Published in final edited form as:
*Mol Aspects Med*. 2013 April ; 34(2-3): 252–269. doi:10.1016/j.mam.2012.07.004.

# The solute carrier family 10 (SLC10): beyond bile acid transport

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## Abstract

The solute carrier (SLC) family 10 (SLC10) comprises influx transporters of bile acids, steroidal hormones, various drugs, and several other substrates. Because the seminal transporters of this family, namely, sodium/taurocholate cotransporting polypeptide (NTCP; *SLC10A1*) and the apical sodium-dependent bile acid transporter (ASBT; *SLC10A2*), were primarily bile acid transporters, the term “sodium bile salt cotransporting family” was used for the SLC10 family. However, this notion became obsolete with the finding of other SLC10 members that do not transport bile acids. For example, the sodium-dependent organic anion transporter (SOAT; *SLC10A6*) transports primarily sulfated steroids. Moreover, NTCP was shown to also transport steroids and xenobiotics, including HMG-CoA inhibitors (statins). The SLC10 family contains four additional members, namely, P3 (SLC10A3; *SLC10A3*), P4 (SLC10A4; *SLC10A4*), P5 (SLC10A5; *SLC10A5*) and SLC10A7 (*SLC10A7*), several of which were unknown or considered hypothetical until approximately a decade ago. While their substrate specificity remains undetermined, great progress has been made towards their characterization in recent years. SLC10A4 may participate in vesicular storage or exocytosis of neurotransmitters or mastocyte mediators, whereas SLC10A5 and SLC10A7 may be involved in solute transport and SLC10A3 may have a role as a housekeeping protein. Finally, the newly found role of bile acids in glucose and energy homeostasis, via the TGR5 receptor, sheds new light on the clinical relevance of ASBT and NTCP. The present mini-review provides a brief summary of recent progress on members of the SLC10 family.

### Keywords

SLC10; bile acid; ASBT; NTCP; SOAT; TGR5

## 1. The Solute Carrier 10 (SLC10) family of membrane transporters

The solute carrier (SLC) family 10 (SLC10) comprises influx transporters of bile acids, steroidal hormones, specific drugs, and a variety of other substrates. Bile acids (BA) are multifaceted molecules that act on several fronts to maintain endocrine homeostasis. They are the major components of bile and in the intestine they work as detergents to aid the solubilization and digestion of dietary nutrients, including vitamins A, D, E, K and cholesterol (Pols et al., 2011). Bile acids also assist in cholesterol solubilization in the gallbladder, thereby preventing formation of cholesterol gallstones (Claudel et al., 2011). In addition to their detergent properties, BA function as complex signaling molecules that



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modulate glucose, lipid and energy metabolism (Pols et al., 2011) and are also believed to participate in the etiology of certain types of cancer (Bernstein et al., 2011; Bernstein et al., 2009; Dvorak et al., 2009). Clearly, rigorous control of BA levels in the body is imperative, and is achieved by regulation of proteins involved in BA synthesis and transport, as well as in BA compartmentalization in the enterohepatic circulation (EHC). The EHC consists of BA recycling between the liver and the intestine, with subsequent return to the liver through the portal circulation and storage in the gallbladder. In the postprandial phase, BA are secreted from the gallbladder into the duodenum and travel down the intestine, where they assist in lipid absorption and are also absorbed passively (Lefebvre et al., 2009). Upon reaching the distal ileum, BA are reclaimed by active apical sodium-dependent bile acid transporter (ASBT)-mediated uptake in the apical brush border membrane of ileocytes. Bile acids that escape ASBT absorption are modified by colonic enterobacteria to yield hydrophobic secondary BA, such as deoxycholic acid (DCA), which are then passively absorbed in the colon. Less than 10% of the BA pool is lost daily via excretion routes, and lost BAs are replaced by *de novo* synthesis from cholesterol in the liver, yielding primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) (Pols et al., 2011). Also in the liver, BA are conjugated with taurine or glycine, resulting in increased water solubility, decreased passive transport and increased BA affinity for specific transporters (Balakrishnan et al., 2006).

Those BA that are internalized by ASBT in the ileocytes are shuttled to the basolateral membrane by the lipid binding protein (ILBP; *FABP6*, also known as ileal bile acid binding protein or IBABP), and are then effluxed into the portal circulation by the heterodimeric organic solute transporter complex OSTα/OSTβ (*OSTalpha* and *OSTbeta*) and, to a lesser extent, the multi-drug resistance-associated protein-3 (MRP3; *ABCC3*). Upon reaching the liver, BA are cleared from the portal circulation sodium-dependently by the sodium/taurocholate cotransporting polypeptide (NTCP; *SLC10A1*) and sodium-independently by the organic anion transporting polypeptides (OATP) OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*). Both NTCP and OATP proteins are expressed in the sinusoidal membrane of hepatocytes. Conjugated or not, BA are then effluxed into the gallbladder by the canalicular bile salt export pump (BSEP; *ABCB11*) and possibly multidrug resistance-associated protein-2 (MRP2; *ABCC2*) and P-glycoprotein (also known as multidrug resistance protein, MDR1; *ABCB1*), thereby completing one EHC cycle. NTCP and ASBT are the chief and rate-limiting mediators of bile acid uptake in the liver and intestine, respectively (Dawson, 2011; Stieger, 2011). A schematic representation of the tissues expressing relevant SLC10 transporters, with special emphasis in tissues involved in the EHC, is presented in figure 1.

While substrate specificity of ASBT is very strict, and taurine-, glycine-conjugated and unconjugated BA remain the only known ASBT natural substrates (Ho et al., 2011), Kolhatkar and collaborators reported the transport of nonsteroidal synthetic compounds in stably transfected Madin-Darby Canine Kidney (MDCK) cells, *i.e.*, ASBT-MDCK cells (Kolhatkar et al., 2012).

In contrast, with ASBT, NTCP substrate specificity is broader and includes steroidal hormones such as estrone-3-sulfate and dehydroepiandrosterone (DHEAS), sulfo-conjugated bile acids, bromosulphthalein and a variety of xenobiotics (Stieger, 2011).

Since their cloning, NTCP and ASBT have been studied extensively. Because they were the seminal and well-characterized members of the SLC10 family until approximately ten years ago, the SLC10 family was frequently referred to as “the sodium bile salt cotransport family” (Hagenbuch and Dawson, 2004). However, this notion was challenged by identification of the sodium-dependent organic anion transporter (SOAT; *SLC10A6*) in rat and human adrenal glands (Geyer et al., 2006). SOAT does not transport the classic bile

acids CA, CDCA and taurocholic acid (TCA). Instead, it transports sulfated forms of bile acids and steroid hormones. In spite of this functional resemblance with NTCP and striking dissimilarity with ASBT, SOAT exhibits higher amino acid sequence identity (41.8%) and similarity (69.7%) with ASBT than with NTCP (33% identity; 62.3% similarity) in humans, which was also observed among rat orthologs. As suggested by Geyer and co-workers, it would be instructive to explore these contradictory characteristics as a way to understand protein regions involved in sulfate recognition, since NTCP and SOAT, but not ASBT, transport sulfated substrates. One common feature shared by all three transporters is their reliance on sodium, as they harness the energy stored in the inwardly directed sodium gradient to actively concentrate solutes in the cell. Moreover, they share topological traits in mammalian species, reputedly exhibiting seven transmembrane domains (TM) oriented with extracellular N-terminus and cytoplasmic C-terminus ($N_{exo}/C_{cyt}$). While initially controversial, the 7 TM topology was either strongly suggested or confirmed by distinct laboratories, using a variety of bioinformatics and experimental approaches (Dawson, 2011). Finally, NTCP, ASBT and SOAT exhibit a highly conserved sequence, namely "ALGMMPL" or the NTCP/ASBT/SOAT signature motif, placed on their third transmembrane domains (Geyer et al., 2006).

In addition to NTCP, ASBT and SOAT, other members of the SLC10 family were identified and assigned to this family due to their high amino acid sequence identity. These additional members include P3/*SLC10A3*, P4/*SLC10A4*, P5/*SLC10A5* and *SLC10A7*. While function and substrate specificity of these proteins remain elusive, great progress has been made recently towards characterizing *SLC10A4* and *SLC10A7* (*vide infra*). For a phylogenetic comparison of all members of the SLC10 family, the reader is directed to the 2006 publication by Geyer and collaborators (Geyer et al., 2006). In the transporter classification system, the SLC10 family is also identified as Bile Acid:Sodium Symporter (BASS) family and belongs to the bile/arsenite/riboflavin transporter (BART) superfamily (Saier et al., 2009).

## 2. Bile acid transport: NTCP (*SLC10A1*) and ASBT (*SLC10A2*)

NTCP and ASBT are highly expressed in the liver and intestine, respectively. Yet, they are also detected in other tissues, where they may participate directly or indirectly in BA recycling. For instance, NTCP was also identified in the luminal membrane of rat pancreatic acini, where it may rescue BA that escape to the terminal acini. However, in this tissue, it may also potentially contribute to the BA-induced acinar cell injury observed in acute pancreatitis (Lange et al., 1986). As suggested by Kim *et al*, in normal physiology, this cytotoxic effect may be prevented by expression of the efflux transporters multidrug resistance protein 1 (MDR1; *Abcb1a*) and multidrug resistance-associated protein 3 (MRP3; *Abcc3*) (Kim et al., 2002).

Proximal tubule cells are the second highest site of ASBT expression, where active ASBT-mediated BA reabsorption minimizes BA loss in the urine (Dawson, 2011). On the apical membrane of cholangiocytes, ASBT allegedly participates in the return of BA from the lumen of bile ducts to the liver through the periductular capillary plexus, for subsequent re-secretion into bile. This process is known as the cholehepatic shunt and relies on passive absorption of unconjugated BA, and on the active (likely through ASBT) absorption of conjugated BA. The cholehepatic shunt is deemed a physiological adaptation to secure circulating BA levels, especially in pathological conditions such as cholestasis (Xia et al., 2006). Activation of the cholehepatic shunt by ursodeoxycholic acid (UDCA) or norUDCA has been postulated as treatment for primary sclerotic cholangitis (PSC) (Glaser and Alpini, 2009).

Both NTCP and ASBT have been examined in humans, rabbits, rats, mice and hamsters (Dawson, 2011; Stieger, 2011). Interspecies, tissue and cell line specificity for these transporters have been described (Anderle et al., 2005; Cheng and Klaassen, 2009; Hilgendorf et al., 2007; Imai et al., 2009; Murray et al., 2011; Sreedharan et al., 2011). Amino acid sequences of NTCP orthologs in humans (hNTCP; ≈ 40 – 50 kDa; 349 amino acids) and rats/mice (rNTCP/mNTCP; ≈ 56 kDa; 362 amino acids; N-glycosylated in rat at Asn5 and Asn11) are nearly 77% identical (Geyer et al., 2006). Splice variants are found for mouse NTCP (317 amino acids) (Cattori et al., 1999) and in rat cholangiocyte ASBT (≈ 19 kDa; 154 amino acids) (Lazaridis et al., 2000), but to our best knowledge, these variants have not yet been reported in humans. Dimerization of NTCP was confirmed just recently (Bijsmans et al., 2012).

Typically, ASBT orthologs contain 348 amino acids and exhibit above 80% overall sequence identity (Geyer et al., 2006); however, apparent molecular masses of mature, glycosylated forms vary slightly among humans (≈ 40 – 50 kDa), hamsters (≈ 43 kDa), rats (≈ 43 kDa) and rabbits (≈ 45 kDa) (Montagnani et al., 2009; Shneider et al., 1995; Wong et al., 1994), possibly due to species-dependent post-translational modifications. For instance, human ASBT (hASBT) has only 1 glycosylation site, namely, Asn10, and rat ASBT (rASBT) is allegedly glycosylated at Asn3 and Asn10 (Geyer et al., 2006). Moreover, a protein with higher molecular mass was detected in rats (99 kDa) (Lin et al., 1990) and rabbits (93 kDa), and may represent dimerized forms of the transporter (Dawson, 2011). Several protein regions were shown to be important for transport in hASBT. Amino acid residues located at extracellular loops 1 (EL1) (Hussainzada et al., 2008a) and 3 (EL3) (Banerjee et al., 2008) were shown to play a putative role in sodium and bile acid binding; TM1 (da Silva et al., 2011), TM6 (Hussainzada et al., 2008b) and TM7 (Hussainzada et al., 2006) appear to line the sodium and bile acid pathways, and TM3 (Hussainzada et al., 2009) and possibly TM4 (Khantwal and Swaan, 2008) may participate in the substrate exit route into the cytoplasm. Descriptions of protein loops may vary, as some authors depict loops in 7TM proteins as ELs 1–3 and ILs 1–3, whereas others choose IL 1,3,5 and EL 2,4,6. To simplify comparisons among orthologs, we adopt the first description, exemplified in figure 2A for the hASBT. Amino acids in EL1 (hASBT, Asp122; rASBT, Asp122; rNTCP, Asp115), and in EL3 (hASBT, Arg256, Glu261, Phe278 and Glu282; rASBT, Glu261; rNTCP, Glu257), were reported as potential sodium sensors. Possible interaction points with bile acids in EL1 (hASBT, Asp124; rASBT, 122), EL3 (hASBT, Leu283 and Glu282; rASBT, Arg256) and EL2 (rASBT, Lys191), and in the C-terminal as well as TM7 are reputedly involved in hASBT or rASBT binding and translocation. As with hASBT, Cys270 in the EL3 of rASBT, as well as the corresponding Cys266 in rNTCP, is not essential for function, albeit residing in the vicinity of functionally important amino acids. In rASBT, the putative phosphorylation sites Ser335 and Thr339, located in a 14-amino acid sorting motif of the C-terminal tail, as well as phosphorylation of Ser226 in rNTCP, were shown to be critical for apical and basolateral sorting, respectively, of these transporters (Dawson, 2011; Geyer et al., 2006; Stieger, 2011). In hASBT, residues in TM6 (Pro234, Gly237 and Gly241) may form a “conformational switch” (Hussainzada et al., 2008b), and in TM7, a hydrophilic cleft formed by Phe287 – Glu297 (Gonzalez et al., 2012), are allegedly required for substrate turnover, whereas Gly50, in TM1, appear to play an important role in hASBT protein stability (da Silva et al., 2011). Based on the 7TM topology, BA malabsorption-associated hASBT mutants L243P, T262M and P290S are located in TM6, EL3 and TM7, respectively, and have impaired function despite detectable protein expression (Dawson, 2011; Geyer et al., 2006).

Recently, Hu and colleagues reported the crystal structure of a *Neisseria meningitis* ASBT (ASBT(NM)) ortholog. The crystal structure was obtained from 3ZUX[1], a mutant constructed from 3ZUY[2], which in turn differs from the native *Neisseria meningitis*

serogroup B strain MC58 ASBT(NM) (Q9K0A9)[3], in that it has eight additional amino acids in the C-terminus. Figure 2B highlights similarities and discrepancies between 3ZUY, ASBT(NM), 3ZUX and hASBT. Based on the crystal structure of 3ZUX, ASBT(NM) exhibits 10 TM domains, arranged as two inverted 5 TM repeats and $N_{cyt}/C_{cyt}$ orientation. Its cytoplasm-facing binding cavity interacts weakly with TCA through Asn295 in TM10 (Hu et al., 2011). This topology differs remarkably from hASBT (7TM), for reasons currently unknown. It is possible that evolutionary distance, low amino acid identity (26%) and differences in membrane composition, *i.e.*, absence of cholesterol and presence of lipopolysaccharides in the outer membrane of gram-negative bacteria, may affect ASBT (NM) packing. In humans, depletion of plasma membrane cholesterol disrupts ASBT activity and association with lipid rafts (Annaba et al., 2008). The role of BA transport in the physiology of *N. meningitis* would require further study, as this bacterial species is not a typical component of the human gut microbiota (Ridlon et al., 2006). Mechanisms used by enterobacteria to handle BA appear to seek evasion, rather than internalization of BA, since these detergent-like molecules are also responsible for the reduction of intestinal bacterial load (Begley et al., 2005). For instance, *E. coli* was shown to efflux BA (Thanassi et al., 1997) or to use BA-induced stress as an indicator of the need to alter their outer membrane, due to environmental threats such as antibiotics (Kus et al., 2011). Furthermore, it remains to be established whether ASBT(NM) strictly transports bile acid. Whether structural features of ASBT(NM) may correlate with mammalian ASBT and be useful in the design of drugs targeting hASBT, remains an open question.

### 2.1. Transcriptional and post-transcriptional regulation of ASBT and NTCP

The detergent properties exhibited by BA render these molecules cytotoxic. To prevent intracellular accumulation, BA bind and activate nuclear receptors (NRs) such as the farnesoid X receptor (FXR). In turn, FXR downregulates BA influx via NTCP, ASBT and OATP1B1 repression, while stimulating expression of phase I and phase II detoxifying enzymes and BA efflux transporters. Finally, FXR activation in the liver downregulates cholesterol 7α-hydroxylase (CYP7A1); the rate-limiting enzyme of the classic and predominant pathway of BA biosynthesis. In the intestine, FXR increases secretion of the fibroblast growth factor 19 (FGF19) (humans)/FGF15 (rodents) hormone, which represses expression of ASBT and NTCP (Eloranta and Kullak-Ublick, 2008). Yet, ASBT and NTCP expression is critical for the EHC and cholesterol homeostasis, and is stimulated by several NR, such as the glucocorticoid receptor (GR), as well as various transcription factors and hormones (Claudel et al., 2011). The GR is both an ASBT and NTCP transactivator (Claudel et al., 2011). In GR deficient mice, disrupted NTCP/*Slc10a1* expression correlates with decreased bile volume in the gallbladder and higher susceptibility to develop cholesterol gallstones (Rose et al., 2011). Another *Slc10a1* gene activator, the peroxisome proliferator-activated receptor-γ coactivator-1α, is a possible enhancer of the GR-mediated NTCP activation by dexametasone (Eloranta et al., 2006). Histone H3 lysine 4 (H3K4) methyltransferase mixed lineage leukemia 3 (MLL3), which is altered in cholestasis, may be an important epigenetic factor involved in NTCP regulation by GR and FXR in humans and mice (Ananthanarayanan et al., 2011). Additional *SLC10A2* positive regulators include the vitamin D receptor (VDR) and the peroxisome proliferator-activated receptor-α (PPARα) (Dawson et al., 2009). The caudal-type homeobox-1 (CDX1) and -2 (CDX2) was recently revealed as a regulator of *SLC10A2* gene expression in the ileum (Ma et al., 2012), and CDX2 was shown to be a potential determinant of ileal ASBT expression in PBAM patients with chronic diarrhea (Balesaria et al., 2008). Also recently, the 3′-untranslated region

[1]Protein Data Bank (PDB) accession #: 3ZUX_A
[2]PDB accession #: 3ZUY_A
[3]UniProtKB/Swiss-Prot accession #: Q9K0A9

(UTR) was reported as a determinant of *SLC10A2* mRNA stability and to be regulated by Hu antigen R (HuR) and tristetraprolin (TTP) (Chen et al., 2011). Both ASBT and NTCP were shown to locate in membrane rafts and to be modulated by cholesterol (Annaba et al., 2008; Molina et al., 2008) as well as by interleukin-6 (IL-6) (Balesaria et al., 2008; Le Vee et al., 2011). It is noteworthy that mice, rats and humans exhibit marked species-specific differences in NTCP, ASBT and CYP7A1 regulation (Eloranta and Kullak-Ublick, 2008).

In the postprandial phase, BA levels at the intestinal lumen and portal blood increase rapidly and BA transporters need to respond swiftly, while concurrently preventing excessive BA influx. Since the slow process of transcription cannot adjust transporter density rapidly enough, post-transcriptional events account for the fine-tuning of transporter density at the cell surface and consequent alterations in BA transport. Accordingly, membrane trafficking upon cyclic adenosine monophosphate (cAMP)-induced ASBT and NTCP dephosphorylation is a reputed way to handle increased demands for BA transport, while intracellular retrieval, mostly via protein kinase C-dependent phosphorylation of ASBT or NTCP, or by NTCP S-nitrosylation (Schonhoff et al., 2011), may prevent cytotoxic BA accumulation. The ubiquitin-proteasome system (UPS) may also participate in this prevention, as well as in acute ASBT and NTCP response to inflammation and in ASBT turnover under basal conditions (Sarwar et al., 2009; Stross et al., 2010; Xia et al., 2006).

### 2.2. ASBT, NTCP and TGR5 effects

Bile acids are agonists of the membrane-bound G protein-coupled receptor (GPCR) TGR5 (also known as G protein-coupled bile acid receptor 1 [GpBAR1] or membrane-type bile acid receptor [M-BAR]), which is a modulator of glucose, lipid and energy metabolism and is highly expressed in the gallbladder and small intestine (Pols et al., 2011). In several rodent and human models, BA treatment resulted in increased energy expenditure, prevention of obesity, amelioration of insulin resistance and attenuation of immune response in colitis and Crohn's disease (Cipriani et al., 2011). Expectedly, due to the essential role of BA transporters in determining BA concentrations, these transporters affect – and may be affected by - numerous metabolic pathways pertinent to TGR5. In figure 3, we present a schematic overview of TGR5 activation and its effects on ASBT and NTCP expression, function, and potential pathophysiological implications.

It is currently known that TGR5 activation results in guanosine triphosphate (GTP)-dependent activation of adenilate cyclase, via G-protein- $_s$, and induction of cAMP. Cyclic AMP (cAMP), stimulates glucagon-like peptide (GLP-1, also known as incretin hormone) in murine (STC-1) and human (NCI-H716) cell models, as well as in mouse colon and distal ileum (Pols et al., 2011). GLP-1 is a hormone secreted by enteroendocrine L-cells in response to a meal, and which induces insulin secretion, inhibition of glucagon release and enhancement of β cell growth. Administration of tauroursodeoxycholic acid (TUDCA) was shown to ameliorate insulin resistance in liver and muscle of obese human volunteers (Kars et al., 2010). In rat primary hepatocytes, TCA improved activity of the insulin receptor and activated the insulin signaling pathway PI3K/PDK-1/AKT, with a consequent stimulation of GPCR-dependent activity of glycogen synthase (Cao et al., 2010). In a rat model of streptozotocin-induced diabetes mellitus, TGR5-mediated insulin secretion in the ileum reduced ASBT at mRNA, protein and functional levels, and decreased *Slc10a2* promoter activity in Caco-2 cells (Annaba et al., 2010b). Recently, Chen and collaborators at GlaxoSmithKline showed that ASBT inhibition with 264W94 elevated GLP-1, decreased hemoglobin A1c (HbA1c) (a marker for abnormally high sugar levels) and reduced plasma glucose in Zucker Diabetic Fatty (ZDF) rats. When administered chronically, 264W94 restored insulin levels and normoglycemia – reputedly due to increased BA residence time in the distal intestine and stimulation of GLP-1 secretion – thus establishing ASBT inhibition as a promising treatment for type 2 diabetes mellitus (Chen et al., 2012). It is

noteworthy that 264W94 (GlaxoSmithKine, Inc.) was originally designed in 2002 for hypolipidemic indications, as a low molecular weight alternative to the ASBT inhibitor 2164U90 (currently, Burroughs Wellcome/GlaxoSmithKline) (Root et al., 2002). However, it was not until 2011 that ASBT inhibition with 264W94 was considered a possible treatment for type 2 diabetes. Interestingly, Kohli and colleagues reported weight loss and improved glycemia in rats and humans that underwent ileal interposition surgery (IIS). In IIS, proximal segments of the jejunum are connected to the ileum, thereby shortening the EHC, while maintaining a functional ASBT. The paradoxical observations that both hepatic BA-induced TGR5 stimulation, as well as a decrease in BA pool via ASBT inhibition, improve glycemia, merits further investigation. It would be informative to discern whether distinct mechanisms underlie the observed effects, *e.g.*, if BA composition would be a determinant factor. In IIS, levels of primary BA were shown to be elevated (Kohli et al., 2010). Discrepancies in the ability of various BA in activating TGR5 have been reported (Pols et al., 2011).

As mentioned above, TGR5 activation increases energy expenditure via cAMP-dependent activation of 2-iodothyronine deiodinase (D2), with consequent conversion of thyroxine (T4) to the active form of thyroid hormone, tri-iodothyronine (T3) (Pols et al., 2011). Feeding female C57Bl/6J mice with conjugated linoleic acids (CLA) (typically found in dairy products, meat of ruminant animals and partially hydrogenated vegetable oils) increased energy expenditure, BA pool size, CYP7A1 and ASBT expression. In contrast, NTCP and BSEP protein levels were reduced. Overall, these mice exhibited increased risk for cholelithiasis (gallstones) (Letona et al., 2011). While not yet confirmed, these effects appear to be consistent with TGR5 activation. Indeed, mice lacking TGR5 exhibited resistance to gallstone formation (Keitel et al., 2009; Vassileva et al., 2006) as well as elevated NTCP expression (Vassileva et al., 2006). In contrast, TGR5 activation by BA in cholangiocytes may have beneficial choleretic effects during cholestasis, as cAMP stimulates ASBT insertion in the apical membrane and BA uptake (Xia et al., 2006), with subsequent reduction in biliary BA concentrations as well as increased BA-induced mucin and cystic fibrosis transmembrane conductance regulator (CFTR)-mediated fluid secretion (Keitel et al., 2009). Bile acids also modulate muscarinic and formyl-peptide (FPR) receptors, and a more detailed discussion on BA effects on these receptors is found in (Keitel and Haussinger, 2012; Pols et al., 2011).

### 2.3. Transporter abnormalities and correlated diseases

**Cholestasis:** Cholestasis is the intra- or extra-hepatic disruption of bile flow. It may be caused by physical blockage of bile ducts due to gallstones, immune diseases, defects in enzymes and transporters involved in BA homeostasis or induction by drugs such as rifampicin, rifamycin SV, glibenclamide, and cyclosporin A. Normally, BA are not present in significant amounts in the urine, systemic circulation or feces, but during cholestasis, BA are detected in all three compartments. Since reduction in biliary BA concentration and consequent cholesterol precipitation is a known mechanism of cholesterol gallstone formation, BA transporters play a vital role in the prevention or etiology of the disease. As gallstones grow or move, they may block bile ducts and interrupt bile flow, resulting in obstructive cholestasis. If left untreated, disruption of bile flow will lead to accumulation of bile contents in the liver, hepatotoxicity, biliary fibrosis and cirrhosis (Hirschfield et al., 2010). Downregulation of uptake transporters, *e.g.*, ASBT, NTCP and OATPs, upregulation of efflux transporters such as BSEP and OSTα/OSTβ and increased BA sulfation and urinary excretion are adaptive responses to cholestasis (Dawson, 2011; Stieger, 2011). Expression of NTCP is reduced in cholestatic mice, humans with primary biliary cirrhosis, cholestatic alcoholic hepatitis, drug-induced cholestasis and in hepatectomized patients (Miura et al., 2011; Stieger, 2011). In contrast with NTCP, a correlation between ASBT and

gallstone disease has not been confirmed (Tonjes et al., 2011). Nonetheless, several lines of evidence have correlated the increased BA fecal excretion resulting from reduced ASBT expression/function, with attenuated cholestatic liver damage. This has been reported in bile duct ligated mice (Soroka et al., 2010), mice deficient in OSTα/OSTβ (Soroka et al., 2011) and phosphatidylethanolamine N-methyltransferase (Li et al., 2011), liver transplantated rats (Cheng et al., 2010b) and humans that are non-obese gallstone carriers (Dawson, 2011). With the rational that ASBT inhibition should increase fecal BA elimination and potentially prevent bile acid return to the liver and hepatic accumulation, Soroka and colleagues suggested that ASBT inhibition, during the early stages of cholestasis, may be a feasible approach to prevent cholestatic liver injury (Soroka et al., 2011). A more comprehensive discussion on the regulation of BA transporters during cholestasis is found in (Wagner et al., 2011).

**Necrotizing enterocholitis:** In necrotizing enterocholitis (NEC), BA accumulation in enterocytes and in the luminal ileum results in epithelial damage and necrosis. ASBT upregulation in NEC models of mice, neonatal rats and human NEC ileal samples, as well as high ASBT-mediated BA uptake and decreased mucin 2 protective mucus layer, were shown to be involved in progression of the disease (Martin et al., 2011).

**Gastrointestinal cancers:** Changes in BA concentration or BA pool composition may underlie the pathogenesis of several cancers. Bernstein and colleagues demonstrated that high BA levels increased risk for cancers in the GI tract, purportedly via acute and chronic mechanisms. Acutely, high exposure to BA generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in DNA damage, mutation and apoptosis. Persistent (chronic) BA exposure reduces the apoptotic capability and may favor the survival of mutated, potentially cancerous cells (Bernstein et al., 2011). A typical Western diet has a high fat and cholesterol content, particularly from consumption of red meat. As a consequence, BA concentrations in the intestinal lumen are noticeably high, in order to emulsify the extra fat. The correlation between a typical Western diet, BA – especially secondary BA - and colorectal cancer have been well established, and Bernstein and colleagues have also confirmed the participation DCA in the etiology of colorectal carcinogenesis in mice (Bernstein et al., 2011). In this study, DCA-induced tumor was inhibited by chlorogenic acid, which is present in high levels in coffee, apples and blueberries. DCA is a secondary bile acid produced from CA de-hydroxylation by colonic bacteria. Thus, it would be feasible to hypothesize that ASBT function is important to the prevention of colorectal cancer, as efficient ASBT-mediated CA uptake should prevent excessive CA from reaching the colon and being metabolized into DCA.

**Barret's esophagus:** Barrett's esophagus (BE) is a pre-carcinogenic condition whereby epithelial cells lining the distal esophagus are damaged, typically as a consequence of chronic gastroesophageal reflux. Allegedly, during reflux, the esophagus is abnormally exposed to gastric contents, proteases and BA, leading to BE which then may progress to esophageal adenocarcinoma (EAC). In esophageal cell biopsies of BE patients, Dvorak and colleagues detected atypical expression of ASBT, IBABP and MRP3 at mRNA and protein levels. This effect is probably a response to increased BA exposure, thus implicating BA in BE pathogenesis and EAC risk. Expression of these transporters increased in BE but was gradually lost as the disease progressed to EAC (Dvorak et al., 2009). Cell damage is often associated with hydrophobic BA such as CDCA or DCA. In cells isolated from a patient with non-dysplastic BE, Goldman *et al* observed that replacement of hydrophobic bile acids with the more hydrophilic glycoursodeoxycholic acid (GUDCA) reduced DNA damage and oxidative stress, and thus GUDCA may possibly prevent EB progress to EAC (Goldman et al., 2010). A putative role for CDX1 and CDX2 as positive ASBT regulators, and their

possible involvement in BE aberrant ASBT expression was revealed recently by Ma and collaborators; both ASBT and CDX2 expression levels increase in BE and decrease as the disease progresses to EAC. This correlation is not observed in the healthy ileum (Ma et al., 2012).

### 2.4. FDA-approved drugs that impact ASBT function or expression

**Vitamin D receptor agonists:** Activation of the vitamin D receptor (VDR) by its natural substrates calcitriol (vitamin D3 or 1α,25-Dihydroxyvitamin D3) and ergocalciferol (vitamin D2 or 1α,25-dihydroxyvitamin D2) promotes intestinal calcium absorption, bone remodeling and phosphate homeostasis. It is also potentially useful for the treatment of osteoporosis and prostate cancer (Gueli et al., 2012). Currently, the synthetic vitamin D analog doxercalciferol (Hectorol®, Genzyme, Cambridge, Massachusetts) is in clinical use for hyperparathyroidism secondary to chronic kidney disease, in patients undergoing dialysis[4]. As mentioned earlier, VDR is a positive ASBT and NTCP regulator, and numerous reports have demonstrated an increase in renal and ileal ASBT mRNA expression and function upon vitamin D treatment (Chow et al., 2011; Chow et al., 2010). With growing clinical use of VDR activators, it would be enlightening to evaluate the health consequences of potentially sustained ASBT overexpression with chronic use of VDR agonists.

**Glucocorticoids:** Crohn's disease is an inflammatory bowel disease (IBD) characterized by chronic inflammation of the terminal ileum and persistent diarrhea. In Crohn's disease patients, ileal ASBT mRNA and protein levels are reduced, leading to bile acid malabsorption. Glucocorticoids such as methylprednisolone (MP) are employed widely in IBD treatment, and are agonists for GR (Jung et al., 2004), which is an ASBT and NTCP transactivator (*vide supra*). As observed by Coon and colleagues, the effect of MP on ASBT expression and function may differ in healthy and inflamed tissue. Specifically, it appears that in the normal rabbit ileal villus MP induces ASBT expression, whereas in the IBD-inflammed ileum, MP inhibits inflammatory mediators that hinder ASBT function (Coon et al., 2010).

**Ampicillin:** Secondary BA are produced by colonic enterobacteria and because antibiotics interfere with the intestinal flora, they may also affect BA homeostasis. Indeed, ampicilin (Amp)-treated mice exhibited reduced FGF15 and elevated ASBT levels, with concomitant reduction in fecal BA excretion. Discontinuation of treatment or long-term Amp administration reverted these effects (Miyata et al., 2011).

**Statins and dihydropyridine calcium channel blockers:** Several FDA approved drugs were currently shown to inhibit ASBT (Zheng et al., 2009a). Using quantitative and qualitative computer models, Zheng and coworkers ranked these drugs based on their inhibition potency reported in the literature. By testing the effect of the most relevant drugs on TCA uptake, using stably transfected MDCK-ASBT cells, the authors revealed that statins and dihydropyridine calcium channel blockers were the most potent ASBT inhibitors (Zheng et al., 2009b). The clinical relevance of this inhibition, especially how it may impact drug-drug interactions, awaits definition.

[4] http://www.hectorol.com/patients.aspx, last accessed on 03/28/2012.

## 2.5. ASBT targeting as therapeutic approach

### 2.5.1. ASBT Inhibition

**Diabetes:** As mentioned earlier, inhibition of ASBT with 264W94 is considered a feasible approach for the treatment for type 2 diabetes mellitus (Chen et al., 2012).

**Chronic constipation:** Inhibition of ASBT causes diarrhea and steatorrhea. What was initially considered an undesirable effect has now turned into a promising treatment for chronic constipation. Presently, the ASBT inhibitor A3309 is being tested with this indication (Chey et al., 2011) and has recently completed phase IIb clinical trials in the U.S.A.[5]

**Dislipidemia:** Bile acids are the major cholesterol catabolites in the body and ASBT inhibition has been consistently shown to reduce plasma cholesterol levels. This approach has been exploited extensively as a potential treatment for hypercholesterolemia. A generally accepted mechanism for the hypocholesterolemic effect of ASBT inhibitors involves the increase in cholesterol catabolism, to compensate for the reduced BA pool, and up-regulation of low-density lipoprotein LDL receptors and hepatic uptake of plasma LDL-cholesterol, in response to cholesterol depletion in the liver (Dawson, 2011). Numerous ASBT inhibitors, with structures that did not always resemble BA, have been developed in the past decade and exert hypocholesterolemic effects. Those include S-8921, PR835 and SC435, as well as a series of benzothiepinic and dihydroquinolinic molecules (Dawson, 2011). In addition to synthetic inhibitors, the natural products Barley cereal (Hoang et al., 2011) and epigallocatechin-3-gallate (EGCG), which is a catechin found in green tea (Annaba et al., 2010a), were shown to have cholesterol lowering properties that correlated with ASBT inhibition.

**Word of caution:** As discussed presently, ASBT inhibition has therapeutic value. However, as Trivedi and Ward noted recently, the risk of colorectal cancer associated with ASBT inhibition needs to be evaluated carefully (Trivedi and Ward, 2012).

**2.5.2. Prodrug approaches—**Due to its strategic anatomic location and its high transport capacity, ASBT is an attractive target for prodrug approaches aiming to increase drug availability (Balakrishnan and Polli, 2006; Rais et al., 2011). Oral administration in rats of the antiviral drug acyclovir conjugated with CDCA via a valine linker, increased acyclovir bioavailability by two-fold compared to acyclovir alone (Tolle-Sander et al., 2004). In addition to this finding, Polli's group and collaborators have recently reported potential prodrug candidates, by conjugation with BA, including a monoanionic gabapentin conjugate (Rais et al., 2011) and enzymatically stable ketoprofen and niacin conjugates, with potential use as sustained release prodrugs (Zheng and Polli, 2010).

## 2.6. NTCP polymorphisms

In the liver, NTCP is the major bile acid transporter, responsible for over 80% of hepatic influx of conjugated bile salts (Eloranta et al., 2006), with less efficient transport of steroid sulfates (Dawson et al., 2009). NTCP was shown to be the only member of the SLC10 family that transports T4, as well as T3 and T4 sulfates (Visser et al., 2010). NTCP also transports a variety of xenobiotics, including; *i)* the HMG-CoA-reductase inhibitors pitavastatin (Fujino et al., 2005), fluvastatin and the active form of the prodrug simvastatin (Greupink et al., 2011); *ii)* propanolol, furosemide and several other drugs described in (Stieger, 2011); *iii)* the antifungal agent micafungin (Yanni et al., 2011); *iv)* Indocyanine

[5] http://clinicaltrials.gov/, last accessed 03.08.2012.

green (ICG), which is clinically employed to evaluate liver function prior to liver surgery (de Waart et al., 2010); *v)* the BA derivatives BALU-3 and BALU-4, designed to treat phalloidin poisoning (Herraez et al., 2009); *vi)* the magnetic resonance contrast agent gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid (Gd-EOB-DTPA) (Leonhardt et al., 2010) and *vii)* the Human Immunodeficiency Virus (HIV)-1 integrase inhibitor raltegravir, for which NTCP transport does not appear to be clinically significant (Moss et al., 2011).

Considering the wide range of physiological and pharmacological NTCP substrates, single nucleotide polymorphisms (SNPs) that interfere with transporter function may potentially affect drug disposition. Functionally deleterious *SLC10A1* alleles were described recently, and were shown to be ethnicity-dependent. Notably, the functionally impairing c.800C>T (p.Ser267Phe)[6] variant is found in Chinese Americans (7.5%) and c.668T>C (p.Ile223Thr) in African Americans (5.5%) and Hispanic Americans (0.55%). The loss of function exhibited by p.Ile223Thr correlated with a reduction in protein expression, whereas p.Ser267Phe exhibited normal protein levels in the cell surface. Interestingly, p.Ser267Phe retained estrone sulfate transport and showed increased rosuvastatin uptake, suggesting that Ser267 may possibly be placed in a protein region specific for BA transport. Moreover, p.Ser267Phe was also found in Korean (3.1%), Chinese (7.4%) and Vietnamese (9.2%) populations (Pan et al., 2011). Less frequent NTCP variants include c.836T>C (p.Ile279Thr) in Chinese Americans (0.5%), c.940A>G (p.Lys314Glu) in Hispanic Americans (0.55%) and c.190G>A (p.Ala64Thr) in Koreans (1%) (Pan et al., 2011). The latter exhibited poor taurocholate and rosuvastatin transport. Thus, it is possible that p.Ala64Thr carriers may require dose adjustments when prescribed rosuvastatin. Non-synonymous NTCP variants, including c.668T>C (p.Ile223Thr), c.800C>T (p.Ser267Phe), c.836T>C (p.Ile279Thr), and c.940A>G (p.Lys314Glu), are addressed in (Stieger, 2011). Presently, gene notation and gene/protein variations follow guidelines of the HUGO Gene Nomenclature Committee (HGNC) (Wain et al., 2002) and the Human Genome Variation Society (HGVS) (den Dunnen and Antonarakis, 2000)[7], respectively.

NTCP and OATPs (*SLCO* family) are the transporters expressed at highest levels in liver tissue (Pan et al., 2011), and typically, OATPs are the major contributors to hepatic uptake of statins (Hilgendorf et al., 2007; Imai et al., 2009). However, the level of OATP contribution depends on the statin molecule tested, the presence of polymorphic *SLCO* alleles and, for overlapping substrates/inhibitors, possibly the levels of transport by NTCP and its variants. In human hepatocytes, NTCP alone showed to be responsible for approximately 35% of hepatic rosuvastatin uptake, which was not observed in rats (Choi et al., 2011). Choi *et al* expressed *SLC10A1*, *SLCO1B1* (alias OATP1B1, OATP-C) and several genetic variants of each transporter in *Xenopus* oocytes, and examined their contribution to pravastatin, pitavastatin, fluvastatin, rosuvastatin, atorvastatin and simvastatin uptake (Choi et al., 2011). The *SLCO1B1* variant c.521T>C (p.Val174Ala) was functionally impaired, but the *SLC10A1* mutant c.800C>T (p.Ser267Phe) exhibited increased rosuvastatin and atorvastatin uptake (Stieger, 2011). The high interindividual variation in *SLC10A1* mRNA (Choi et al., 2011) and *SLCO1B1* suggests that SNPs may not be uncommon for these transporters. Thus, it is feasible to conjecture that both variants, the OATP1B1 loss-of-function and the NTCP gain-of-function, as well as other variants of each transporter, may coexist. In such a scenario, NTCP and its variants may become a more

[6]Notation (den Dunnen and Antonarakis, 2000; updated version in http://www.hgvs.org/mutnomen/recs-prot.html, last accessed on 06/01/2012): “c” = variant described in the coding DNA sequence; “>” = substitution at DNA level. For instance, in c. 800C>T, a cytosine (C) was replaced with a thymine (T) at at nucleotide 800, which is located in the DNA coding region; “p” = variant described in the protein sequence. For example, in p. Ser267Phe, the amino acid serine (Ser), located at position 267 of the protein sequence, is replaced with phenylalanine (Phe).

[7]Updated version in http://www.hgvs.org/mutnomen/recs-prot.html, last accessed on 06/01/2012.

important contributor for rosuvastatin and atorvastatin distribution. The clinical consequences of these variants are still undetermined and merit further examination *in vivo*. Moreover, rats exhibited striking differences in tissue-specific levels of gene expression compared to humans (Stieger, 2011), suggesting that extrapolation of statin studies to humans based on rat models should be evaluated carefully.

### 2.7. ASBT polymorphisms

Similarly to *SLC10A1*, *SLC10A2* genetic variation is influenced by ethnicity. However, functionally disruptive SNPs are rare, as revealed by systematic screening and functional characterization of human *SLC10A2* alleles (Ho et al., 2011; Pan et al., 2011). Among *SLC10A2* variants, the most prevalent was the functionally silent 511G>T (Ala171Ser), found in European American (6%), African American (1.7%), Chinese American (4.5%) and Hispanic American (3.9%) populations (Ho et al., 2011). Also without functional consequences, 475G>A (V159I) was found in European, African and Hispanic Americans with frequencies ranging from 0.5% to 1.6%. Pan and colleagues also reported this variant with high frequency in Korean (25%) populations (Pan et al., 2011).

Loss of function variants include c.790A>G (p.Met264Val), c.868C>T (p.Pro290Ser), c. 728T>C (p.Leu243Pro) and c.785C>T (p.Thr262Met) (Ho et al., 2011). p.Pro290Ser was originally identified in one patient with Crohn's disease and p.Met264Val, in healthy volunteers and patients with inflammatory bowel disease. The variants p.Leu243Pro and p.Thr262Met are associated with primary bile acid malabsorption (PBAM); an inherited disease characterized by diarrhea, steatorrhea, increased fecal excretion of BA and sterols as well as decreased plasma cholesterol levels (Dawson, 2011). Reduced TCA uptake was observed for c.292G>A (p.Val98Ile), which is present in European, African and Hispanic Americans with frequencies ranging from 1.1% to 2.2%, and the infrequent c.431G>A (p.Cys144Tyr), found in 0.5% in European Americans (Ho et al., 2011). Renner *et al* (Renner et al., 2009) and Pan *et al* (Pan et al., 2011) have identified several other genetic variants in promoter, intron, exon 3′-UTR and 5′-UTR regions of the *SLC10A2* gene, but their impact on ASBT function is yet undetermined (Dawson, 2011; Pan et al., 2011).

Variants with potential clinical relevance are described in the pharmacogenomics knowledge database (PharmGKB)[8], and include g.26469C>T and g.9943C>T[9]. The first variant was identified in patients with prostate cancer during a phase II trial of docetaxel and thalidomide and is currently listed in PharmGKB as a potential risk factor for toxicity induced by these drugs. The latter, *i.e.*, g.9943C>T, was associated with decreased anthracycline-induced cardiotoxicity in childhood neoplasm. Screening of patients with colorectal cancer negated a correlation between the disease and the ASBT variants c. 507C>T (p.Leu169Leu) and c.511G>T (p.Ala171Ser) (Grunhage et al., 2008). Likewise, g. 13360A>G[10], located in the *SLC10A2* mRNA[11] intron, was initially thought to be a genetic determinant of gallstone disease, but this correlation was not confirmed in a subsequent meta-analysis study (Tonjes et al., 2011).

The reason for scarcity of functionally important *SLC10A2* variants is unclear. It would be tempting to speculate that it may be due to ASBT's role as the major BA transporter in the intestine(Dawson, 2011). Disruption of ASBT/*SLC10A2* function or gene expression results in a drastic increase in fecal BA elimination and decrease in plasmatic bile acid levels, as

[8] https://www.pharmgkb.org/rsid/rs2301159, last accessed on 03/28/2012.
[9] Notation: "g" = variant described in the genomic sequence (den Dunnen and Antonarakis, 2000): "g.26469C>T" is an alternative name for the NCBI "reference SNP cluster" (refSNP) identifier rs2301159 and "g.9943C>T" is an alternative name for rs9514091.
[10] Alternative names include the refSNP rs9514089 and the HGVS name G_016648.1:g.13360A>G.
[11] NCBI RefSeq NM_000452.2

observed in *Slc10a2* knockout mice and in PBAM patients. In the knock-out mice, this effect does not seem to be compensated by alternative mechanisms of BA absorption, such as passive absorption in the jejunum or in the colon (Dawson et al., 2003). While extrapolation from mice to humans should be done with caution due to fundamental differences between the two species, these observations support the notion that ASBT is the chief and rate-limiting step of bile acid absorption in the intestine (Annaba et al., 2010b). In contrast, we find a higher number of *SLC10A1* SNPs, possibly because the resulting phenotype may be partly compensated for by other transporters capable of shuttling bile acids in the liver, such as OATP1B1 and OATP1B3.

## 3. The sodium-dependent organic anion transporter (SOAT; *SLC10A6*)

The sodium-dependent organic anion transporter (SOAT) was first identified from an alignment search based on sequence similarities with NTCP and ASBT. Then, Geyer and coworkers (Geyer et al., 2007) cloned the *Slc10a6/SLC10A6* genes from rat and human adrenal gland cDNA. In both species, SOAT is highly expressed in heart and lung as well as in various tissues. In humans, the testis is the predominant site, followed by placenta, pancreas and mammary gland. As conjectured by Geyer, high expression in testis and placenta may imply SOAT participation in androgen/estrogen production and progesterone synthesis from PREGS, respectively (Geyer et al., 2007). Bioinformatic search also detected SOAT in *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus* (Sreedharan et al., 2011). Similarly to NTCP and ASBT, SOAT is an integral glycoprotein with putative seven transmembrane domains and $N_{exo}/C_{cyt}$ orientation. In rat chromosome, the *Slc10a6* gene is localized at 14p22 and *SLC10A6* in humans is at 4q21, and contains six exons (Geyer et al., 2006). As mentioned earlier, SOAT does not transport BA, but it does transport the sulfoconjugated BA taurolithocholic acid-3-sulfate (TLCS) in addition to sulfoconjugated steroid hormones (DHEAS, E1S, and PREGS) and the sulfoconjugated methylpyrenes (SMP) 2-SMP and 4-SMP. SOAT is relevant for homeostasis of sulfo-steroidal hormones and sulfo-BA. Sulfation is likely relevant for SOAT substrate recognition, as all known substrates are sulfated. Sodium specificity does not appear to be as strict in SOAT as in ASBT and NTCP, as $Li^+$ (but not choline chloride) can still elicit transport. Among inhibitors of SOAT activity are 3α-monohydroxylated bile acids and the 1-metylpyrene metabolite 1-SMP, which is present in cigarette smoke (Geyer et al., 2007). Finally, *SLC10A6* was identified as one of the genes augmented in clones of irinotecan-resistant melanoma cells (Gao et al., 2008). Its potential role in drug resistance warrants further examination.

## 4. P3/SLC10A3 (*SLC10A3*)

The gene encoding SLC10A3 (also known as DXS253E) was identified in 1998 and cloned from teratocarcinoma and placental cDNA libraries (Geyer et al., 2007). *SLC10A3* is a 4 kb gene, linked to the human X chromosome, located in an unmethylated CpG island, which is a region with high density of CpG nucleotides, *i.e.*, guanine and cytosine base pairs. In mammalians, CpG islands are typically found in the vicinity of promoter regions associated with housekeeping genes, and unmethylation of CpG-enriched regions indicates gene expression. Due to its gene location, putative ubiquitous expression (placenta, lung, brain, kidney, stomach (Splinter et al., 2006)) and high levels of interspecies conservation (Geyer et al., 2006; Hagenbuch and Dawson, 2004), SLC10A3 is believed to be a housekeeping protein. Since it exhibits amino acid identity with ASBT and NTCP of approximately 27%, SLC10A3 was included in the SLC10 family (Hagenbuch and Dawson, 2004). Search at the Uniprot website[12] indicates that SLC10A3 consists of 477 amino acids, with a calculated

[12] http://www.uniprot.org/uniprot/P09131#section_comments, last accessed on 03/28/2012.

mass of approximately 50 kDa, but the protein expression has not yet been demonstrated and remains at the transcript level. Furthermore, its function and substrate specificity are unknown. Alternative splicing generates three variants of the *SLC10A3* gene[13]. A correlation between increased *SLC10A3* expression and chemotherapy resistance was reported recently in ovarian cancer models (Cheng et al., 2010a).

## 5. P4/SLC10A4 (*SLC10A4*)

Identified originally on a expressed sequence tag (EST) search, the protein encoded by the *SLC10A4* gene was still considered hypothetical in 2004 (Hagenbuch and Dawson, 2004). Since then, great progress has been made in cloning and characterizing SLC10A4, although its substrate specificity is yet unknown. In humans and rats, SLC10A4 has 437 amino acids, a putative 7 TM topology and conserved glycosylation (Asn6, Asn20, Asn26, Asn181, and Asn195) and phosphorylation (Ser405, Thr417, and Tyr419) sites. Heterologous expression in Human Embryonic Kidney 293 (HEK293) cells and *Xenopus* oocytes revealed a 47 kDa protein. In central nervous system (CNS) samples, however, an additional 73 kDa band was observed, possibly representing the mature, glycosylated form of the protein (Geyer et al., 2008; Splinter et al., 2006). At the mRNA level, *SLC10A4* is distributed widely in human tissues including placenta, pancreas and brain. The protein was detected in the cytoplasm and on the plasma membrane of CHO cells. Geyer *et al* observed a clear difference in mRNA distribution in rats/mice and humans, with highest mRNA levels in human brain and small intestine and rat/mice brain (Geyer et al., 2008). Rat *Slc10a4* was also detected in monoaminergic neurons and secretory granules of mast cells, in the substantia nigra, which is a midbrain region enriched with dopamine neurons (Zhou et al., 2011), and in cholinergic regions of the mouse retina (Siegert et al., 2009). Immunohistochemistry of rat CNS showed SLC10A4 in cholinergic regions and co-localized with the cholinergic markers choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT) and high-affinity choline transporter (CHT1) (Burger et al., 2011). Despite strong phylogenetic relationship with NTCP (Geyer et al., 2006), SLC10A4 does not transport typical NTCP substrates such as BA or sulfo-sterols. It does not transport the CHT1 substrate choline chloride either (Geyer et al., 2008). Interestingly, SLC10A4 exhibits vesicular expression, in contrast with NTCP, which is expressed on the plasma membrane (Burger et al., 2011). Overall, SLC10A4 is majorly expressed in neuronal tissues and mast cells, and may potentially participate in vesicular storage or exocytosis of neurotransmitters or mastocyte mediators (Burger et al., 2011). Clearly, unambiguous SLC10A4 characterization emerges in the horizon.

## 6. P5/SLC10A5 (*SLC10A5*)

Also considered hypothetical in 2004, *SLC10A5* was cloned and characterized by Fernandes and colleagues, in 2007 (Fernandes et al., 2007). Using a FLAG-*SLC10A5* construct and expression in *Xenopus* oocytes, the authors identified cell surface protein exhibiting a 42 kDa and an 84 kDa band, likely representing the non-glycosylated and the glycosylated forms of the protein, respectively. Despite high (>70%) sequence identity among human, mouse and rat orthologs, mRNA tissue distribution was shown to vary markedly among these species. In humans and mice, *SLC10A5* was detected in the liver and kidney, with remarkably higher hepatic expression in humans. In rats, expression was broader, and included the brain, heart and small intestine. While functional significance of the SLC10A5 protein remains to be elucidated, its cell surface expression and tissue distribution supports a role as solute transporter.

[13]NCBI accession numbers: NM_001142391, NM_001142392 and NM_019848

## 7. SLC10A7

The gene encoding SLC10A7 was first identified by large scale sequencing analysis of a cDNA library of human fetal brain, and was named C4orf13. Godoy and coworkers reassigned it as *SLC10A7*, and showed it to encode a protein of 330 – 436 amino acids, molecular mass of ~27–37 kDa, which is expressed in numerous tissues and species (Godoy et al., 2007). However, a specific pattern of expression appears non-identifiable. Among all members of the SLC10 family, SLC10A7 was shown to have the highest amino acid sequence identity (20%) among mammalians and bacteria. Moreover, *SLC10A7* appears to have unusual gene structure, with 12 coding exons, while *SLC10A2* and *SLC10A6* have six and *SLC10A1* has five exons (Geyer et al., 2006). SLC10A7 topology is also atypical, with putative 10 TM and $N_{cyt}/C_{cyt}$ orientation. SLC10A7 does not transport BA or steroid sulfates. More recently, the *SLC10A7* gene was shown to be disrupted in a chromosomal region exhibiting abnormalities, in a patient with hypergonadotropic hypogonadism (Tzschach et al., 2009). However, the clinical relevance of this finding awaits definition. By the time of this writing, SLC10A7 substrate specificity was yet unknown.

## 8. Conclusion

Characterization of SLC10 proteins has steadily increased over the past decade as new family members have been identified. While NTCP and ASBT were already established as key players in BA homeostasis, new findings on the impact of BA on glucose and energy homeostasis shed new light on the clinical significance of these transporters and of bile acids in general. Moreover, a third SLC10 member, namely, SOAT, was identified as an important steroidal hormone transporter. ASBT's participation in pathophysiological processes such as NEC or as a potential target for drug or prodrug approaches, further establishes SLC10 as a complex protein family of pharmacological importance. In light of the potential role of NTCP and its SNPs in drug disposition, we anticipate increased clinical and pharmaceutical emphasis on this transporter. Inclusion of NTCP in pre-clinical testing may be required to evaluate potential NTCP-associated risks, such as cholestasis. Finally, additional members of this family appear relevant for neuronal processes (SLC10A4), solute transport (SLC10A5 and SLC10A7) and housekeeping processes (SLC10A3), although they still await unambiguous characterization.

## Acknowledgments

We acknowledge the National Institutes of Health (NIH) for financial support through the grants NIDDK DK061425 (to P. W. S.) and DK067530 (to J. E. P.), as well as the University of Maryland for continuing support. We also thank Drs. C. S. Raman, Sairam Mallajosyula and Robyn Moore for informative discussions on ASBT topology, and Dr. Paresh Chothe for helpful feedback on the transporter physiology figure. We declare no conflict of interest.

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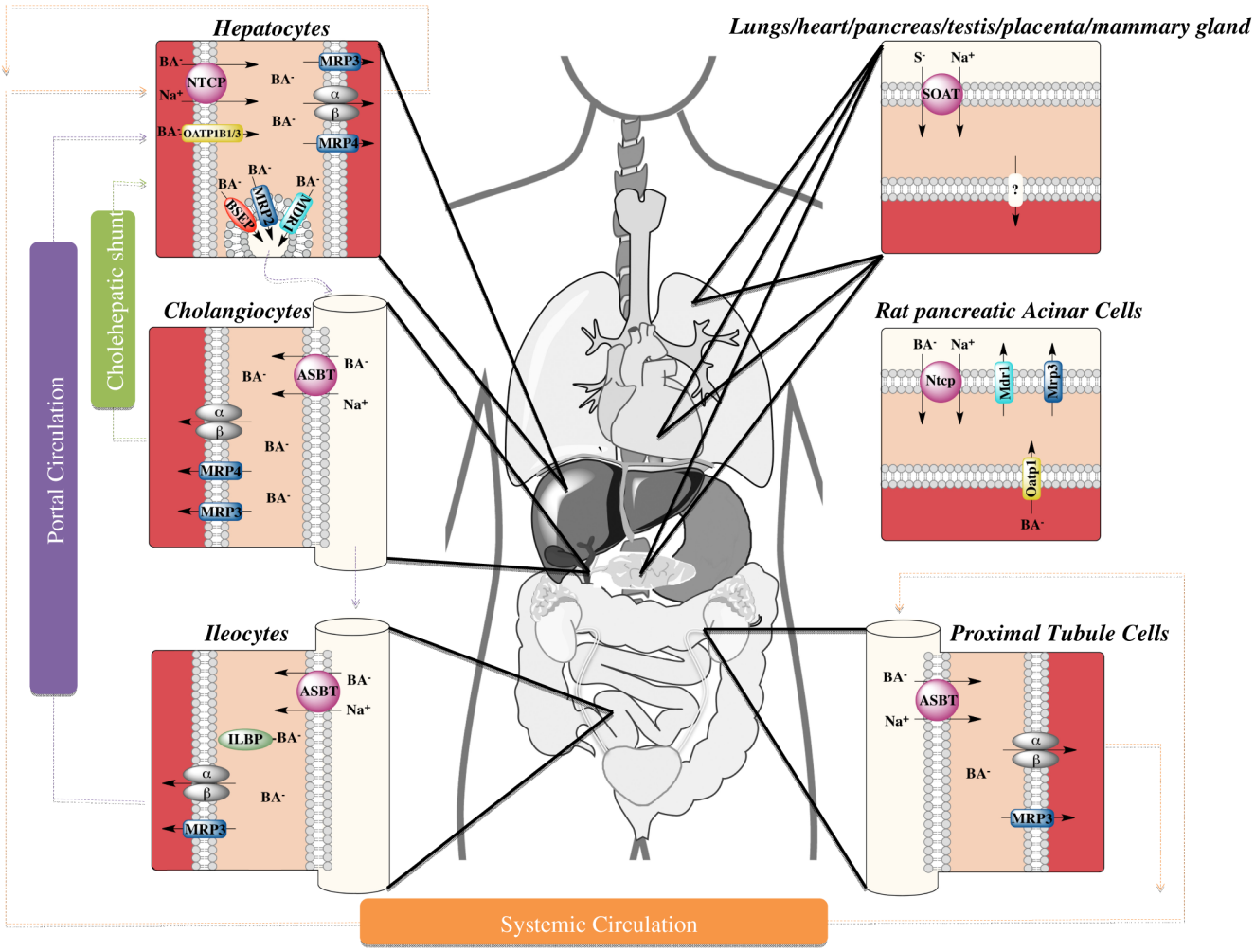


**Figure 1. Schematic tissue distribution and activity of established members SLC10 family**

Tissues involved in bile acid (BA) or sulfated ($S^-$) substrates are highlighted. *Top right square:* Sodium-dependent transport of sulfated substrates ($S^-$) as well as tissues with high SOAT expression in humans. Efflux transporters of SOAT substrates are not fully elucidated but may include the breast cancer resistant protein (BCRP, *ABCG2*) in the placenta, for efflux of sulfated estrogens from the fetus. *Top left square*: Bile acids are synthesized and conjugated to taurine or glycine in the liver and are actively effluxed from the liver into bile by the canalicular ATP-binding cassete (ABC) transporters BSEP, MRP2 and possibly, MDR1; they travel down the biliary tract (cholangiocytes) and are stored in the gallbladder. In response to meal ingestion, BA are secreted into the duodenum and travel down the intestine, where they are absorbed passively until they reach the distal ileum and are actively reclaimed by ASBT in the brush border membrane of ileal enterocytes (ileocytes). BA that escape ASBT absorption reach the colon, where they are modified by enterobacteria and are absorbed passively into the portal circulation (not shown). In the ileocytes (*bottom left square*), BA are shuttled to the basolateral membrane by the cytosolic ILBP, and are effluxed into the portal blood by OST/and MRP3. Because of the high electrochemical gradient generated by BA influx, here the facilitative transporter OST/functions as a BA efflux transporter, although it can function bi-directionally. Next, BA reach the liver through the portal circulation and are taken up, both by sodium-dependent NTCP, as well as via sodium-independent OATP1B1 and OATP1B3, in the sinusoidal membrane of hepatocytes.

A fraction of BA that escape canalicular efflux spill over into the systemic circulation assisted by the sinusoidal efflux transporters MRP3, MRP4 and OST/. From the systemic circulation, BA reach tissues where they act as signaling molecules, *e.g.*, by binding and activating specific receptors such as nuclear receptors and the membrane-bound TGR5 receptor. Systemically circulating BA also reach the kidney (*bottom right square*), undergo glomerular filtration, are reclaimed by ASBT in the apical membrane of proximal tubule cells, and are effluxed back into the systemic circulation by the basolaterally-expressed MRP3 and OST/. They return to the liver where, again, canalicular BA efflux in the hepatocytes direct BA to travel down the bile ducts and reach the gallbladder. In the bile ducts, a small fraction of BA undergo cholehepatic shunt, *i.e.*, are absorbed actively - putatively through ASBT - or passively into cholangiocytes (*center left square*) and return to the liver through the periductular capillary plexus. Efflux from cholangiocytes is mediated by MRP3, MRP4 and OST/. Additional transporters, *e.g.*, MRP2 in hepatocytes and MRP2/3 in enterocytes may possibly be involved in efflux of modified BA, such as sulfates and glucuronides. NTCP expression in the luminal membrane of rat pancreatic acinar cells (*center right square*) suggests another possible mechanism of BA salvage, whereby BA that reach the lumen through OATP1 uptake and MRP3 and MDR1 efflux, or by spillage from the bile ducts into the terminal acini, are reclaimed by NTCP.

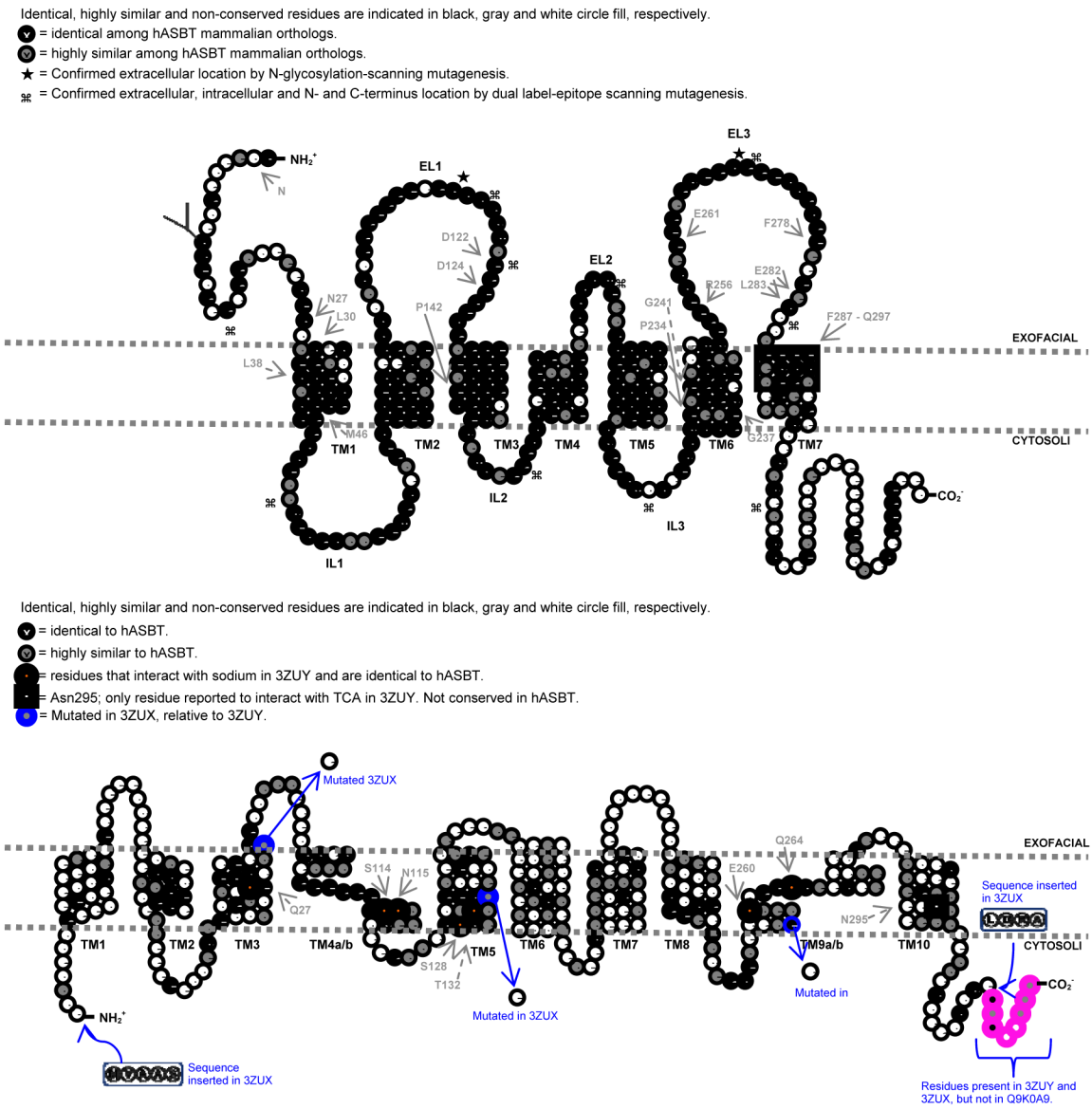


**Figure 2. Secondary structure of hASBT and 3ZUY (ASBT(NM)), highlighting primary stucture similarities and differences between hASBT and mammalian ASBT species, as well as between proteins reported by Hu and colleagues (Hu et al., 2011)**

Transmembrane (TM) domains, extracellular (EL), intracellular (IL), N- and C-terminal loops are indicated. Amino acids putatively relevant for transport are indicated by arrows, with full arrows pointing directly to the amino acid, whereas dotted arrows point to regions in the proximity to the amino acid. Amino acid sequences employed to build secondary structure representations were retrieved from public databases, namely, from the National Center for Biotechnology Information (NCBI): human ASBT (RefSeq ID: NP_000443.1); from the Universal Protein Resource Knowledgebase: Neisseria meningitidis serogroup B strain MC58 (UniProtKB/Swiss-Prot ID: Q9K0A9); from the Worldwide Protein Data Bank: 3ZUY (PDB ID: 3ZUY_A) and 3ZUX (PDB ID: 3ZUX_A). Primary sequence alignments were performed in ClustalW NPS@: Network Protein Sequence Analysis (Combet et al., 2000). A, hASBT topology highlighting its 7 TMs, $N_{exo}/C_{cyt}$ orientation, glycosylation site (Y) and amino acids reputedly relevant for transport of sodium (N27, L38,

M46, D122, F287, F278 and E261) and bile acid (N2, D124, P142, E282, L283, P234, G237, G241, and the TM7 hydrophilic cleft formed by F287 – Q297). Amino acids for which topology was confirmed by N-glycosylation (★) and dual label epitope insertion mutagenesis ( ⌘) are highlighted. Alignment of hASBT with other mammalian species, namely, orangutan, dog, wild boar, mouse, rat, hamster and rabbit were compared and are represented here as one letter amino acid codes, enclosed in black circles for identical, gray circles for highly similar and white circles for nonconserved residues. These sequences were also retrieved from NCBI, under the RefSeq ID's: orangutan (*Pongo abelii*; NP_001125080.1), dog (*Canis lupus familiaris*; NP_001002968.1), wild boar (*Sus scrofa*; NP_001231392.1), mouse (*Mus musculus*; NP_035518.1), rat (*Rattus norvegicus*; NP_058918.1), hamster (*Cricetulus griseus*; NP_001233749.1) and rabbit (*Oryctolagus cuniculus*; NP_001076233.1). B, Secondary structure of 3ZUY (derived from (Hu et al., 2011) supplementary Fig. 5), highlighting its 10 TMs, $N_{cyt}/C_{cyt}$ orientation and amino acids reputedly relevant for transport of sodium (Q77, E260 and Q264) and bile acid (N295). Amino acids that differ between 3ZUY, Q9K0A9 (*i.e.*, native ASBT in MC58 *Neisseria meningitis*), and the mutant employed to generate the crystal structure, 3ZUX, based on ClustalW alignment, are also indicated. Amino acids in 3ZUY were aligned with hASBT, and are indicated by black circles for identical, gray circles for highly similar and white circles for non-conserved residues.

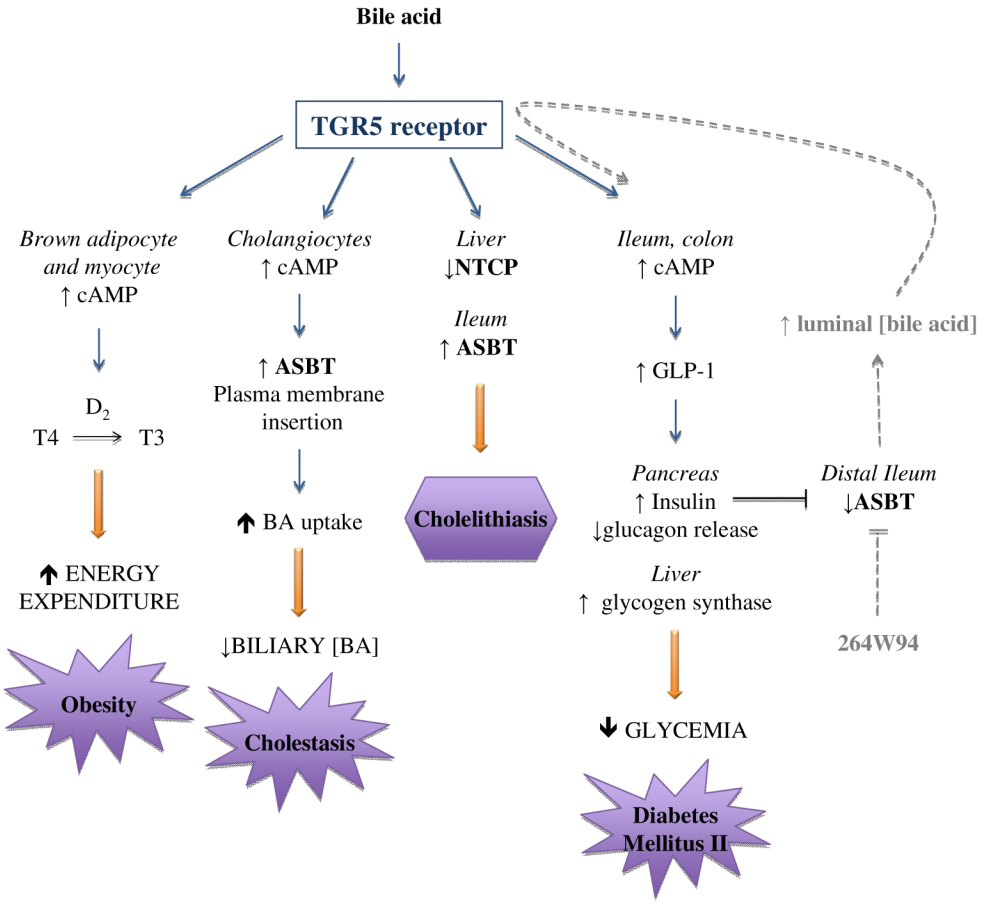


**Figure 3. Bile acid activation of the TGR5 receptor, possible effects on SLC10 proteins and potential clinical implications**

Bile acids are natural ligands of the membrane-bound receptor TGR5, which is a GPCR expressed in various tissues and whose activation elevates cAMP levels. In brown adipose and muscle tissue, this increase in cAMP was shown to activate the thyroid hormone (T3) and increase energy expenditure, with potential implications for obesity (Pols et al., 2011). In cholangiocytes, it is possible that cAMP-induced ASBT translocation to the cell surface and increased BA uptake will reduce biliary BA concentrations during cholestasis. In mouse liver and ileum, TGR5 activation reduced NTCP, elevated ASBT levels and increased the risk of cholelithiasis. In the colon and ileum, cAMP stimulates GLP-1 secretion with downstream improved glycemia (Pols et al., 2011). Arrows denote activation of downstream pathways, while flat-end arrows indicate inhibition. Gray clouds indicate pathophysiological conditions that may be positively affected by TGR5 activation, while gray hexagons denote potential negative outcomes of TGR5 activation. Dashed arrows indicate the possible mechanism involved in ASBT inhibition by 264W94, which elevates ileal and colonic BA levels resulting in TGR5 activation and GLP-1 secretion (Chen et al.). Interestingly, insulin secretion consequent to GLP-1 activation was shown to reduce ASBT expression (Annaba et al.), and may potentially function as a positive feedback mechanism of ASBT inhibition with 264W94 treatment.

**Table 1**

Current members of the SLC10 family (original version in (Hagenbuch and Dawson, 2004)).

| SLC10 - Sodium bile salt cotransport family | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Human gene name** | **Protein name** | **Aliases** | **Predominant substrates** | **Transporter type/coupling ions*)** | **Tissue distribution and cellular/ subcellular expression** | **Link to disease** | **Human gene locus** | **Sequence accession ID** | **Splice variants and their features** |
| SLC10A1 | NTCP | LBAT | bile acids | C/$Na^+$ | liver (basolateral), pancreas | | 14q24.1 | NM_003049 | |
| SLC10A2 | ASBT | IBAT, ISBT, NTCP2 | bile acids | C/$Na^+$ | ileum, kidney, billiary tract (apical) | (primary bile acid malabsorption) | 13q33 | NM_000452 | |
| SLC10A3 | P3 | | | O | ubiquitous | | Xq28 | NM_019848<br>NM_001142392<br>NM_001142391 | 3 splice variants |
| SLC10A4 | P4 | | | O | brain (cholinergic neurons), placenta, liver | | 4p12 | NM_152679 | |
| SLC10A5 | P5 | | | O | liver, kidney | | 8q21.13 | NM_001010893 | |
| SLC10A6 | SOAT | P6 | estrone-3-sulfate, dehydroepiandrosterone sulfate, pregnenolone sulfate | C/$Na^+$ | testis, placenta, pancreas | | 4q21.3 | NM_197965 | |
| SLC10A7 | P7 | C4orf13 | | O | liver, testis | | 4q31.22 | NM_001029998<br>NM 032128 | 2 splice variants |

*C: Cotransporter; E: Exchanger; F: Facilitated transporter; O: Orphan transporter