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EMERGING INTEREST IN BILE ACID TRANSPORTERS IN PATHOPHYSIOLOGY AND PHARMACOLOGY

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REVIEW

ABSTRACT. BILE ACIDS form a large family of compounds with marked organotropic characteristics. These allow them to be kept within the enterohepatic circulation, which includes the liver, biliary system, intestine and portal vein. This circuit is based on the existence of plasma membrane proteins able to efficiently transport bile acids from the sinusoidal blood into bile and from the intestinal lumen to the portal blood. The carriers involved in bile acid transport belong to different protein families (SLC10A, SLCO, ABCB, ABCC, and ABCG). The energy driving such transport varies from substrate gradient to sodium-cotransport, anion-exchange or ATP-hydrolysis. The abnormal functioning of some of these transporters accounts for diseases such as cholestasis or intestinal malabsorption, whereas impaired hepatobiliary function, resulting in bile acid accumulation, induces changes in the expression and/or sorting of these proteins. Owing to the broad substrate specificity of some of these transporters they have been implicated in several interactions between drugs, toxins, food components and endogenous substances. In addition to other pharmacological and pharmaceutical uses of bile acids, their organotropism has resulted in a large list of compounds that have been conjugated with them. The benefits of these drugs include their ability to target pharmacologically active agents toward the liver, the biliary system and the intestine. Moreover, pharmacological modulation of the expression/function of some of these transporters is of great importance. Thus, cholesterol-lowering drugs able to interact with bile acid transporters, inhibit enterohepatic circulation of bile acids, hence increasing fecal elimination and stimulating cholesterol metabolism, have been developed.

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1. BILE ACIDS PHYSIOLOGY

1.1. WHAT ARE BILE ACIDS?

The term bile acid (BA) defines a large family of compounds with three shared properties: they have a typical steroid core consisting of four rings and a side chain that, if not derivatized, ends in a carboxyl group (FIG. 1). Moreover, the molecule includes one or several hydroxyl groups, usually located on the steroid ring. In humans, the most abundant species are primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA), synthesized directly by hepatocytes from cholesterol, and the secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA), generated in the intestine by bacterial biotransformation of primary BAs (FIG. 1). Small amounts of other BAs, such as ursodeoxycholic acid (UDCA), are also present in the human BA pool. Before being secreted into bile, most BA molecules are biotransformed by the liver to their amidated forms by conjugation with glycine or taurine. Under normal conditions, the amount of BAs undergoing further biotransformation to dianionic glucuronidated or sulfated derivatives is negligible, although this may become important in cholestasis (for review, see refs. [1,2]).

1.2. BILE ACID HOMEOSTASIS

BAs are involved in several important functions in the liver and the intestine. The best known physiological roles of BAs are carried out in bile — namely, osmotic generation of the so-called BA-dependent bile flow and solubilization of cholesterol — and in the intestine, where BAs are needed for the emulsification, digestion and absorption of fat and liposoluble vitamins. However, lately interest in these molecules has grown considerably owing to the fact that BAs are indeed involved in a more diverse range of physiological functions, suggesting that alterations in their homeostasis have important pathophysiological implications. Moreover, their biological properties have led them to being used as pharmacological tools.

The physiological relevance of BAs explains why along evolution several mechanisms have been selected to prevent their fecal elimination. Thus, these molecules are recovered by the intestine and transferred to the portal blood. Only 5% of the BA pool is lost each day in feces and this fraction is restored by de novo synthesis. Once portal blood takes BAs back to the liver sinusoids, these molecules undergo re-uptake, metabolic repair and re-secretion into bile by hepatocytes. Thus, most of the BA pool is maintained in the so-called enterohepatic circuit [3]. This circulation is based on the function of carrier proteins located at the plasma membrane of both hepatic and intestinal cells. BA homeostasis is maintained by a tightly controlled cross-talk between feed-back and feed-forward mechanisms that regulate the expression of the enzymes involved in the metabolic pathways responsible for BA synthesis and biotransformation, as well as BA transporters located in the enterohepatic circuit.

Under normal circumstances, a small fraction of the amount of BAs that is secreted into bile canaliculi is reabsorbed in the upper part of the biliary tree, toward the periductular capillary plexus through cholangiocytes to undergo subsequent re-uptake by hepatocytes. This constitutes an intrahepatic circuit named the cholehepatic shunt pathway [4], which has been reported to play a role in bicarbonate-rich hypercholeresis induced by BA species with a relatively high pKa' value, such as UDCA [5,6]. These BAs are secreted in anionic form but may be reabsorbed in protonated form by non-anionic diffusion, inducing biliary bicarbonate secretion as the result of H^+ removal and CO_2 hydration, which is catalyzed by carbonic anhydrase [7,8]. The intrahepatic cycling of BAs also probably involves carrier-mediated transport across the plasma membrane of biliary epithelial cells. Under circumstances of bile retention and, hence BA accumulation in bile ducts, this transport system may constitute a pathway for the efflux of BAs toward the blood, from where they can follow alternative excretory pathways.

In this respect, the kidney plays a double role in BA homeostasis: on one hand, this organ is able to carry out the recovery of BAs from the glomerular filtrate, which reduces the renal loss of these mole-

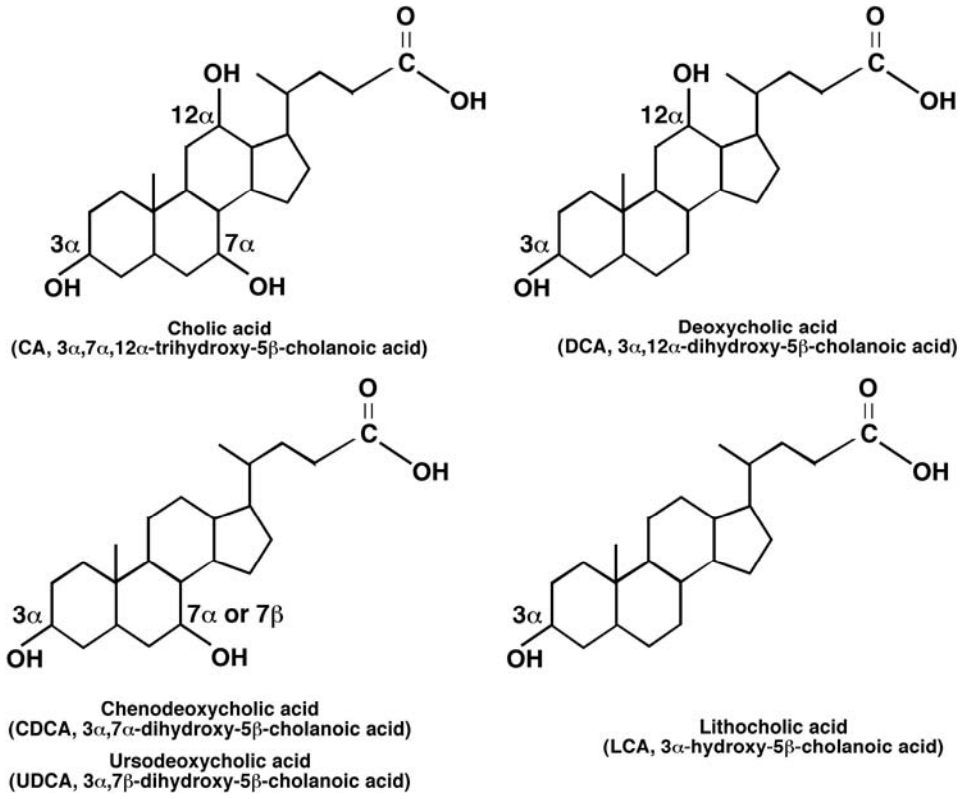

FIGURE 1. SCHEMATIC REPRESENTATION OF MAJOR BILE ACIDS IN HUMANS.

cules in healthy individuals. On the other hand, the kidney can become a major route for the excretion of sulfated and glucuronidated BA derivatives in cases of accumulation of these compounds in the body due to an impairment in hepatobiliary function. Both roles involve the function and regulation of the expression of BA carrier proteins located in this organ.

Finally, the presence of BA transporters in the placenta should be considered. Although the fetal liver is able to synthesize BAs, this organ does not yet have the ability to eliminate them into bile, and hence the maternal liver must eliminate fetal BAs once they have been transferred across the placenta. This vectorial transfer of BAs in the fetus-to-mother direction is possible due to the existence in this organ of transport proteins responsible for mediating this process.

The present review article will address several aspects of these BA transporters as regards their role in the physiology and pathophysiology of all the aforementioned organs, as well as the emerging interest in these proteins as molecular targets for the development of pharmacological strategies aimed at treating very different diseases, varying from hypercholesterolemia to cancer.

2. CARRIER PROTEINS WITH THE ABILITY TO TRANSPORT BILE ACIDS

At physiological pH values, both in blood and within cells, most BA molecules are in anionic form. In this form, BAs are poorly diffusible across lipid membranes [9]. Thus, although minor diffusion across cell membranes, at least for the BA species with the highest pKa value is possible, most

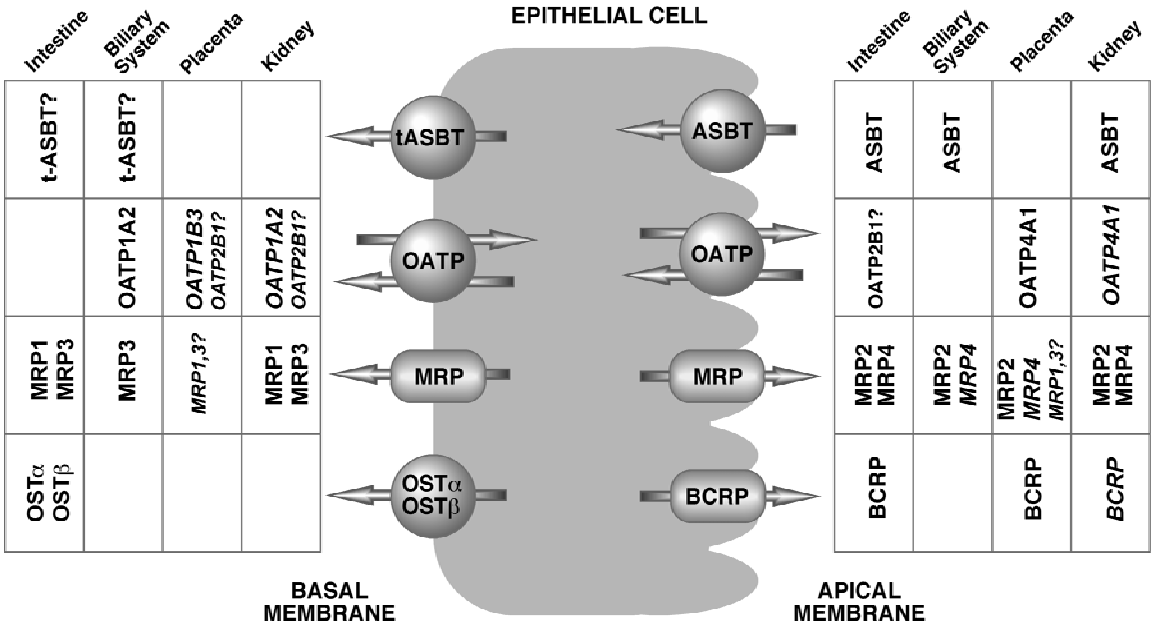

FIGURE 2. SCHEMATIC REPRESENTATION OF THE PLASMA MEMBRANE CARRIERS INVOLVED IN BILE ACID TRANSPORT IN HUMAN EPITHELIAL CELLS OF THE INTESTINE, BILIARY SYSTEM, PLACENTA AND KIDNEY. The name of the transporter is in italics when actual location has not been unambiguously detected. For MRP1 and MRP3 in human placenta, question marks reflect the uncertainty of whether they are located in the trophoblast, in the fetal endothelial cells, or in both cell types. For t-ASBT, question mark indicates that this variant has been described in rat but not yet in humans. For OATP2B1, question mark indicates the doubt of its ability to transport bile acids.

trans-membrane traffic of BAs occurs via BA transporters. For more extensive information about BA transporters and their regulation, the review by Kullak-Ublick et al. [10] is recommended.

BA transporters include proteins with very different structures and characteristics that can first be classified from a physiological point of view as transporters mainly involved in BA uptake by cells or transporters involved in the efflux of these molecules out of cells (FIG. 2). It should be noted that this classification is not strict, since some carriers are potentially bi-directional, even in the same cells. Moreover, some transporters account for BA uptake in certain cells but contribute to BA efflux in others [11].

2.1. THE SLC10A FAMILY

The most efficient transport systems involved in BA uptake are two carriers with functional characteristics of BA:Na^+ co-transporters; that is, BA uptake is actively driven by these proteins due to the energy of the inward sodium gradient. One of these transporters is the Na^+/taurocholate co-transporting polypeptide (NTCP, gene symbol *SLC10A1*), which seems to be specifically located at the sinusoidal plasma membrane of hepatocytes [12,13] (FIG. 3). Negligible expression of NTCP has been found in rat [14] and human [15] placenta, which is consistent with functional evidence suggesting that the carrier-mediated uptake of BAs across the basal plasma membrane of the trophoblast is not sensitive to the presence of sodium gradients [16].

The other BA:Na^+ co-transporter was first termed the "ileal bile acid transporter" (IBAT) because it was first described at the apical membrane of hamster [17], rat [18], and human [19] ileal mucosa cells. However, this protein has also been located at the apical membrane of rat cholangiocytes [20] and cells of the rat [21] and human [22] renal proximal tubule. Thus, this transporter is now referred to as the apical sodium-dependent bile acid

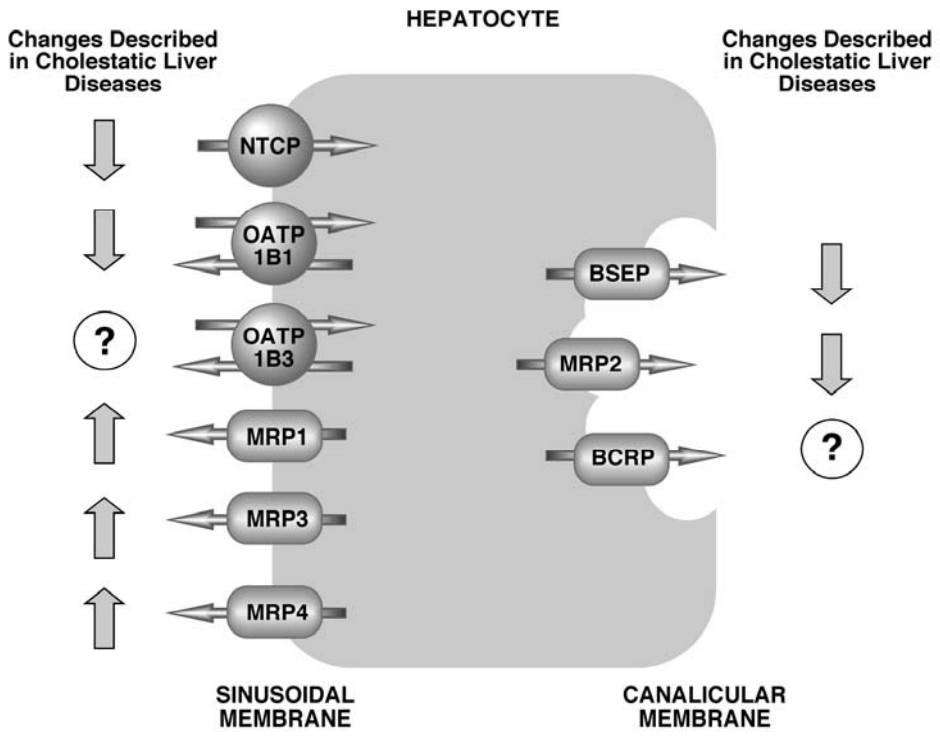

FIGURE 3. SCHEMATIC REPRESENTATION OF THE PLASMA MEMBRANE CARRIERS INVOLVED IN BILE ACID TRANSPORT BY HUMAN HEPATOCYTES. Up- or down-arrows indicate general changes in the expression levels in several cholestatic liver diseases. It should be noted that these changes may vary depending on species, experimental model and, liver disease associated with human cholestasis.

transporter (ASBT, gene symbol *SLCA102*). A short or truncated variant of ASBT (t-Asbt) has been suggested to be expressed at the basal membrane of rat cholangiocytes and ileal cells and to be involved in the sodium-independent efflux of BAs toward the adluminal pole of these epithelia [23]. Whether a similar human truncated ASBT variant also exists is not known. This function may be shared, at least in the intestine, by the heteromeric organic solute transporter, Ostα-Ostβ, which is also expressed at the basolateral plasma membrane of ileal enterocytes and which is able to carry out Na^+-independent BA transport [24].

2.2. THE OATP FAMILY

In the liver of non-mammalian vertebrates, such as the small skate, the ability to carry out efficient Na^+-dependent BA uptake is not yet present. However, the hepatocytes of these animals are able to carry out BA uptake, although this occurs via Na^+-independent transporters [25]. These proteins belong to the family of organic anion-transporting polypeptides (OATP) [26], which could therefore be considered as being more primitive along evolution than the SLCA10 family, although they are also more versatile, since they are able to transport a large variety of structurally different compounds, including BAs and conjugated [27] or unconjugated bilirubin [28]. Three isoforms of this family have been shown to transport BAs and to be expressed in human hepatocytes, including OATP-A or OATP1A2, according to the new nomenclature recently adopted by the HUGO Gene Nomenclature Committee [29] (gene symbol *SLCO1A2*), OATP-C/OATP1B1 (*SLCO1B1*) and OATP8/ OATP1B3 (*SLCO1B3*). Although OATP-A/ OATP1A2 has been shown to be able to transport BAs [30], owing to the low expression of OATP-A/OATP1A2 in normal adult hepatocytes [26] the role of this trans-

porter in the uptake of BAs by the liver from sinusoidal blood is probably minor as compared to that of NTCP, OATP-C/OATP1B1 and OATP8/OATP1B3. However, the expression of OATP-A/OATP1A2 in biliary epithelial cells [31] may play a role in the re-absorption of BAs from bile toward the periductular capillary plexus.

In addition to the transporters mentioned above, a role in BA uptake by hepatocytes of other transporters - belonging or not to the OATP family - cannot be ruled out. Thus, although OATP4A1 (previously OATP-E), whose mRNA has been detected in human liver as well as in other human tissues [32], is believed to be a thyroid hormone transporter, among the substrates of both human OATP-E/OATP4A1 [33,34] and its rat ortholog Oatp-E/Oatp4a1 [34] there are also some BAs.

Functional evidence suggests that a Na^+-independent carrier-mediated system, probably involving one or several OATPs, would account for BA uptake from fetal blood across the basal membrane of the human trophoblast [16,35]. Moreover, BA transport across the opposite membrane of this epithelium, i.e., the maternal-facing brush-border membrane, is mediated by ATP-dependent mechanisms [36], although the existence of ATP- and Na^+- independent processes, probably mediated by OATPs, has been also suggested [37,38].

Measurement of the abundances of mRNA in human placenta has revealed that this is higher for OATP8/OATP1B3 than for OATP-C/OATP1B1 and OATP-A/OATP1A2 [28]. Therefore, OATP8/OATP1B3 is so far a major candidate in the mediation of BA uptake from the fetal blood by the placenta. However, a role for other members of this family in this function cannot be ruled out, such as OATP-B/OATP2B1, which has been localized at the basal membrane of human trophoblasts [39]. However, there is some controversy regarding the substrate-specificity of this transporter. While the rat ortholog Oatp9/Oatp2b1, which is also expressed in the placenta of this species [14], seems to be able to transport taurocholate, among other substrates [40], no ability of OATP-B/OATP2B1 to transport BAs has been found, at least when this protein is expressed in *Xenopus laevis* oocytes [39].

OATP-E/OATP4A1 is also highly expressed in human placenta [33]. The ortholog Oatp12/Oatp4a1 is also abundantly expressed in rat placenta [14]. Since this carrier has mainly been detected at the apical surface of the human syncytiotrophoblast [33] its role, if any, in the transplacental transfer of BAs would be probably involved in the efflux of these molecules from the trophoblast toward the maternal blood.

In the kidney, several Oatps/OATPs have been identified at mRNA level; some of them with the ability to transport BAs. These include rat Oatp1/Oatp1a1, Oatp2/Oatp1a4, and human OATP-A/OATP1A2, OATP-B/OATP2B1, and OATP-E/OATP4A1. At protein level, Oatp1 has been identified in the apical plasma membrane in the S3 segment of the proximal tubule [41]. These transporters could play a role in the re-absorption of freely filtered organic compounds and in the urinary excretion of certain organic compounds due to uptake/secretion by proximal tubular cells. This role may depend upon the compound considered, and the physiological or pathological circumstances that determine the magnitude and direction of gradients as well as changes in the expression of the carriers involved, as will be discussed below. Another group of organic transporters, named OATs, which belong to a protein family (SLC22) other than that of OATPs, may also be involved in BA transport across the basolateral membrane of proximal tubules. Thus, the ability of mouse Oat3 (Slc22a8) to transport taurocholate has been reported [42].

The expression of Oatp3/Oatp1a5 in the apical membrane of rat small intestine has been described [43]. Moreover, its human ortholog OATP-B/OATP2B1 has been suggested to play a role in the absorption of anionic compounds across the apical membrane of human intestinal epithelial cells [44].

2.3. THE ABCB FAMILY

Several members of the superfamily of ATP-binding cassette (ABC) proteins are able to actively transport BAs out of cells in an ATP-dependent

manner. One of these efflux pumps, the sister of the P-glycoprotein or bile salt export pump (BSEP; gene symbol *ABCB11*), is the main mechanism of BA secretion into bile [45], even in lower vertebrates [46]. BSEP uses the energy of ATP hydrolysis to transport monoanionic BAs across the canalicular membrane with high affinity (in the 10 μM range) [47,48]. This protein has not been located in other epithelia involved in BA transport. Although the expression of BSEP in human [15,49] and rat placenta [14] has been detected, its abundance is so low that a major physiological role of the protein in BA transport across the placenta is unlikely. Similarly, although RT-PCR analysis has revealed the presence of BSEP transcripts in porcine large and small intestinal mucosa, as well as in brain, but not in kidney [50], the physiological role of this protein in these tissues remains unclear.

2.4. THE ABCC FAMILY

Several isoforms of the ABCC family of multidrug resistance-associated proteins (MRPs) are able to transport BAs. Thus, MRP2 (*ABCC2*), which is located at the canalicular membrane of hepatocytes, and plays an important role in the export of drugs and conjugated bilirubin into bile [51,52]. The ability of rat Mrp2 to also transport dianionic sulfated [53] and glucuronidated [54] BAs has been reported.

Other human and rat proteins of the MRP family are also able to transport monoanionic and dianionic BAs in a glutathione-dependent or independent manner. These include MRP1 (*ABCC1*), [55,56], MRP3 (*ABCC3*) [57-59], and MRP4 (*ABCC4*) [60,61]. Regarding their role in BA transport by hepatocytes, it should be noted that all these transporters are located at the basolateral membrane and their level of expression is low in healthy individuals. Therefore, under physiological circumstances they probably play a minor role in the overall traffic of BAs across the sinusoidal pole of hepatocytes.

The expression of MRP1 in the body is widespread, and its levels are particularly high in intestine and kidney, as well as in brain, lung, and testis [62]. MRP1 has also been detected in the basolateral membranes of polarized epithelial cells, including those of mouse renal distal and collecting tubules and intestinal crypts [63]. In contrast, MRP2 is located at the apical membranes of epithelial cells mainly in the liver, intestine, and kidney tubules [64,65]. MRP2 expression is highest towards the proximal segments of rat intestine and decreases towards the distal ileum. This, and its apical localization, suggest that MRP2 may play a role in secreting compounds, including glucuronidated or sulfated BAs, into the intestinal lumen as part of a detoxification pathway in this tissue. MRP4 is also expressed at the apical membranes of epithelial cells of human jejunum [66] and renal proximal tubules [67] and may thus contribute, together with MRP2, to BA excretion or the prevention of premature absorption of BAs that, with the bile, reach the upper segments of the intestine.

In contrast to MRP2, rat MRP3 expression is higher in the ileum and colon as compared to that seen in duodenum and jejunum [68]. Owing to the pattern of MRP3 expression in the intestine, and the facts that: (i) this protein is located at the basolateral membrane [69], and (ii) it is able to transport mono- and di-anionic BAs [57,58], it could be speculated that MRP3 might be also involved in BA re-absorption by the intestine.

The list of members of the MRP family able to transport BAs and expressed in human placenta include MRP1, MRP2 and MRP3 [70], and Mrp1, Mrp2 and Mrp3 in rat placenta [71]. Moreover, at least in rats, Mrp4 expression levels are markedly higher in placenta than in liver, although lower than in kidney [72].

2.5. THE ABCG FAMILY

Another ABC transporter also located at the apical membrane and with the ability to transport steroids, including BAs, with higher efficiency for sulfated derivatives [73,74], is the breast cancer resistance protein (BCRP; gene symbol *ABCG2*), which is highly expressed in placenta [75], thus accounting for one of the names of this protein, ABC placental protein (ABCP). However, the rele-

vance of BCRP in overall BA trans-placental transfer is not yet known. Although to a lesser extent, this protein is also expressed at the apical membrane of small intestinal and colonic epithelia, and in hepatocyte canalicular membranes [76].

3. RELATIONSHIP BETWEEN BILE ACID TRANSPORTERS AND DISEASE

The overall dynamics of BAs in the organism is closely dependent upon the correct function of BA transporters. On the other hand, the function of these transporters is regulated in response to several pathological situations, such as cholestatic liver diseases, through changes not only in gene expression and, hence in the amount of proteins synthesized, but also in the fate of these proteins. This includes post-translational modifications, intracellular trafficking, as well as insertion into and retrieval from the plasma membrane. These processes are triggered by a complex system of diverse intra- and extra-cellular signals.

3.1. EFFECT OF IMPAIRED HEPATOBILIARY FUNCTION ON THE EXPRESSION OF BA TRANSPORTERS

Among the factors affecting the function of BA transporters, BAs themselves play a major role. These molecules are able to reach the nucleus in a molecular species-selective manner [77,78] and activate a hierarchical network of nuclear receptors and transcription factors. Moreover, they can trigger intracellular signaling cascades that may induce post-translational changes affecting the fate of these transporters.

The most important regulatory element that responds to elevations in BA levels is the farnesoid X receptor (FXR), which is efficiently activated by some BAs, such as CDCA, but poorly activated by others, including UDCA. Upon activation, FXR binds to DNA as a heterodimer with the retinoid X receptor (RXR). This results in a direct up-regulation of BSEP, MRP2, OATP8/OATP1B3 and the small heterodimer partner (SHP), which in turn induces the down-regulation of NTCP, ASBT and OATP-C/OATP1B1. SHP is another nuclear receptor that blocks the transcription factors LRH1/FTF (liver receptor homolog 1 or fetal transcription factor) and HNF4α (hepatocyte nuclear factor). The action of the latter is necessary for the expression of key enzymes in BA synthesis: CYP7A1 and CYP8B1 (for a review, see ref. [10]).

Cholestasis-induced BA intracellular accumulation above certain levels results in profound changes in the expression of BA transporters (FIG. 3). This may vary depending on the species, the experimental models and, in humans, on the nature of the liver disease responsible for the cholestasis. A reduction in the expression of transporters involved in BA uptake by the liver has been reported in several cholestatic liver diseases [10,79,80]. Moreover, the expression levels of MRP2 and BSEP are reduced in patients with cholestasis [79-81]. On the other hand, the expression of MRP1 (*ABCC1*) and MRP3 (*ABCC3*), which is very low in adult hepatocytes, may be markedly increased both in hepatocytes and cholangiocytes in response to experimental and clinical cholestasis [81-84] and endotoxemia [85]. Up-regulation of these pumps in pathological conditions might favor the elimination from liver cells to blood of potentially toxic cholephilic compounds, including BAs, when they cannot be secreted into bile. The kidney, where MRP2 is up-regulated, would subsequently excrete these compounds in urine [86].

Intestinal MRP3 is also regulated by exposure to BAs. A 3-fold increase in mRNA levels of MRP3, but not MRP2, has been detected in human intestinal cells (Caco-2) following exposure to several BAs for 48 h [87].

At least in rat placenta, the expression levels of the BA carriers Oatp1/Oatp1a1, Oatp2/Oatp1a4 and Oatp1a4/Oatp1b2, which are low under physiological conditions [14], increase markedly during maternal cholestasis, and even more so when pregnant rats are treated with UDCA [71]. This change could enhance the defensive barriers against the inverted gradient of BAs, an alteration that may favor the entry of these compounds into the trophoblast. Thus, up-regulation of these transporters may result in an enhanced ability of the placenta to return BAs

back to the maternal blood. Indeed, whereas common bile duct ligation results in an increase in serum BA concentrations above 20-fold in the mother, this increase is only of approximately 2-fold in their fetuses [71].

3.2. ROLE OF IMPAIRED BILE ACID TRANSPORT IN CHOLESTASIS

Among the group of severe genetic cholestatic liver diseases occurring early on in life known by the common name of progressive familial intrahepatic cholestasis (PFIC), only one subtype, PFIC-2 is due to a primary impairment in BA transport into bile. This results from the existence of mutations in chromosome 2 affecting the expression of functional BSEP [88,89]. At least ten different missense mutations have been described, some of them leading to altered expression and/or sorting of the protein to the canalicular membrane, accounting for a deficient secretion of BAs [90]. PIFC-4 is not primarily due to abnormal expression of BSEP, but probably to impaired function of this pump by the inhibitory action of flat cholenoic acids [91], which are accumulated in the liver of these patients as a result of a defect in the enzymes Δ^4-3-oxosteroid 5β-reductase [92] or 3β-hydroxy-Δ^5-C27-steroid dehydrogenase/isomerase, both involved in BA biosynthesis [93,94]. Like cholenoic acids, several xenobiotic and endogenous compounds able to induce the inhibition of BSEP may be involved in the etiology of intrahepatic cholestasis [95]. In this respect, intrahepatic cholestasis of pregnancy has recently been suggested [96] to be due, at least in some cases, to trans-inhibition of BSEP from the intracanalicular lumen by sulfated progesterone metabolites, which have been found elevated as a primary event in pregnant women with asymptomatic hypercholanemia of pregnancy (AHP), that is, serum BA concentrations higher than normal but no other clinical or biochemical abnormalities [97].

In contrast to PFIC, benign recurrent intrahepatic cholestasis (BRIC) is a group of less serious diseases characterized by recurrent episodes of cholestasis. One of the variants of this disease, BRIC2, is probably associated with mutations in the regulatory regions of the *ABCB11* gene coding for BSEP [98].

Impaired expression of canalicular MRP2 is associated with the Dubin-Johnson syndrome, characterized by impaired bilirubin secretion, although normally with minor and indirect effects on overall BA homeostasis. However, the Dubin-Johnson syndrome is not homogeneous and in some patients an abnormality of BA clearance coexists with an abnormality in bilirubin clearance [99].

No other liver diseases have so far been associated with genetic alterations leading to abnormal function of other basolateral or canalicular BA transporters.

3.3. DRUG-INDUCED CHOLESTASIS DUE TO BSEP INHIBITION

Drug-induced cholestasis is a frequent form of acquired liver disease. The molecular pathogenesis of these alterations often resides in the inhibitory effect of cholestatic drugs on the normal function of BSEP [100]. Thus, cyclosporin A, rifamycin SV, rifampicin, and glibenclamide have been reported to be able to carry out competitive cis-inhibition of BA transport by rat [95] and human [47] BSEP.

Troglitazone is a thiazolidinedione insulin-sensitizer drug initially developed for the treatment of type 2 non-insulin-dependent diabetes mellitus, but no longer used in clinical practice owing to its toxicity, which includes cholestatic liver toxicity. Both troglitazone and troglitazone sulfate have been found to cis-inhibit BA transport by rat Bsep [101].

The widely used immunosuppressor cyclosporin A has also been found to cause cholestasis. In addition to other interactions with OATPs, which will be commented below, and disruption of the pericanalicular F-actin cytoskeleton, the cholestatic effect of this drug is also accounted for by its ability to inhibit BA transport across the rat canalicular plasma membrane, in part due to alterations in the localization of Bsep at canalicular level, which results in its relocation back into the cell [102].

During clinical trials, bosentan, the first orally

active endothelin receptor antagonist, caused asymptomatic transaminase elevations in some patients due to dose-dependent and reversible liver injury, which was accompanied by a significant increase in serum BA levels. This has been reported to be mediated, at least in part, by inhibition of BSEP by bosentan and its metabolites, causing the intracellular accumulation of cytotoxic BAs and subsequent BA-induced liver cell damage [103].

The non-steroid anti-inflammatory drug sulindac is secreted into bile in unconjugated form and is absorbed by cholangiocytes, inducing hypercholeresis. At high flux rates, sulindac competitively inhibits canalicular BA transport; such inhibition may contribute to the propensity of sulindac to induce cholestasis in patients treated with this drug [104].

3.4. REPERCUSSIONS OF IMPAIRED INTESTINAL BILE ACID TRANSPORT

Primary BA malabsorption is a congenital disease characterized by diarrhea, steatorrhea, and growth failure. The enterohepatic circulation of BAs in these patients is almost absent due to impaired intestinal absorption even though other absorptive processes are normal [105]. The reason is that the altered mechanism only affects the expression of functional ASBT [19]. Indeed, at least four polymorphisms affecting the *SLC10A2* gene have so far been identified in families with this disease [106].

4. USE OF BILE ACID DERIVATIVES IN DRUG TARGETING

4.1. USEFULNESS OF THE PHARMACOLOGICAL STRATEGY

The benefits of using BAs as conjugating agents in pharmacology include their ability to behave as "Trojan horses" to vectorize drugs toward the liver, the biliary system and the intestine. This strategy is aimed at enhancing the liver uptake, biliary secretion, and intestinal absorption of the conjugated drug [for a review, see 107]. Moreover, by inhibiting the enterohepatic circulation of endogenous BAs, hence increasing their fecal elimination and stimulating cholesterol metabolism, BA derivatives may be useful as cholesterol-lowering drugs.

4.2. TARGETING OF SMALL ORGANIC AND INORGANIC MOIETIES

Since the first BA conjugates or their analogues were proposed as shuttles for drugs towards tissues located in the enterohepatic circuit [108-110], the list of BA derivatives that can be included in this group has expanded considerably. Some examples of small moieties conjugated with BAs are: HMG-CoA reductase inhibitors [111]; non-steroidal selective modulators of glucocorticoid receptors [112]; and sulfonamide derivatives with strong inhibitory effects on isozymes I, II and IV of carbonic anhydrase [113,114].

The formation of complexes between BAs and the magnetic resonance agent gadolinium has permitted contrast agents to be obtained with enhanced ability to be transported toward the hepatobiliary system [115]. A similar strategy has been assayed to concentrate into bile the chelating agent EDTA, which otherwise exhibits a predominantly extrahepatic distribution and marked renal clearance [109].

BA-drug complexes have been also designed to deliver active agents to the intestine. Thus, UDCA-5-aminosalicylate conjugate, which is poorly absorbed by the small intestine and is hydrolyzed into both constituents of the complex by intestinal bacteria, has been proposed as a useful compound to deliver the anti-inflammatory agent 5-aminosalicylic acid to the colon for the treatment of inflammatory bowel diseases, ulcerative colitis and Crohn's disease [116].

Several BA derivatives have been proposed as efficient germicides. Thus, compounds obtained by the acylation of BAs with long-chain fatty acids (C12-C16) induce inhibitory effect on the growth of some strains of Gram-positive and -negative bacteria [117]. Moreover, cationic BA derivatives have been described to exert a potent and broad-spectrum activity against multidrug-resistant Gram-negative and -positive bacteria [118]. The antima-

larial activity of several CA-derived 1,2,4,5-tetraoxanes has been reported. The cytotoxicity of these compounds is not limited to *Plasmodium falciparum*; they are also able to inhibit the proliferation of human melanoma (Fem-X) and human cervix carcinoma (HeLa) cell lines [119].

Cholylsarcosine is a synthetic conjugated BA that is efficacious and safe for enhancing fat absorption and nutritional status in patients with residual colon, whereas natural conjugated BAs improve steatorrhea to a smaller extent and considerably worsen the diarrhea [120]. Replacement therapy with cholylsarcosine reduces urinary oxalate excretion and improves hyperoxaluria, oxalate nephrolithiasis and nutritional status in patients with short bowel syndrome with colon in continuity [121].

Portal hypertension, a life-threatening complication of liver cirrhosis, results from increased intrahepatic resistance and increased portal blood inflow through a hyperdynamic splanchnic system. The increased intrahepatic vascular tone is the result of an enhanced activity of endogenous vasoconstrictors and a deficiency in nitric oxide (NO) release by sinusoidal endothelial cells. Thus, the ideal drug for the treatment of portal hypertension should act by specifically decreasing intrahepatic vascular resistance. NCX-1000 is the prototype of a family of NO-releasing derivatives of UDCA able to selectively release, from parenchymal and non-parenchymal hepatic cells, biologically active NO into the liver microcirculation, without detectable effects on the systemic circulation. This suggests that these new drugs may provide a novel therapy for the treatment of patients with portal hypertension [122].

4.3. BILE ACID TRANSPORTERS IN ENTEROHEPATIC TUMORS

The potential use of BAs in drug targeting for anticancer chemotherapy is of great interest. However, this strategy requires the functional expression of BA transporters in targeted tumors. Therefore, a key question arises as to whether tumors derived from tissues that normally express these proteins, such as enterohepatic tumors, maintain (at least to a certain extent) the ability to take up BA through these specific mechanisms. Owing to heterogeneity in tumor phenotypes, the positive answer suggested by the available evidence cannot be extended to all enterohepatic tumors. Studies using tumor cell lines of enterohepatic origin have revealed that, although markedly reduced in some cases, the expression of carrier proteins and the ability to take up cholephilic organic anions that are typically substrates of OATPs and NTCP are still present in several, but not all, of them [30, 123-125]. Several of these transporters have also been found in human hepatocellular carcinomas, where the expression of NTCP, but not that of OATPs, seems to be markedly reduced [126].

Using RT real-time quantitative PCR, we have recently measured the mRNA abundance of some of these transporters in biopsies collected from intestinal adenomas and carcinomas. The amounts of these mRNAs in tumor tissues were compatible with a functional level of protein expression and, indeed, when total mRNA from these cells was injected to *Xenopus laevis* oocytes it conferred them the ability to take up natural BAs and cytostatic BA derivatives (unpublished results). Moreover, cells isolated from the livers of rats undergoing a protocol of hepatocarcinogenesis induction were able to carry out selective and sodium-independent uptake of BA derivatives, suggesting that OATPs could account for this transport [127]. It is important to note that although the efficiency of tumor cells in taking up BAs is probably low, due to reduced expression levels of BA transporters as compared with normal epithelial cells, it should be recalled that tumor cells are not polarized and hence drug uptake might not be accompanied by similar extent of efflux out of the cells to a similar extent. As an example in support of this hypothesis, when overall drug accumulation was analyzed after administering BA derivatives to nude mice that had previously been ortothopically implanted with murine liver tumor cells, the amount of drug in the tumor was higher than that found in the adjacent healthy tissue from the same liver [128]. Moreover, previously expected relationship between tumor cell

load and sensitivity to the cytostatic effects of these compounds has been reported [129].

The reasons for synthesizing cytostatic BA derivatives were originally dual: to use them against tumors located in tissues of the enterohepatic circuit [130] and to enhance their water miscibility [131]. However, another interesting aspect of BA derivatives in anticancer chemotherapy would be to extend their use to regional chemotherapy of tumors located outside the enterohepatic circuit. In these cases, the pharmacological advantage would also be based on the organotropic characteristics of BA derivatives. Thus, the fraction of drug administered that may reach the general circulation after escaping from the site of administration near the tumor would be expected to be efficiently taken up and eliminated into bile by the liver [132,133].

4.4. COUPLING BILE ACIDS TO CYTOSTATIC AGENTS

The possibilities of targeting cytostatic agents to enterohepatic tumors by coupling of BAs to organic and inorganic cytostatic moieties are very broad. Among organic antitumor drugs, chlorambucil [134] as well as other organic moieties have been investigated. In this line of research, several synthetic derivatives of UDCA (HS-1183), and CDCA (HS-1199 and HS-1200), have been synthesized and found to be effective inductors of apoptosis in several human tumor cell lines [135].

Regarding inorganic moieties, our group has explored the usefulness of BA-conjugates containing DNA-reactive transition metals. With this aim, a novel family of compounds named Bamets, from BA (bile acid) and MET (metal), has been developed. The reason for selecting this tandem was the small size of the expected molecule. This would increase the probability of maintaining the substrate properties as regards BA transporters and reactivity, and hence the antiproliferative effect of these metals, in particular platinum(II), such as in cisplatin (*cis*-diamminedichloro-platinum(II)) [136]. Bamets containing transition metal atoms other than platinum, such as gold [137], are less efficient cytostatic agents than those containing Pt(II) in the reactive moiety [130].

Two types of variable have been assayed regarding the organic moiety of these molecules. These are the BA moiety and the nature of the linker placed between this and the transition metal atom alone or as part of the cisplatin-derivative [130]. More recently, other groups have expanded the list of variations in the Bamet family by synthesizing several carboplatin-BA derivatives [138]. Two of the best studied and most promising compounds of the Bamet family are *cis*-diammine-chlorocholylglycinate-platinum(II) (Bamet-R2) and *cis*-diammine-bisursodeoxy-cholate-platinum(II) (Bamet-UD2). Both compounds are able to induce an "in vitro" cytostatic effect and "in vivo" inhibition of tumor growth [128] and to interact with several BA transporters [139]. These properties, together with the possibility of efficiently loading them into liposomes due to their amphipathic characteristics [140], permits resistance to cisplatin to be overcome [128]. Moreover, they have several very interesting characteristics, in particular Bamet-UD2: they are efficiently eliminated in bile [132] and, at therapeutic doses, they have no toxic effect on the liver, kidney, nerve or bone marrow of laboratory animals [141].

4.5. DELIVERY OF OLIGONUCLEOTIDES, PEPTIDES AND POLYSACCHARIDES

Oligonucleotides can be conjugated to BAs via, for instance, the hydroxyl group located at C3 in major BA molecules. These complexes have been proposed as useful tools for liver-specific gene therapy [142]. With this aim, different backbone-modified antisense oligonucleotides directed against the hepatitis C virus genome conjugated with BAs have been obtained and assayed [143,144]. Surprisingly, conjugation with BAs has also been seen to be effective in enhancing antisense oligonucleotide internalization by cells, such as neutrophils, that do not express BA transporters, which suggests that other routes are probably involved in the uptake of these complexes [145]. Conjugation to BA moieties has also been used as a strategy to protect sequence-specific oligonucleotides aimed at interacting with molecular targets located in the small or large intestine from

nuclease activity during gastrointestinal transit [146].

Small peptides have been also conjugated to BAs to improve their pharmacokinetic properties [147]. Thus, N-LCA acylated insulin [Lys(B29)-lithocholyl des-(B30) human insulin] has been shown to disrupt neither the important conformational features of the insulin molecule nor its hexamer-forming ability. Indeed, binding studies have shown that the affinity of N-lithocholyl insulin for the human insulin receptor is not significantly diminished. However, the subcutaneous injection of this compound results in slow absorption into the bloodstream and prolongs its half-life once there [148].

A different strategy for the delivery of peptides consists of the use of polymeric molecules that act as carriers. Some of the polymers developed have also included BA moieties to improve the physical-chemical and biological properties of the preparations [149-150].

Heparin administration is usually limited to intravenous or subcutaneous injection. However, a new method for oral delivery of this polysaccharide for treating patients at a high risk of deep vein thrombosis or pulmonary embolism has been proposed. Heparins of various sizes were conjugated with DCA to enhance the oral absorption of the drug in the gastrointestinal tract. Investigation of these compounds revealed that indeed they have a high anticoagulant effect when administered orally [151].

4.6. BAS IN PHARMACEUTICAL PREPARATIONS AS ABSORPTION ENHANCERS AND PERMEATORS

The physical-chemical characteristics of BAs can be used to obtain absorption-enhancing agents. For example, the inclusion of a BA, such as DCA, in hydrogel preparations has been considered as a promising alternative as drug carrier system for topical administration of drugs and cosmetics [152]. In this respect, non-toxic BA derivatives, such as cholylsarcosine, rather than natural BAs have been proposed as enhancers of intestinal absorption, which is particularly important in certain pathological circumstances, such as in "short bowel syndrome", or for increasing the intestinal absorption of peptides with therapeutic activity [153]. Moreover, 3α,7α-dihydroxy-12-oxo-5β-cholanate has been proposed as a blood-brain barrier permeator. Thus, at least in rats this compound promotes the analgesic action of morphine and enhances the hypnotic effects of pentobarbital [154].

5. PHARMACOLOGICAL INHIBITION OF BILE ACID TRANSPORTERS

5.1. INHIBITION OF ASBT

Interest in inhibiting intestinal BA absorption in attempts to reduce serum cholesterol levels accounts for the development of a large number of inhibitors of the Na^+-dependent BA transport across the brush-border membrane of intestinal mucosa cells, some of which are reviewed here.

2164U90 is a benzothiazepine-based compound [(3R,5R)-trans-3-butyl-3-ethyl-2,3,4,5-tetrahydro-5-phenyl-1,4-benzothiazepine-1,1-dioxide] [155] that was first found to be able to competitively inhibit Na^+-dependent BA transport across the plasma membrane of rat, monkey and human ileal cells, with Ki values lower than 10 μM [155,156], and to stimulate the elimination of exogenously loaded BAs. Moreover, 2164U90 inhibited the increase in the levels of lipoproteins VLDL plus LDL induced by diets containing cholesterol-CA (in rats) and cholesterol-CA-coconut oil (in mice) [155].

In contrast, S-8921 (methyl-1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate) is able to inhibit ASBT by performing mixed competitive and non-competitive inhibition, with an overall Ki value for hamster Asbt expressed in COS7 cells of 66 μM. Owing to this ability, when administered in the diet this drug caused a dose-dependent decrease in serum cholesterol concentrations, accompanied by increased fecal excretion of BAs in hamsters that had not been loaded with cholesterol and BAs [157]. In rats, S-8921 has a hypocholesterolemic action by inhibiting cholesterol absorption from the intestine and enhancing its elimination from the body. S-8921 does not inhibit the absorption of cholesterol

from rat jejunum; it clearly inhibits the active absorption of tauro-CA and glyco-CA from rat ileum, and it does not inhibit the passive absorption of CA from the rat jejunum [158]. Based on the promising results of this drug in preventing atherosclerosis in heterozygous Watanabe heritable hyperlipidemic rabbits [159], phase I trials have begun in 2000 [160].

SC-435 (1-[4-[4[(4R,5R)-3,3-dibutyl-7-(dimethylamino)-2,3,4,5-tetrahydro-4-hydroxy-1,1-dioxido-1-benzothiepin-5-yl]phenoxy]butyl]-4-aza-1-azoniabicyclo [2.2.2] octane methanesulfonate) is a more recently developed strong inhibitor of ASBT, with Ki values of less than 10 nM, and it is minimally absorbed by the intestine. In miniature pigs, this compound has been found to significantly reduce plasma LDL cholesterol through enhanced LDL receptor-mediated LDL apoB clearance, secondary to increased expression of cholesterol 7α-hydroxylase (CYP7A1) [161]. SC-435 also reduces LDL-cholesterol concentrations and attenuates the progression of atherosclerosis in guinea pigs [162]. In mice, the SC-435-induced inhibition of Asbt also resulted in significant increases in hepatic mRNA levels for cholesterol 7α-hydroxylase and HMG-CoA reductase [163]. Thus, the reduction in LDL cholesterol and LDL apolipoprotein B in miniature pigs fed a typical human diet was enhanced by combining SC-435 with the HMG-CoA reductase inhibitor atorvastatin [164].

Another compound with ability to inhibit BA transport by rat and human ASBT, with Ki values below 1 μM, and hence able to induce "in vivo" up-regulation of CYP7A1 is named 264W94 [165].

Olsalazine, a derivative of 5-aminosalicylic acid that is currently being used for the treatment of colitis, has been found to induce diarrhea, associated with a marked inhibition of intestinal absorption of BAs [166].

Molecular modeling techniques in combination with the measurement of the inhibitory activity of 17 different ASBT inhibitors, some of them with Ki values lower than 10 μM, have afforded the basic structure of the pharmacophore model, which could be useful in the design of novel and more effective ASBT inhibitors [167].

5.2. INHIBITION OF OATPS

Although considered here as BA transporters, some of these proteins exhibit a broad substrate specificity with a large degree of overlap. This accounts for the possibility of transporter-mediated drug-drug interactions among substrates.

Regarding hepatocytes, this is particularly relevant for OATPs. Some of the interactions so far identified include the competition for OATP-C/OATP1B1 of cyclosporin A and gemfibrozil with HMG-CoA reductase inhibitors, such as cerivastatin (for a review, see [168]).

This type of interaction also occurs between drugs, toxins and normal food components. Among the examples of drug-toxin transporter-mediated interactions is the ability of drugs such as bromosulfophthalein, cyclosporin A and rapamycin to inhibit the OATP-C/OATP1B1-mediated uptake of the mycotoxin from *Amanita phalloides*, phalloidin. This inhibition protects the hepatocytes from the severe damage caused by this toxin [169].

In the small intestine, some components of grapefruit and other fruit juices, in particular citrus juice, are able to inhibit the uptake of drugs, such as fexofenadine and others, whose uptake is mediated by members of the OATP family [170].

Regarding transporter-mediated interactions between drugs and endogenous substances, it should be noted that the inhibition of OATP-C/OAPT1B1 by several drugs, including indinavir, saquinavir, cyclosporin A, and rifamycin SV, is an important mechanism for drug-induced unconjugated hyperbilirubinemia [171]. Similarly, the elimination of substances such as the neuroactive steroid dehydroepiandrosterone sulfate across the blood-brain barrier via OATP-mediated transport may be impaired by the presence of endogenous compounds (bilirubin, BAs), drugs or toxins, which may competitively inhibit dehydroepiandrosterone sulfate transport from brain to the circulating blood across the blood-brain barrier [172].

6. OTHER USES OF BILE ACIDS IN PHARMACOLOGY

Natural BA molecules or their derivatives are also arousing pharmacological interest, with a broad spectrum of applications. Among natural BAs, CDCA, first, and UDCA, later, have been used with success to dissolve cholesterol gallstones [173,174]. UDCA has become more popular due to its low toxicity and efficiency, not only in cholelithiasis but also in many different cholestatic liver diseases because of its multiple beneficial actions, which include: (i) protection of cholangiocytes against the toxic effects of more hydrophobic BAs, (ii) stimulation of the so-called BA-dependent and BA-independent bile flow, even in situations of impaired biliary secretion, (iii) stimulation of the detoxification of hydrophobic BAs, (iv) immunomodulation, (v) enhancement of defenses against oxidative stress, and (vi) inhibition of apoptosis induced by several agents in hepatocytes. Through one or more of these mechanisms, UDCA slows the progression of primary biliary cirrhosis and improves a number of other cholestatic disorders (for a review, see [175]). Some of these beneficial properties may be also useful to protect organs other than the liver, such as the brain [176] or the placenta [177].

Major BAs and their derivatives are natural detergents with microbicidal activity. In this respect, they have been proposed for numerous uses, either topically [118, 178-180] or orally to reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhosis [181].

Pharmacological applications for minor BA molecules, with or without chemical modifications, have also been reported. An intranasally applied solution of monoketo-CA has a hypoglycemic effect, but only in rats with diabetes. The same substance has no effect on blood glucose levels in normoglycemic rats [182].

Modulation of the activity of metabolic pathways by manipulating the expression of enzymes under the control of nuclear receptors is a promising pharmacological strategy. In this respect, BAs and their derivatives have been assayed. LCA induces its metabolism through interaction with the vitamin D receptor (VDR). Several-fold less toxic but stronger activators of VDR have been synthesized. LCA-acetate is the most potent of these VDR agonists [183].

Certain natural 6α-hydroxylated BAs are receptor-specific activators of nuclear liver X receptor alpha (LXRα), which regulates the expression of the gene coding for cholesterol 7α-hydroxylase, the rate-limiting enzyme in the major pathway of BA synthesis. Synthetic 6α-hydroxylated BA analogs have been synthesized with LXRα-selective agonistic activity and they have been proposed to be useful for the modulation of cholesterol catabolism in hypercholesterolemia [184].

Several 6α-alkyl-substituted analogues of CDCA have been synthesized and evaluated as potential FXR ligands. Among them, 6α-ethyl-CDCA was shown to be a very potent and selective FXR agonist [185]. This is particularly interesting, since activation of the FXR-SHP regulatory cascade promotes the resolution of liver fibrosis in rodent models, and hence FXR ligands might offer a novel therapeutic option to treat liver fibrosis [186].

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