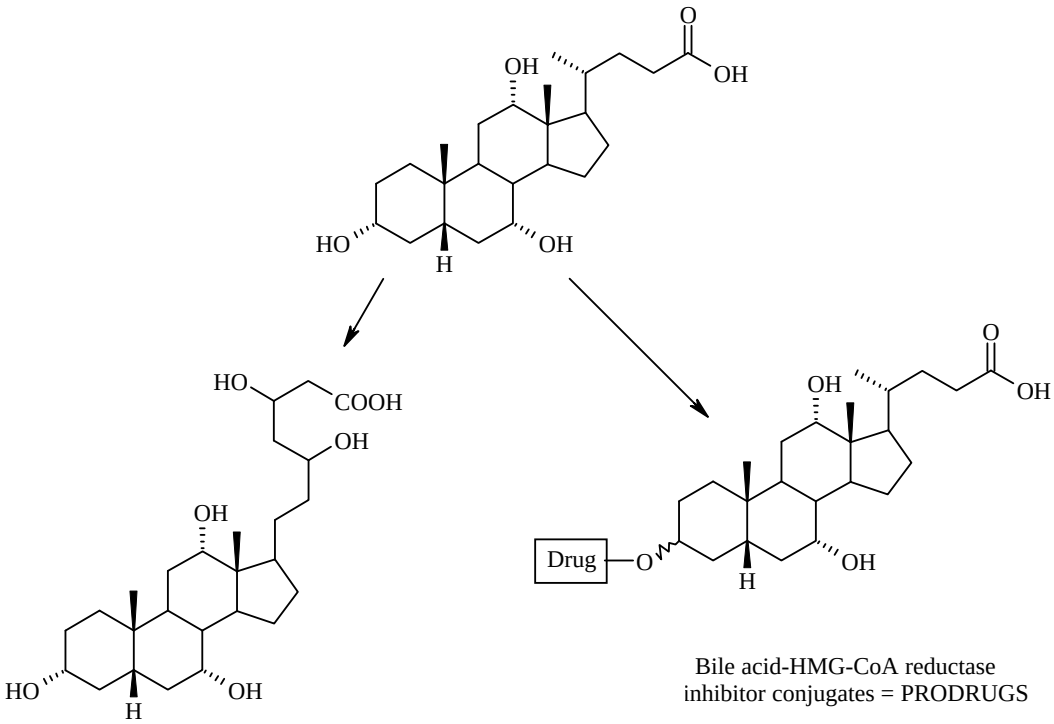


Later, however, Kramer, Wess, and co-workers observed *in vivo* in rats that covalent conjugation of oligodeoxynucleotides of different length *via* a linker at position C_3 resulted in an increased biliary excretion of the conjugates compared to the unconjugated ODNs. It seems as the transport mechanisms responsible for the hepatic uptake and elimination of ODN conjugates differ from the low molecular weight drug conjugates being yet unidentified. For liver specific targeting of ODNs conjugation to more than one bile acid molecules is probably needed [112]. This was further confirmed by an investigation on normal mixed backbone phosphodiester/phosphorothioate ODNs (n-ODNs) and two bile acid-conjugated mixed backbone ODNs (1BA-ODN and 2BA-ODN, respectively) on normal Wistar rats and Wistar TR rats, the latter of which lack a functional multidrug resistance-associated protein 2 (Mrp2) at the canalicular membrane of the liver [113]. In contrast to normal rats, the n-ODN excretion into bile by the mutant rats was significantly decreased. These rats, however, excreted the ODN conjugated with two cholic acid molecules (2BA-ODN) into bile. Since by normal rats the n-ODNs were secreted *via* Mrp2 into bile, in the absence of the protein further excretory transport systems with affinity for bile acids seem to be relevant [113].

Modified antisense oligodeoxynucleotides directed against the hepatitis C virus (HCV) conjugated to bile acids and their derivatives as potential liver specific antisense therapeutics have been proposed by Lehmann *et al.* [114, 115].

The rate-limiting step in cholesterol biosynthesis is the transformation of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) to mevalonate catalyzed by HMG-CoA reductase [116]. One approach aiming at lowering the plasma cholesterol levels is to inhibit the activity of this enzyme. There are several drug substances functioning on this basis, such as lovastatin, simvastatin, and pravastatin [117]. In addition to inhibiting the cholesterol synthesis in the liver and intestine, these drugs have prevented biosynthesis in other tissues as well, which has induced undesirable side-effects for some patients. Kramer, Wess, and colleagues have combined bile acids and HMG-CoA reductase inhibitors with a view of targeting these compounds to liver exploiting the bile acid transport system [117]. They have utilized two approaches: preparation of bile acid-HMG-CoA reductase inhibitor hybrids and conjugation of HMG-CoA reductase inhibitors to C_3-position of the bile acid (see Scheme 3).

Scheme 3. Liver-specific HMG-CoA reductase inhibitors by combining with bile acid structural elements [117].



Bile acid-HMG-CoA reductase inhibitor hybrids

Bile acid-HMG-CoA reductase inhibitor conjugates = PRODRUGS

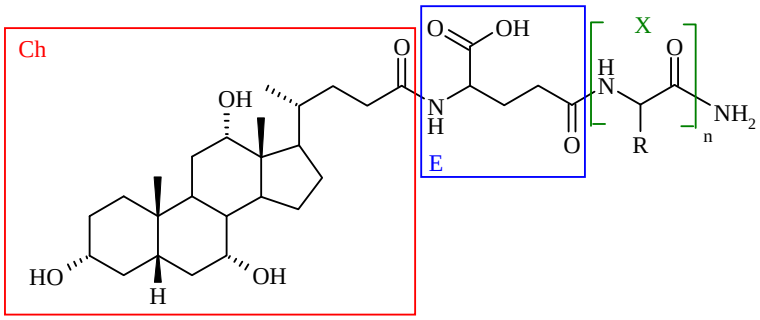
In the bile acid-HMG-CoA reductase inhibitor hybrid molecules the hexahydronaphthalene moiety of lovastatin was replaced by a modified bile acid. The 3,5-dihydroxy heptanoic acid side chain either in the open ring or lactone form was conserved. The effect of substituents at positions C_3, C_7, and C_{12} on steroid skeleton, the effect of the presence or absence of a methyl group at position C_{21}, as well as the side chain configuration were investigated. Compounds containing methylbutanoic residue at position C_{12} and possessing the side chain configuration of lovastatin showed higher inhibition on HMG-CoA reductase and cholesterol biosynthesis than did their diastereomers. Furthermore, the demethylated compound was more active than the one containing methyl group at C_{21}. The

demethylated compounds, however, were not delivered as actively by EHC as the methylated ones [8, 117, 118]. The prepared hybrid molecules have observed to interact with transport mechanisms in the liver and intestine, which opens possibilities in targeting the inhibition of cholesterol biosynthesis more precisely to liver and intestine in contrast to the existing drug substances. In addition to Kramer, Wess, and co-workers, at least two research groups have developed bile acid-derived HMG-CoA inhibitor hybrids of their own [119, 120].

In the other approach, the 3,5-dihydroxyheptanoic acid moiety of HMG-CoA reductase inhibitors (HR780 and lovastatin) was covalently linked *via* an amide bond to C_3-aminoalkoxy substituted bile acids [104, 106, 117, 118]. The conjugates were not expected to inhibit the target enzyme until the dihydroxyheptanoic acid moiety was hydrolyzed from the bile acid promoiety. Compared to the parent drug the conjugates showed increased affinity for the hepatocyte bile acid transport systems. Alike behavior was observed for the intestinal transport system in brush border membrane vesicles [8, 117, 121]. Secretion profile similar to natural bile acids was detected contrary to the parent drug itself. Evidence of intracellular release of the active drug substance from the bile acid promoiety was perceived in the form of a major polar metabolite. Furthermore, after the intravenous administration of the conjugates to rats, the concentration of the conjugated drug in extrahepatic tissues was considerably lower compared to its concentration in the liver expressing selectivity.

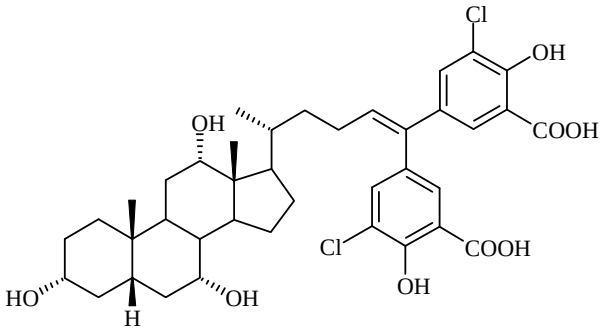
Other research groups have also been active in the area of designing and synthesizing bile acid-drug conjugates targeted towards bile acid transport proteins with the aim of improving absorption or systemic bioavailability as well as targeting the drug substance. For example, Kim and colleagues [122] have conjugated a renin inhibitory heptapeptide ditekiren at C_3 position of cholic or taurocholic acid *via* a spacer molecule. These conjugates were observed to exhibit ASBT inhibition, yet they were not transported by the protein. Swaan *et al.* [100] attached eleven peptides with an amino acid backbone consisting of 2-6 residues to the C_{24} position of cholic acid *via* an amide bond. The general formula for the conjugates can be expressed as $ChEX_n$, where Ch corresponds to cholic acid, E to γ-L-glutamic acid, and X to any amino acid (see Figure 6). The affinity towards the intestinal bile acid carrier was observed to decrease when an amino acid was replaced by a dipeptide. Further increase of the peptidyl chain did not markedly affect on the transport activity. Peptides with four amino acid residues were transported by the intestinal bile acid carrier. Even the hexapeptides had moderate affinity for the carrier protein. Moreover, it seemed that by coupling small peptides to cholic acid the metabolic degradation of the resulting conjugates was reduced [100].

Figure 6. General structural formula of bile acid-peptide conjugates studied by Swaan *et al.* [100].



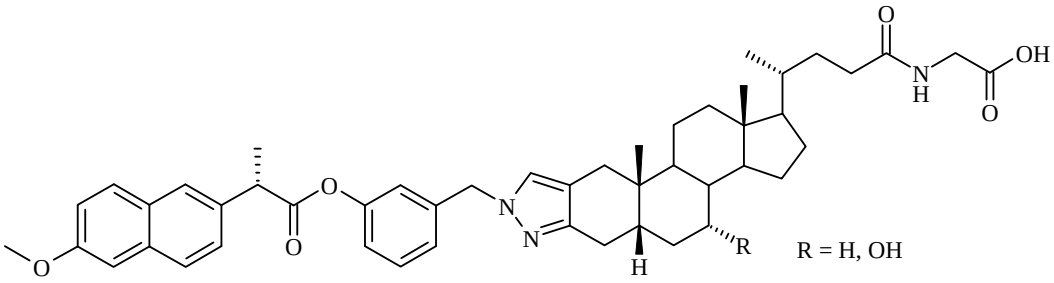
Recombinant human insulin has covalently been attached to deoxycholic acid derivatives with the aim of synthesizing orally active insulin analogs [123, 124]. Conjugation to bile acid might, but increase the metabolic stability against peptidases as well as absorption from the intestine enabling oral administration of insulin instead of parenteral injections, prolong the biological activity of native insulin in the physiological conditions. Indeed, results confirming this scenario have already been obtained [124]. Hybrid molecules with HIV-1 protease inhibitory activity with potential to be transported by the ileal bile acid transporter were prepared by conjugating amino acids, amino acid analogs, and/or small peptides at the C_{24} position of cholic acid [125]. Of all tested compounds, only cholyl D-Asp-β-benzyl ester showed modest HIV-1 protease inhibitory activity. Transport of the conjugates was observed to be influenced by charge and hydrophobicity around the C_{24} position of the sterol nucleus. Another hybrid molecule with anti-HIV activity, a cholic acid analog of cosalane (see Figure 7) was designed to target the ileal bile acid transporter and thus facilitate the poor oral absorption of cosalane, an anti-HIV agent [126]. The bile acid analog retained antiviral activity but was less potent than cosalane itself. This was believed to be, at least partially, due to decrease in lipophilicity of the steroidal moiety compared to the parent compound. Three hydroxyl groups, however, were believed to increase the affinity of the hybrid towards the transport system, which is why they were preserved in the molecule [126].

Figure 7. The structure of cholic acid analog of cosalane designed to improve the poor oral bioavailability of cosalane, an anti-HIV agent [126].



To investigate steroidal pyrazole templates as drug carriers, a series of novel C_2-C_3 annulated bile acid pyrazoles were synthesized by Bhat *et al.* [127, 128]. These were further conjugated with drugs and drug surrogates, such as naproxen (see Figure 8). The annulated pyrazoles and their drug conjugates showed better affinity to NTCP than they did for ASBT. It was, however, clearly demonstrated that the bile acid analogs derived from heteroannulation onto the bile acid A ring at C_2-C_3 were able to act as substrates for bile acid transport systems and are potential vehicles for drug targeting [127, 128].

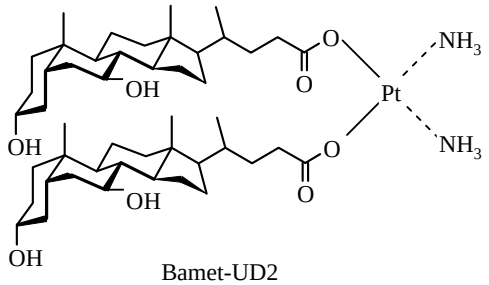
Figure 8. Structures of C_2-C_3 annulated bile acid pyrazoles conjugated with naproxen [128].



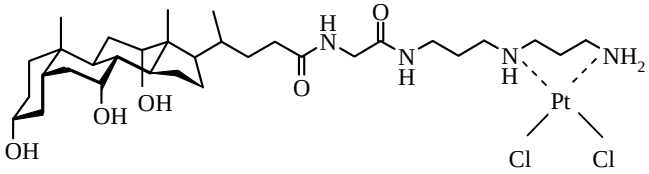
Rapid clearance of drugs from the systemic circulation combined with the poor systemic absorption represents the major obstruction to effective clinical use of therapeutic and/or prophylactic compounds. These impediments may require administration of larger doses of the drug resulting in greater variability in drug exposure and undesirable side effects. Moreover, frequent drug administration to maintain a certain minimum systemic level of the drug may be necessary. In addition to the exploitation of the bile acid transport system for organ-targeted drug delivery, improvement of absorption, or increase of metabolic stability, it has found use in enhancing systemic bioavailability of orally transmitted drugs. This is delivered by effective trafficking of the bile acid-drug conjugates from the intestine to the liver and excretion into the bile providing a circulating reservoir of the conjugated drug, which is slowly released into the systemic circulation for maintaining sustained concentrations. Simultaneously, conjugation to a bile acid may enhance the translocation of the drug from the intestinal lumen to the systemic circulation. Examples of drug substances conjugated to bile acids in order to increase the systemic bioavailability include GABA (γ-aminobutyric acid) and its analogs, used as therapeutics, for example, for epilepsy, chronic pain, and behavioral disorders, as well as L-DOPA (L-dihydroxyphenylalanine), inhibitors of cathecol O-methyl transferase (COMT), and/or inhibitors of L-aromatic amino acid decarboxylase (AADC), commonly used in the treatment of Parkinsonism [129-134].

Severe toxicity and development of resistance to cisplatin have led researchers all over the world to developing numerous cisplatin analogs. With a view of using bile acids as shuttles for delivering platinum-related cytostatic agents to liver tumors, several bile acid derivatives, which have a platinum(II) atom as the DNA-reactive part, have been synthesized and their ability to interact with DNA, their cytotoxicity, as well as their liver organotropism have been investigated by Marin and colleagues [135-150], who have named their compounds as Bamets, “Ba” standing for bile acid and “met” for metal. Figure 9 represents two examples of the Bamet family, Bamet-UD2 and Bamet-D3, respectively. Other examples of linking a bile acid transport fragment to cisplatin analogs have been presented, for example, by Paschke *et al.* [151]. Further, a new class of Re(I) and Tc(I) carbonyl complexes of natural bile acids with a potential of acting in radiodiagnostic and radioimmunotherapeutic applications for liver, bile duct, gall bladder, and intestine have been reported by Campazzi and co-workers [152]. A series of bile acid-polyamine amides conjugated with 3’-azido-3’-deoxythymidine (AZT) as potential antitumor prodrugs as phosphoramidates were synthesized by Wu and colleagues [153].

Figure 9. The chemical structures of Bamet-UD2 (left) and Bamet-D3 (right), respectively [144].



Bamet-UD2



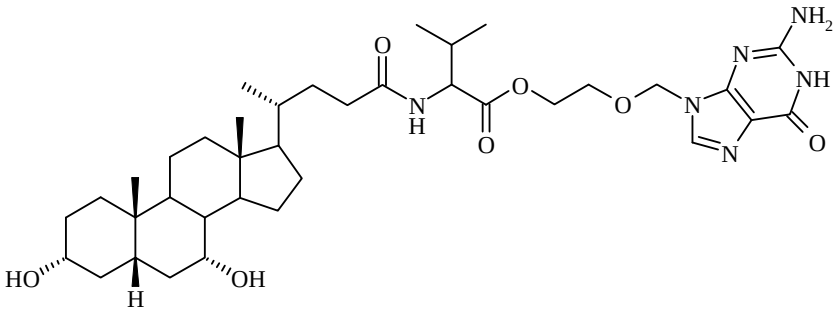
Bamet-D3

Bile acid derivatives conjugated with metal ion-chelated complexes have been successfully used as contrast agents in magnetic resonance imaging (MRI) [154-156]. For example, conjugates of gadolinium(III) complexes to bile acids were prepared with the aim of directing contrast agents for MRI to hepatocytes [157]. Polyaminopolycarboxylic ligands were selected as the chelating subunits for Gd(III) ion and cholic acid, cholylglycine, and cholyltaurine acted as the bile acid moieties. In first generation conjugates the Gd(III) complexes were attached to the carboxyl group of cholic acid, in second generation conjugates the complex was linked at C_3 position of the bile acid, and in third generation conjugates at ε nitrogen of the cholyllysine moiety. A second generation conjugate, in which the Gd(III) complex was conjugated to cholic acid through 3α-OH group, seemed to enter hepatocytes *via* Ntcp [157]. It has been shown that NTCP is not expressed in some human hepatoma cell lines [158], which is why discovery of a contrast agent transported into hepatocytes by that particular transporter, might allow direct diagnosis of hepatic diseases, such as hepatocellular carcinoma [157].

One of the most interesting recent applications in the field of using bile acids in prodrugs is undoubtedly acyclovir valylchenodeoxycholate (see Figure 10), which is a conjugate of valacyclovir (L-valine ester prodrug of acyclovir, an antiviral drug that inhibits herpes virus proliferation) and chenodeoxycholic acid [25, 97, 159]. Since ASBT exhibits micromolar affinity and higher capacity compared to PepT1 intestinal transporter, conjugation to a bile acid moiety was believed to enhance the intestinal absorption of acyclovir even more than its conjugation with valine. Four bile acid-containing acyclovir prodrugs were synthesized (acyclovir valylchenodeoxycholate, acyclovir valylcholate, acyclovir valyldeoxycholate, and acyclovir valylursodeoxycholate), two of which exhibited affinity for ASBT. The valacyclovir conjugate of chenodeoxycholate possessed the greatest affinity, which is why it was chosen for further investigation. Liberation of acyclovir from the prodrug was demonstrated by chemical hydrolysis. The permeability was increased by both ASBT-mediated uptake and enhanced passive membrane permeability. Even though acyclovir valylchenodeoxycholate showed more favorable *in vitro* uptake properties compared to valacyclovir, in rats the oral bioavailability was only 2-fold (48 %) greater than after administration of acyclovir [97], while valacyclovir has improved the oral bioavailability of acyclovir in humans for 54 % (3-fold improvement) [9, 160]. Because the bile acid carrier proteins are mainly located in the terminal ileum, a possible explanation for this is prodrug hydrolysis in the stomach and proximal intestine [97]. Since the prodrug acyclovir valylchenodeoxycholate was designed to target the human apical sodium-dependent bile acid transporter, a pharmaceutical formulation approach where sodium is fabricated

with or coadministered with the active agent, was developed with the view of further enhancing the uptake [161].

Figure 10. Structure of the prodrug acyclovir valylchenodeoxycholate [97, 159].



Conclusions

Taken together, the efficiency and organ specificity of enterohepatic circulation of bile acids with numerous transport proteins is an intriguing target in designing prodrugs with a view of improving intestinal absorption, increasing the metabolic stability of pharmaceuticals, specifically targeting drugs to organs involved in enterohepatic circulation, as well as sustaining therapeutically or prophylactically reasonable systemic concentrations of the active agents. Examples include bile acid-drug hybrid molecules and bile acid-derived prodrugs. These may be targeted to transport proteins in the hepatocytes, such as bile acid conjugates with chlorambucil, oxaprolyl peptides, cholesterol-level lowering agents, antisense oligodeoxynucleotides, cytostatic agents, or MRI contrast agents, just to mention a few. Peptides and peptide drugs have been linked with bile acids resulting in improved absorption from the intestine combined with increased metabolic stability and prolonged period of influence. Several antiviral agents, acting against *e.g.* HIV or herpes virus, have been attached to bile acids in order to enhance their oral bioavailability. The systemic concentrations of drugs, such as those used for the treatment of Parkinsonism, have suggested to be sustained by conjugation to bile acids. With the versatile derivatization possibilities, rigid steroidal backbone, enantiomeric purity, availability, and low cost combined to the unique physiology, bile acids have proven to be inspiring tools in the design of pharmacological applications with infinite amount of objectives.

Acknowledgements

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