Review

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Interactions of Na^+/taurocholate cotransporting polypeptide with host cellular proteins upon hepatitis B and D virus infection: novel potential targets for antiviral therapy

https://doi.org/10.1515/hsz-2022-0345
Received December 8, 2022; accepted April 13, 2023;
published online April 28, 2023

Abstract: Na^+/taurocholate cotransporting polypeptide (NTCP) is a member of the solute carrier (SLC) family 10 transporters (gene symbol *SLC10A1*) and is responsible for the sodium-dependent uptake of bile salts across the basolateral membrane of hepatocytes. In addition to its primary transporter function, NTCP is the high-affinity hepatic receptor for hepatitis B (HBV) and hepatitis D (HDV) viruses and, therefore, is a prerequisite for HBV/HDV virus entry into hepatocytes. The inhibition of HBV/HDV binding to NTCP and internalization of the virus/NTCP receptor complex has become a major concept in the development of new antiviral drugs called HBV/HDV entry inhibitors. Hence, NTCP has emerged as a promising target for therapeutic interventions against HBV/HDV infections in the last decade. In this review, recent findings on protein–protein interactions (PPIs) between NTCP and cofactors relevant for entry of the virus/NTCP receptor complex are summarized. In addition, strategies aiming to block PPIs with NTCP to dampen virus tropism and HBV/HDV infection rates are discussed. Finally, this article suggests novel directions for future investigations evaluating the functional contribution of NTCP-mediated PPIs in the development and progression of HBV/HDV infection and subsequent chronic liver disorders.

Keywords: bile salt transport; entry inhibitor; HBV; NTCP; receptor; transporter.

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1 Introduction

The hepatitis B virus (HBV) belongs to the Hepadnaviridae family and infects hepatocytes of humans, nonhuman primates (chimpanzee, gorilla, orangutan, gibbon), and two other small mammals (tree shrew and woodchuck) (Bonvicino et al. 2014). The HBV infection of humans can be either transient (<6 months) or chronic and lifelong, depending on the ability of the host immune response to clear the infection. This is accompanied by hepatitis and liver dysfunction. Furthermore, the disease can progress to chronic infection, cirrhosis, or even hepatocellular carcinoma (Iannacone and Guidotti 2022; Yuen et al. 2018). According to the latest global epidemiological statistics, about 250 million people are chronically infected with HBV, associated with approximately 1 million lethal cases per year due to liver failure, fibrosis, and cancer. About 5% of all chronic HBV carriers are additionally infected with the hepatitis D virus (HDV), a satellite virus of HBV that bears the identical envelope proteins. The coinfection of HBV carriers with HDV causes a more rapid disease progression and increased mortality rates (Hughes et al. 2011). Based on this, HBV/HDV infections are considered to be a severe global health problem (Polaris Observatory 2018).

Although the current anti-HBV therapies are complex and result in the suppression of HBV replication in the majority of patients, HBV surface antigen loss and seroconversion are rarely successful despite long-term antiviral treatment (Kim et al. 2022). In addition, therapeutic options against HDV infection are greatly limited. Therefore, there is an unmet global medical need in drug development for novel potent anti-HBV and -HDV drugs. Strategies targeting the virus entry process, *inter alia*, are promising for combination therapies (Kim et al. 2022; Mokaya et al. 2018).

Due to its distinct tissue tropism and the highly specific viral and cellular factors involved in virus entry, HBV/HDV infections are ideal candidates for pharmacological entry inhibition (Kirstgen et al. 2021a,b; Lucifora et al. 2013;

Nkongolo et al. 2014; Schulze et al. 2007, 2010). In the last few years a number of pharmacological studies have already addressed this strategy, mostly by blocking the interaction of virus surface proteins with the host receptor Na^+/taurocholate cotransporting polypeptide (NTCP) (Nkongolo et al. 2014; Schulze et al. 2010; Watashi et al. 2014). Following this concept, bulevirtide (Hepcludex®, formerly known as Myrcludex B) recently received conditional approval from the European Medicines Agency as a treatment option for HDV infections, and, thus, represents the first-in-class entry inhibitor acting via NTCP inhibition (Bogomolov et al. 2016; Petersen et al. 2008). Myrcludex B is a synthetic peptide mimicking the preS1-domain of the HBV envelope L-protein, and, by high-affinity binding to NTCP, prevents virus-NTCP complex formation and virus entry into hepatocytes (Petersen et al. 2008; Tsounis et al. 2021). Since these processes depend strongly on many host-derived proteins that form a protein complex with NTCP, a better understanding of the NTCP structure and protein–protein interactions (PPIs) between NTCP and relevant entry cofactors will help to identify and design novel targets for virus entry inhibition (Fukano et al. 2021; Gad et al. 2022; Hu et al. 2020; Iwamoto et al. 2019; Palatini et al. 2022, 2021).

2 NTCP structure and functions

2.1 NTCP structure

Insights into the structure and transport mechanism of NTCP have come from various sources in the past 20 years: (1) single point mutation studies on human NTCP or rat Ntcp and their functional characterization (Fu et al. 2017; Ho et al. 2004; Ruggiero et al. 2021; Saeki et al. 2002; Yan et al. 2012, 2013; Zahner et al. 2003; Zakrzewicz et al. 2022); (2) X-ray crystal structures of two prokaryotic homologues of NTCP, named $ASBT_{NM}$ (Hu et al. 2011) and $ASBT_{Yf}$ (Zhou et al. 2014); (3) AlphaFold protein structure prediction of human NTCP (Varadi et al. 2022; Zakrzewicz and Geyer 2022) and (4) very recently, four independent cryo-electron microscopy (cryo-EM) structures of human NTCP (Asami et al. 2022; Goutam et al. 2022; Liu et al. 2022; Park et al. 2022). In the following, only the crystal structures of the bacterial ASBT proteins and the cryo-EM structures of human NTCP are described more in detail.

The $ASBT_{NM}$ structure (PDB: 3ZUY) adopts an inward-facing conformation with one taurocholic acid molecule and two Na^+ ions bound (Hu et al. 2011). Regarding $ASBT_{Yf}$, an inward-facing apo conformation (PDB: 4N7W) was obtained for the wild-type protein and an apo outward-facing structure for an E254A mutant that destroys one of the Na^+ binding sites (PDB: 4N7X) (Zhou et al. 2014). All three structures exhibit similar membrane topology with 10 transmembrane domains (TMDs) and cytoplasmic N- and C-termini. The TMDs 1–5 and 6–10 are topologically similar but oppositely orientated within the membrane, revealing an internal two-fold pseudosymmetry. Each repeat unit has an N-terminal V-shaped motif made of TMDs 1 and 2, as well as TMDs 6 and 7, respectively. These motifs (TMDs 1, 2, 6, 7) form the panel domain of the protein. In addition, each repeating unit contributes to the core domain of the protein with TMDs 3–5 and 8–10, respectively. Within the core domain, TMDs 4 and 9 are discontinuous and cross each other (Hu et al. 2011; Zhou et al. 2014). Both inward and outward open conformations of the bacterial ASBT proteins have been confirmed by different disulfide cross-linking mutants of $ASBT_{Yf}$. These revealed 4N7W-like inward-facing conformations for Y113C/P190C (PDB: 7CYG) and V110C/197C (PDB: 7CYK) mutants in the free form, and a 4N7X-like outward-facing conformation in the cysteine cross-linked form of the Y113C/P190C mutant (PDB: 6LH1) (Wang et al. 2021a). In addition, two inward-facing 4N7W-like structures were generated from the P10C/S291C mutant $ASBT_{Yf}$ protein after cysteine cross-linking in the apo state (PDB: 6LH0) and with glycine and two Na^+ ions bound (PDB: 6LGY) (Wang et al. 2021b).

The recent cryo-EM structures of human NTCP show a high similarity with these bacterial ASBT structures (Figure 1A) (Asami et al. 2022; Goutam et al. 2022; Liu et al. 2022; Park et al. 2022). All are organized in similarly structured core and panel domains and most residues interacting with the sodium ions are highly conserved (see below). However, two striking differences occur. (I) The TMD 1 of the bacterial proteins is missing in all human NTCP structures. Instead, the N-terminus is at the extracellular side and the panel domain has only three TMDs, namely, 1, 5, and 6. The core domain is composed of TMDs 2–4 and 7–9, and TMDs 3 and 8 represent the discontinuous helices that cross over in the middle of the membrane. Consequently, the human NTCP protein opens a large amphiphilic cavity framed by TMDs 6 and 9 to the cytoplasm and laterally to the hydrophobic core of the plasma membrane (Asami et al. 2022; Goutam et al. 2022; Liu et al. 2022). This laterally open cavity is covered by TMD 1 in all bacterial ASBT proteins. (II) In addition to one inward-facing conformation stabilized by nanobody Nb87 (PDB: 7PQG (Goutam et al. 2022)) and several outward-facing conformations of the wild-type NTCP (PDB: 7WSI (Asami et al. 2022); PDB: 7FCI (Park et al. 2022)) or its Q261A mutant (PDB: 7VAD (Asami et al. 2022)), two of the structures were surprisingly captured in an open-pore conformation (PDB: 7PQQ (Goutam et al. 2022); PDB: 7ZYI (Liu et al. 2022)). One of these structures was stabilized by

nanobody Nb91 (Goutam et al. 2022) and opened a wide pore through the transporter, exposing the sodium binding sites near the crossover region of TMDs 3 and 8 simultaneously to both sides of the membrane (Goutam et al. 2022). The other open-pore structure was resolved by Liu et al. and revealed a tunnel through the protein connecting the extracellular milieu to the cytosolic site and laterally to the inner leaflet of the bilayer (Liu et al. 2022). At the narrowest section of this tunnel, two strong EM densities were visible that were interpreted as two bile salt molecules sitting at two distinct binding sites (S_{out} and S_{in}) inside of the tunnel. This open pore conformation and the double binding site for bile salts are in clear contrast to the bacterial ASBT proteins that all contain a narrow pocket for only one single bile salt molecule that is shielded from either the extracellular side (inward-facing conformation) or the intracellular milieu (outward-facing conformation). The physiological relevance of this open-pore conformation state of human NTCP in the bile salt transport cycle is still controversial and needs further investigation.

The core and panel domains within NTCP are connected by a cytoplasmic α-helical "bridge" located between TMDs 1 and 2 (Figure 1A) (Asami et al. 2022; Liu et al. 2022; Park et al. 2022) and a flexible extracellular fragment bridging TMDs 6 and 7 (Goutam et al. 2022). These two structural elements are also present in other SLC transporters, such as the proton-coupled oligopeptide transporter family SLC15 (Killer et al. 2021). Although the functional role of these elements is unclear, they might take part in lipid sensing or PPIs (Killer et al. 2021). In addition, they are probably involved in the conformational changes during the transport cycle by moving the panel domain against the core domain (Goutam et al. 2022). It is imaginable that this movement contributes to the transport process of bile salts from the extra- to the intracellular side of hepatocytes, a process that is strictly dependent on sodium cotransport. The NTCP transports two sodium ions together with each bile salt molecule (Hagenbuch and Meier 1996; Weinman et al. 1998). Previous biochemical and structural studies on the bacterial ASBT proteins have identified several highly conserved amino acids that are essentially involved in the binding and translocation of Na^+ ions. Two sodium binding sites conserved among the bacterial ASBT proteins and NTCP have been identified: Na1 formed by S105, N106, S119,

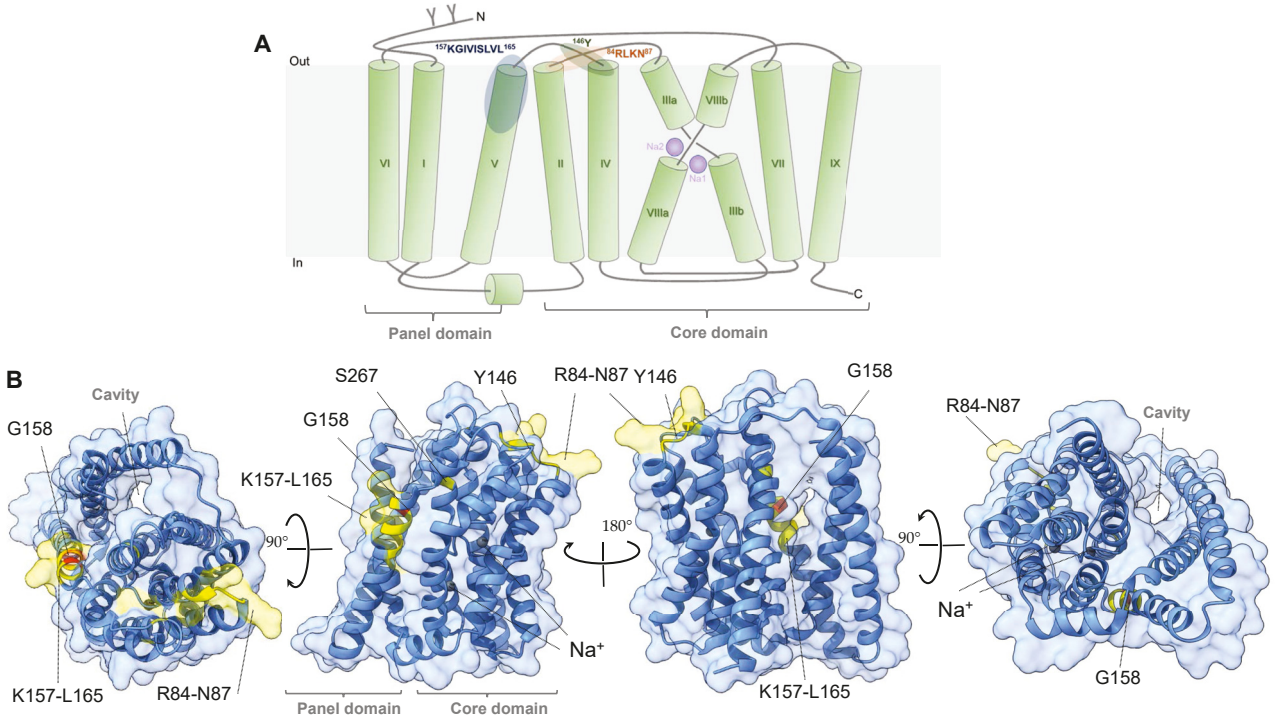

Figure 1: Na^+/taurocholate cotransporting polypeptide (NTCP) is a sodium-dependent bile salt transporter and high-affinity hepatitis B/D virus (HBV/HDV) receptor. (A) Schematic representation and membrane topology of human NTCP and its nine transmembrane domains (TMDs I–IX), which form a panel domain (TMDs I, IV and V) and a core domain with two inverted triple-helix repeats (TMDs II–IV and VII–IX). Two sodium ions are colored purple. The cell surface-exposed virus preS1-peptide binding regions of human NTCP are highlighted: $_{157}$KGIVISLVL$_{165}$, $_{84}$RLKN$_{86}$, and Y146. (B) Transparent surface presentation of human NTCP (PDB: 7ZYI). Amino acids involved in preS1-peptide binding are labeled. Amino acid position S267 that is relevant for bile salt binding and transport is indicated. The glycine at position 158 that is essential for preS1-peptide binding is highlighted in red.

T123, and E257 in human NTCP, as well as by S114, N115, S128, T132, and E260 in $ASBT_{NM}$; Na2 formed by Q68 and Q261 in human NTCP, as well as by Q77 and Q264 in $ASBT_{NM}$ (Hu et al. 2011; Liu et al. 2022). Both Na^+ binding sites are near the crossing motif of TMDs 3 and 8 in NTCP, or TMDs 4 and 9 in $ASBT_{NM}$, respectively (Figure 1A) (Asami et al. 2022; Goutam et al. 2022; Liu et al. 2022; Park et al. 2022).

2.2 NTCP functions

2.2.1 NTCP is a bile salt transporter

The NTCP is a multitasking membrane protein, which plays fundamental roles in the physiological hepatic bile salt transport, the development and progression of cholestatic liver diseases, and HBV/HDV virus entry into hepatocytes (Anwer and Stieger 2014; Appelman et al. 2021; Döring et al. 2012; Jetter and Kullak-Ublick 2020; Salhab et al. 2022). Its primary function is related to bile salt transport across the sinusoidal membrane of hepatocytes and, thereby, the regulation of bile salt concentrations in the peripheral blood circulation (Kullak-Ublick et al. 2000; St-Pierre et al. 2001; Vaz et al. 2015). Uptake of bile salts into hepatocytes occurs largely in a sodium-dependent manner via NTCP (Hagenbuch and Meier 1996; Weinman et al. 1998). Nearly all physiological bile acids are substrates of NTCP, including cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, sarcosine cholic acid, glycocholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, glycoursodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, tauroursodeoxycholic acid, and taurolithocholic acid. In addition, sulfated steroid hormones, such as estrone-3-sulfate, dehydroepiandrosterone sulfate, and pregnenolone sulfate, as well as sulfated jodothyronines are transported via NTCP (Grosser et al. 2018, 2021; Hagenbuch et al. 1991; Kersseboom et al. 2017; Schroeder et al. 1998; Visser et al. 2010). The NTCP also transports some clinically used drugs, such as rosuvastatin, pitavastatin, fluvastatin, and atorvastatin (Bi et al. 2013; Fujino et al. 2005; Greupink et al. 2011; Ho et al. 2006). In addition, NTCP transports the drug conjugate taurocholate-chlorambucil (Kullak-Ublick et al. 1997).

Based on the resolved structural conformations of the bacterial ASBT proteins and human NTCP, different transport-associated dynamics have been proposed for these SLC10 proteins. Some of them are described here. Jardetzky proposed an alternating-access mechanism to describe the transporter-mediated movement of substrates across the plasma membrane more than 50 years ago (Jardetzky 1966). According to this model, a membrane carrier alternates between an outward open and an inward open state, in which the centrally located substrate binding site is accessible to either the outside or inside of the cell. In addition, occluded conformational states have been described for many membrane carriers, in which the substrate binding site is inaccessible from either side of the membrane (Quistgaard et al. 2016). In the case of sodium-dependent cotransporters, both outward and inward conformations are expected to be fully thermodynamically reversible, whereby the directionality of the substrate flux is determined by the combined electrochemical potential of the substrate and the co-substrate (Drew and Boudker 2016). In the case of the bacterial proteins $ASBT_{NM}$ and $ASBT_{Yf}$, the transition between the resolved inward-facing (PDB: 4N7W, 3ZUY, 7CYG, 7CYK, 6LH0) and outward-facing (PDB: 4N7X, 6LH1) conformations best reflects an alternating access mechanism previously described as an elevator-type transport mechanism (Garaeva and Slotboom 2020; Wang et al. 2021a,b; Zhou et al. 2014). During this elevator-like conformational transition, the substrate binding site that is mostly confined to the core domain (also called the transport domain) undergoes a large rigid-body rotation against the relatively immobile panel domain (also called the scaffold domain) (Drew and Boudker 2016). It was proposed for the bacterial ASBT proteins that the two Na^+ ions and the bile salt molecule bind to binding sites near the crossover region of TMDs 4 and 8. These binding sites have alternating access to the extra- and intracellular milieu in the outward- and inward-facing conformations, respectively. During the transport cycle, the Na^+ ions might bind first, thereby, facilitating bile salt binding to the dual-accessibility region. Alternatively, the bile salt molecule might bind first and subsequent sodium binding induces transition to the inward-facing conformation, from where both substrates are released to the intracellular milieu. Finally, release of the substrates might turn the transporter back to its outward open conformation (Lu et al. 2021; Wang et al. 2021a,b; Zhou et al. 2014). During the transport cycle, the Na^+ ions seem to bind to several different binding sites along their ion translocation path (Alhadeff et al. 2015) and the bile salt molecule occupies an additional inward-facing binding site, before it is released to the intracellular milieu (Hu et al. 2011).

Cryo-EM structures on human NTCP have provided deeper insight into the conformational transitions of this human protein more recently. In analogy to the inward-facing structures of $ASBT_{NM}$ and $ASBT_{Yf}$, human NTCP in complex with nanobody Nb87 adopted an inward-facing state, where the core and panel domains are tightly packed against each other on the extracellular side of the membrane, thereby, occluding the cavity from the outside (Goutam et al. 2022). In addition, the Q261A Na^+ binding site

mutation of human NTCP, in analogy to the E254A mutation of $ASBT_{Yf}$, revealed an outward open conformation (Asami et al. 2022). This conformation was also obtained from wild-type human NTCP (Asami et al. 2022; Park et al. 2022). The transition between these inward- and outward-facing conformations of human NTCP would fit well with an elevator-type accessible transport mode of NTCP, as previously described for the bacterial homologues. However, when the NTCP was reconstituted in nanodisc structures and complexed with nanobody Nb91 for cryo-EM, it revealed an open-pore conformation (Goutam et al. 2022). Such an open-pore conformation was also found by Liu et al., with the nanodisc reconstituted NTCP complexed with Fab12 (Liu et al. 2022). This conformation is apparently in conflict with the typical alternating-access transport mechanisms supported by the inward- and outward-facing conformations of the bacterial ASBT proteins and some of the human NTCP structures. Finally, it is not clear whether this open-pore conformation represents an intermediate state of the protein between the outward and inward open conformation. Furthermore, it is unclear how such a conformation would support thermodynamically active transport.

However, based on these structural realities, two different transport mechanisms have been proposed. Goutam et al. indicated a relatively large cavity between the core and the panel domains in the inward-facing state, forming an amphiphilic open pore for bulky bile salt integration and translocation (Goutam et al. 2022). Thereby, more subtle conformational changes between the core and panel domains, namely, a transition to the open-pore conformation, may actively drive the transport of one bile salt molecule together with 2 Na^+ ions through the protein (Goutam et al. 2022; Park et al. 2022). According to this hypothesis, this pore is transiently open in the presence of substrate and the thermodynamically coupled sodium and closes upon release of the substrates into the cytoplasm in the inward-facing state. Another proposed transport mechanism of NTCP is purely based on the open pore conformation of the protein (Liu et al. 2022). Accordingly, one bile salt molecule binds to S_{out} and, thereby, prevents Na^+ ion leakage by sealing the open pore. Subsequently, this bile salt molecule is shifted from S_{out} to S_{in}, while the transporter is reloading two sodium ions and one additional bile salt molecule to S_{out} from the outside. Then, only the bile salt molecule bound to S_{in} is released together with the two Na^+ ions to the intracellular milieu, while the bile salt molecule at S_{out} still prevents ion leakage. In this scenario, smaller conformational changes driven by the downhill Na^+ gradient are sufficient to move the substrate through the tunnel structure (Liu et al. 2022).

However, it has to be emphasized that such an open-pore state, in which the substrate binding sites are accessible from both sides of the membrane, is quite unusual for an active transporter. In almost all other membrane carriers, simultaneous access from both sides of the membrane to the substrate-binding site is excluded by strict conformational transition between inward- and outward-facing conformations that allow alternating access of the substrate to a central binding site (Drew and Boudker 2016).

In addition, it has to be emphasized that NTCP and bacterial ASBT are the only elevator-like proteins proposed for which only monomeric crystal and cryo-EM structures have been determined. Supposing an elevator-like transport mechanism, it is still unclear whether the panel domain in NTCP, $ASBT_{NM}$, and $ASBT_{Yf}$ is enough to anchor and support elevator-like structural transitions of the large 6 TMD core domain on its own.

2.2.2 NTCP is the high-affinity HBV/HDV entry receptor

During the life cycle of HBV in infected hepatocytes, the circular, partially double-stranded virion DNA is converted in the host nucleus to a covalently closed circular DNA that assembles into a minichromosome, the template for viral mRNA synthesis (Tsukuda and Watashi 2020). At the same time, envelope proteins enter the endoplasmic reticulum and assemble into subviral particles or transfer to multivesicular bodies where the virion is assembled and released by exosomes (Iannacone and Guidotti 2022; Yuen et al. 2018). The HBV surface consists of three envelope proteins called large (L), medium (M), and small (S), which are essential for virus attachment to hepatocytes in the initial infection process. The C-terminal S-domain is common to all three envelope proteins. The M-protein also contains an extra N-terminal preS2-domain, and the L-protein comprises a preS1-domain in addition to the preS2- and S-domains (Iannacone and Guidotti 2022; Yuen et al. 2018). The HDV, as an HBV satellite virus, depends on the HBV surface proteins for packing, release, and transmission. Accordingly, HDV also bears the preS1 domain-containing L-protein that is essential for the interaction with NTCP.

The HBV/HDV entry into hepatocytes is a multistep process and consists of the following general phases: (a) heparan sulfate proteoglycan-dependent low affinity attachment of HBV/HDV to the cell surface of hepatocytes; (b) high-affinity binding to extracellular domains of NTCP; (c) endocytosis of the virus-receptor complex; and (d) endosomal escape of the virion (Figure 2) (Herrscher et al. 2020a, b; Yan et al. 2012). The most recent milestone in understanding the HBV/HDV entry process was the identification of NTCP as the high-affinity entry receptor for HBV

in 2012 (Yan et al. 2012) and for HDV in 2013 (Drexler et al. 2013). Thereby, the 2–48 N-terminal amino acids of the myristoylated preS1-domain of the large HBV surface protein (myr-preS1$_{2–48}$) are absolutely essential for virus interaction with NTCP (Meier et al. 2013; Petersen et al. 2008).

Although several cryo-EM structures of human NTCP have recently been reported (see above), the structural insights into the virus-NTCP interactions and exact virus binding sites within NTCP have not been determined thus far (Asami et al. 2022; Goutam et al. 2022; Liu et al. 2022; Park et al. 2022). However, comprehensive mutational analyses of the NTCP protein have previously defined two protein regions, namely, $_{84}$RLKN$_{87}$, located in an extracellular loop between TMDs 2 and 3, and $_{157}$KGIVISLVL$_{165}$, belonging to TMD 5, which are involved in the interaction with preS1 (Figure 1) (Fukano et al. 2021; Iwamoto et al. 2019; Yan et al. 2013, 2014; Zakrzewicz et al. 2022). In addition, tyrosine 146, that belongs to extracellular loop bridging TMDs 4 and 5, is involved in the direct interaction with preS1 (Zakrzewicz et al. 2022). Since all these regions and amino acids mentioned above are exposed to the extracellular milieu and not directly to the pore where bile salts bind to NTCP, single amino acid mutations within these regions (e.g. G158R or Y146 A/E) mostly abolished preS1-peptide binding to NTCP, but retained its bile salt transport function (Asami et al. 2022; Müller et al. 2018; Yan et al. 2013; Zakrzewicz et al. 2022). Based on this, it is not surprising that some inhibitors of NTCP selectively block virus-binding to NTCP without tackling its physiological bile salt transport function (Chen et al. 2022; Kirstgen et al. 2020; Passioura et al. 2018).

By contrast, some other amino acids, namely, serine 267 and valine 263, are located more inside the pore and are essential for both functions of NTCP (Binh et al. 2019; Fu et al. 2017; Hu et al. 2016; Uchida et al. 2021). The NTCP V263A/I mutants and the genetic variant S267F abolished the transporter function of NTCP, at least for bile salts (Fu et al. 2017; Ruggiero et al. 2021). In addition, these variants made NTCP a less efficient receptor for HBV entry with a subsequent

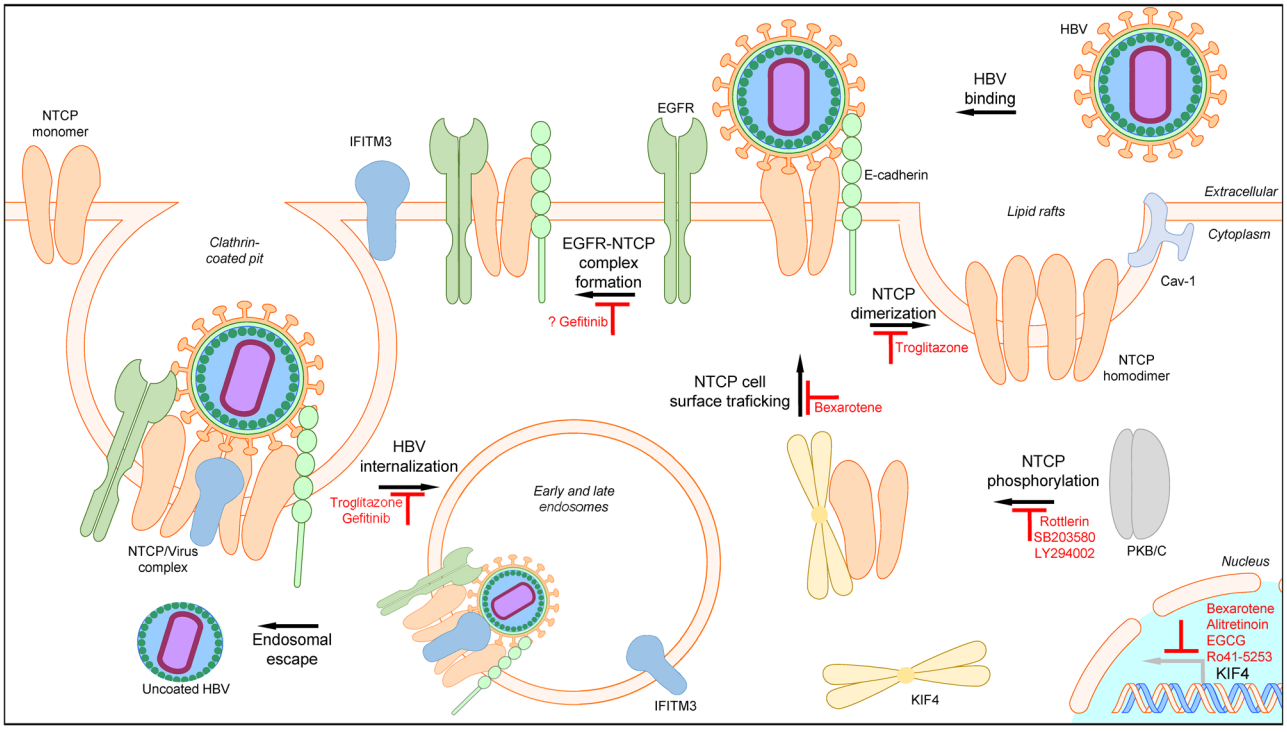

Figure 2: Schematic representation of the HBV/HDV entry pathway and cellular NTCP protein-protein interactions (PPIs) relevant for the virus internalization. HBV particles attach to hepatocytes through cell-surface factors, including heparan sulfate proteoglycans (not shown). Afterwards, HBV binds with high affinity to the NTCP receptor and entry into hepatocytes occurs via clathrin-dependent endocytosis. All these steps are tightly regulated by PPIs of NTCP with other membrane proteins, such as E-cadherin, epidermal growth factor receptor (EGFR), and interferon-induced transmembrane protein 3 (IFITM3). Endocytosis of the virus-NTCP complex most probably requires NTCP in an oligomeric state. Trafficking toward the plasma membrane and cell surface expression of NTCP are post-translationally controlled by phosphorylation and PPI with kinesin family member 4 (KIF4). NTCP-mediated HBV/HDV internalization as well as trafficking of NTCP to the plasma membrane can be blocked by pharmacological inhibitors, such as troglitazone, bexarotene, and others (shown in red).

decreased susceptibility to HBV and HDV infection in humans (Fu et al. 2017; Liu et al. 2018; Ruggiero et al. 2021). This clearly indicates that these amino acids are important for bile salt transport and preS1 binding to NTCP (Binh et al. 2019; Liu et al. 2018, 2017). This data is supported by the recent cryo-EM structure of NTCP that was generated in the presence of glycochenodeoxycholic acid. Within this structure, S267 directly interacts with the substrate (Liu et al. 2022).

Following the attachment of the virus particles to NTCP via the preS1-domain, the virus-receptor complex undergoes endocytosis. This process also involves numerous protein cofactors and PPIs with NTCP (Figure 2) (Fukano et al. 2018, 2021; Hu et al. 2020). Membrane-localized proteins, such as epidermal growth factor (EGF) receptor (EGFR) or E-cadherin, are recruited to the virus-NTCP complex, thereby triggering its clathrin-mediated endocytosis, a process that requires a phosphorylation and, thus, activation of EGFR (Fukano et al. 2021; Herrscher et al. 2020a; Hu et al. 2020; Iwamoto et al. 2019). In addition to the interaction with EGFR, NTCP self-assembly seems to play a pivotal role, not only in the regulation of NTCP biology and NTCP transporter function but also in facilitating a successful entry of the virus-receptor complex into the early endosomal trafficking pathway toward lysosomes (Fukano et al. 2018, 2021; Qin et al. 2022). Although the detailed mechanism underlying HBV/HDV post-attachment entry remains largely unknown, it is quite clear that the endosomal network, with its residing proteins, plays a key role in this process (Iwamoto et al. 2020). The knockdown of adaptor molecules belonging to the EGFR endocytosis machinery, such as adaptor-related protein complex 2 subunit α 1 (AP2A1) and receptor pathway substrate 15 (EPS15), as well as the NTCP cofactor interferon-induced transmembrane protein 3 (IFITM3), all significantly affected HBV or HDV infection rates (Iwamoto et al. 2020; Palatini et al. 2022) (Figure 2 and Table 1).

3 Protein–protein interactions in NTCP biology

3.1 NTCP dimerization/oligomerization

A number of cellular and membrane proteins in both prokaryotic and eukaryotic systems have oligomeric properties (Kumari and Yadav 2019). These oligomers are composed of multiple subunits (polypeptide chains), which may be the same (in homo-oligomeric proteins) or different (in hetero-oligomeric proteins). The common mechanisms of protein oligomerization are domain swapping and ligand-induced dimerization, which has also been reported for NTCP (Kumari and Yadav 2019; Fukano et al. 2021; 2018). The NTCP forms homodimers, in which the individual subunits are functionally active in transporting bile salts in a sodium-dependent manner (Bijsmans et al. 2012; Noppes et al. 2019; Qin et al. 2022). Interestingly, although NTCP homodimers are the predominant form of NTCP *in vivo*, recent cryo-EM studies have reported NTCP structures only in a monomeric state (Asami et al. 2022; Goutam et al. 2022; Liu et al. 2022; Park et al. 2022). This suggests that either the purification process of detergent-solubilized NTCP or the embedding into nanodisc structures preceding cryo-EM favors the monomeric state. Nevertheless, *in vivo* NTCP homodimerization occurs early in the secretory pathway and persists after its sorting to the plasma membrane. Furthermore, it has been shown in a series of coprecipitationexperiments that the C-terminus of NTCP is not involved in NTCP dimerization, as demonstrated with NTCP mutants lacking amino acids 308–349 of the C-terminus (Y307X mutant) (Table 1) (Bijsmans et al. 2012). Interestingly, after co-expression with this NTCP Y307X mutant or the NTCP homolog SLC10A4, which are both localized mainly in intracellular structures in cell culture, wild-type NTCP is intracellularly trapped, most probably due to the formation of heterodimers early in the secretory pathway. Consequently, the plasma membrane expression and bile salt transport function of NTCP are hampered (Bijsmans et al. 2012; Noppes et al. 2019). These data clearly point to functional homo- and heterodimerization of NTCP and a clear effect on plasma membrane sorting and function.

Domains important for NTCP homodimerization were first identified by Fukano et al. (Fukano et al. 2018), who demonstrated that peptides corresponding to amino acids 221–240 or 271–290 of NTCP were able to reduce its homodimerization (Table 1). It is well-known that protein dimerization occurs between TM helices of single-pass membrane proteins and often involves certain sequence motifs, such as “GXXXG/A” (Teese and Langosch 2015). In the case of NTCP, two potential GXXXG/A dimerization motifs are present, namely, $_{60}$GXXXA$_{64}$ in TMD 2 and $_{233}$GXXXG$_{237}$ in TMD 7 (Table 1 and Figure 3) (Palatini et al. 2021). It is noteworthy that the $_{233}$GXXXG$_{237}$ motif lays within one of the NTCP fragments (amino acids 221–240) mentioned above. Interestingly, G60LXXXA64L and G233LXXXG237L mutants revealed that both GXXXG/A motifs are important for the proper folding and plasma membrane sorting of NTCP, and, indirectly, for NTCP glycosylation, homodimerization, and its function as a bile salt transporter and HBV/HDV receptor (Palatini et al. 2021). However, it is still a matter of debate whether the homodimerization of NTCP is a

Table 1: The list of human NTCP interacting partners identified.

NTCP cofactor (Uniprot)	Interaction occurs at	Functional consequences of the interaction	Confirmed by
NTCP (Q14973)	Plasma membrane	– NTCP coprecipitated with NTCP → NTCP homodimerization (Bijsmans et al. 2012; Noppes et al. 2019) – Transport negative NTCP E257N mutant still coprecipitated with wild-type NTCP → individual subunits of the NTCP dimer are functional (Bijsmans et al. 2012) – NTCP/NTCP-Y307X and NTCP-Y307X/NTCP-Y307X coprecipitated, and these dimers were retained in the ER → NTCP dimerization occurs already in the ER and the C-terminus is not involved in dimerization (Bijsmans et al. 2012) – NTCP F274A mutation maintained preS1 binding, but reduced preS1 internalization, NTCP dimerization, HBV infection and bile salt transport → F274 involved in NTCP dimerization (Fukano et al. 2021) – G_{60}LXXXA_{64}L (TMD2) and G_{233}LXXXG_{237}L (TMD7) reduced homodimerization of NTCP, the G_{233}LXXXG_{237}L mutant in contrast to G_{60}LXXXA_{64}L did not reach the plasma membrane, both mutants showed reduced bile salt transport and HBV infection → both GXXXG/A motifs are important for proper folding, sorting, glycosylation and dimerization of NTCP (Palatini et al. 2021) – Troglitazone inhibited NTCP oligomerization, preS1 internalization, and HBV/HDV infection → troglitazone = entry inhibitor (Fukano et al. 2018) – NTCP peptides 221–240 and 271–290 inhibited NTCP dimerization, preS1 internalization and HBV infection → these domains are involved in NTCP dimerization (Fukano et al. 2018) – NTCP G144A/G148A mutation reduced NTCP oligomerization → these amino acids are involved in NTCP dimerization (Fukano et al. 2021)	Y2H, co-IP, IF
SLC10A4 (Q96EP9)	Intracellular compartments	– SLC10A4 coprecipitated with NTCP and *vice versa*, heterodimerization with SLC10A4 retained NTCP in intracellular compartments and reduced bile salt transport → SLC10A4 hampers plasma membrane sorting of NTCP (Bijsmans et al. 2012; Noppes et al. 2019)	IF, co-IP
SOAT (Q3KNW5)	Plasma membrane	– SOAT (SLC10A6) coprecipitated with NTCP and *vice versa*, plasma membrane sorting and bile salt transport unaffected → homo- and heterodimerization is a common feature of SLC10 carriers (Bijsmans et al. 2012; Noppes et al. 2019)	IF, co-IP
EGFR (P00533)	Plasma membrane, endosomes	– The NTCP peptide 131–150 and NTCP G144A/G148A mutations abolished interaction with EGFR and reduced HBV and HDV infection, EGFR knockdown reduced HBV and HDV infection, but retained preS1 binding → NTCP interaction with EGFR via $_{144}$GXXXG_{148} is required to fully support HBV/HDV entry (Fukano et al. 2021; Iwamoto et al. 2019)	KD, co-IP, IF
KIF4 (O95239)	Cytoplasm	– NTCP co-localized with KIF4 across microtubule filaments and coprecipitated with KIF4, KIF4 depletion reduced surface expression of NTCP and HBV/HDV infection → KIF4 is a critical regulator of NTCP trafficking to the plasma membrane (Gad et al. 2022)	IF, co-IP
IFITM3 (Q01628)	Endosome, plasma membrane	– IFITM3 coprecipitated with NTCP in hepatoma cells, IFITM3 knockdown had no effect on bile salt transport and preS1 binding, but reduced HBV and HDV infection → IFITM3 promotes virus entry at a post attachment step (Palatini et al. 2022)	Y2H, co-IP, IF
SPP1 (P10451)	Secreted	– Prominent hit from Y2H screen, but not yet confirmed by co-IP (Palatini et al. 2022)	Y2H
TOMM6 (Q96B49)	Mitochondrial outer membrane	– Prominent hit from Y2H screen, but not yet confirmed by co-IP (Palatini et al. 2022)	Y2H
CD63 (P08962)	Cell membrane, lysosome membrane, late endosome, exosome, cell surface	– Prominent hit from Y2H screen, but not yet confirmed by co-IP (Palatini et al. 2022)	Y2H
KRTCAP2 (Q8*N*6L1)	ER membrane	– Prominent hit from Y2H screen, but not yet confirmed by co-IP (Palatini et al. 2022)	Y2H

Table 1: (continued)

NTCP cofactor (Uniprot)	Interaction occurs at	Functional consequences of the interaction	Confirmed by
SELK (Q9Y6D0)	Cell membrane, ER	– Prominent hit from Y2H screen, but not yet confirmed by co-IP (Palatini et al. 2022)	Y2H
STOM (P27105)	Cell membrane, cytoplasm, lipid rafts	– Stomatin overexpression increased NTCP-mediated bile salt transport without affecting cell surface expression of NTCP (Appelman et al. 2020) – Stomatin knockdown increased plasma membrane expression of NTCP and bile salt transport rates (Appelman et al. 2020) – NTCP lacking its C-terminus (Y307X) can still interact with stomatin (Appelman et al. 2020)	IF, co-IP, SGU
CLCC1 (Q96S66)	ER, Golgi apparatus and nucleus membranes	– Neither upregulation nor downregulation of CLCC1 had an effect on bile salt transport via NTCP (Appelman et al. 2020)	IF, SGU
E-CAD (P12830)	Cell membrane, cell junction, endosome, Golgi apparatus	– Overexpression of E-cadherin increased HBV infection in HepaRG cells, E-cadherin knockdown reduced preS1 binding and HBV infection of primary human hepatocytes → E-cadherin is important for trafficking of glycosylated NTCP to the plasma membrane (Hu et al. 2020)	KD, co-IP
CANX (P27824)	ER and ER membrane	– Calnexin depletion reduced NTCP expression and decreased bile salt transport (Robin et al. 2018)	MS, co-IP

CANX, calnexin; CLCC1, chloride channel CLIC-like protein 1; co-IP, co-immunoprecipitation; E-CAD, E-cadherin; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; IF, immunofluorescence; IFITM3, interferon-induced transmembrane protein 3; KIF4, kinesin family member 4; KRTCAP2, keratinocyte-associated protein 2; PHH, primary human hepatocytes; PPI, protein–protein interaction; SELK, selenoprotein K; STOM, stomatin; SGU, sucrose gradient ultracentrifugation; SOAT, sodium-dependent organic anion transporter; SPP1, secreted phosphoprotein 1 (osteopontin); TOMM6, mitochondrial import receptor subunit TOM6 homolog; KD, knockdown; Y2H, yeast-two hybrid screen.

strict prerequisite for its HBV/HDV receptor function and the internalization of the virus/NTCP complex into hepatocytes. It was recently demonstrated that HBV/HDV preS1-peptide binding to NTCP and virus entry into hepatocytes can be significantly reduced with either peptide- or mutation-based approaches targeting potential dimerization motifs of NTCP (Figure 2) (Fukano et al. 2018; Palatini et al. 2021). In addition, the NTCP oligomerization process seems to be tightly regulated by several other membrane-associated proteins, for instance, EGFR (Iwamoto et al. 2019). The latter was identified as an important host cofactor that is involved in NTCP-mediated virus entry. Furthermore, it was suggested that NTCP oligomerization is initiated downstream of the NTCP-EGFR interaction and then triggers HBV internalization (Fukano et al. 2018, 2021). One of the amino acids that seems to be essential for this process is phenylalanine 274, which, after mutation to alanine, caused a loss of HBV susceptibility. Furthermore, the oligomerization of NTCP and HBV internalization were disrupted by F274A mutation, but without affecting the viral attachment to the cell surface (Table 1) (Fukano et al. 2021; Fukano et al. 2018). However, neither troglitazone, an inhibitor of NTCP oligomerization, nor F274A mutation affected the interaction of NTCP with EGFR (Figure 2) (Fukano et al. 2021; 2018). These data indicate that inhibiting the oligomerization of NTCP can serve as a new therapeutic strategy for HBV/HDV entry inhibitors. Troglitazone has been registered as an antidiabetic and anti-inflammatory drug that activates peroxisome proliferator-activated receptors (PPARs), particularly PPARα and PPARγ (Aljada et al. 2001b; Devchand et al. 2018). Troglitazone is also known to influence NFκB signaling, an important regulator of immune responses, which are essential in fighting HBV infections (Aljada et al. 2001a). Therefore, this compound has adverse effects related to its multi-target mode of action. Hence, it demonstrated high hepatotoxicity and serious adverse events experienced by patients, which finally resulted in the withdrawal of the drug from the market. Nevertheless, the promising *in vitro* data on troglitazone-mediated inhibition of NTCP oligomerization and HBV infection will stimulate further studies focusing on the thiazolidinedione drug class (including troglitazone, pioglitazone, lobeglitazone, and some others), which should particularly focus on the increased target specificity toward NTCP and reduced hepatotoxicity. Notably, troglitazone analogs with poor toxicity toward hepatocytes have already been synthesized and successfully tested as potential antiproliferative agents for the treatment of metastatic solid tumors (Komatsu et al. 2014; Murakami et al. 2014). These modified troglitazone-based thiazolidinedione analogs, for instance, efatutazone, could be considered as potential candidates.

Taking together, HBV/HDV entry inhibitors can target not only virus-binding to NTCP, but also NTCP oligomerization and interaction with cofactors that are essential for entry of the virus-NTCP receptor complex.

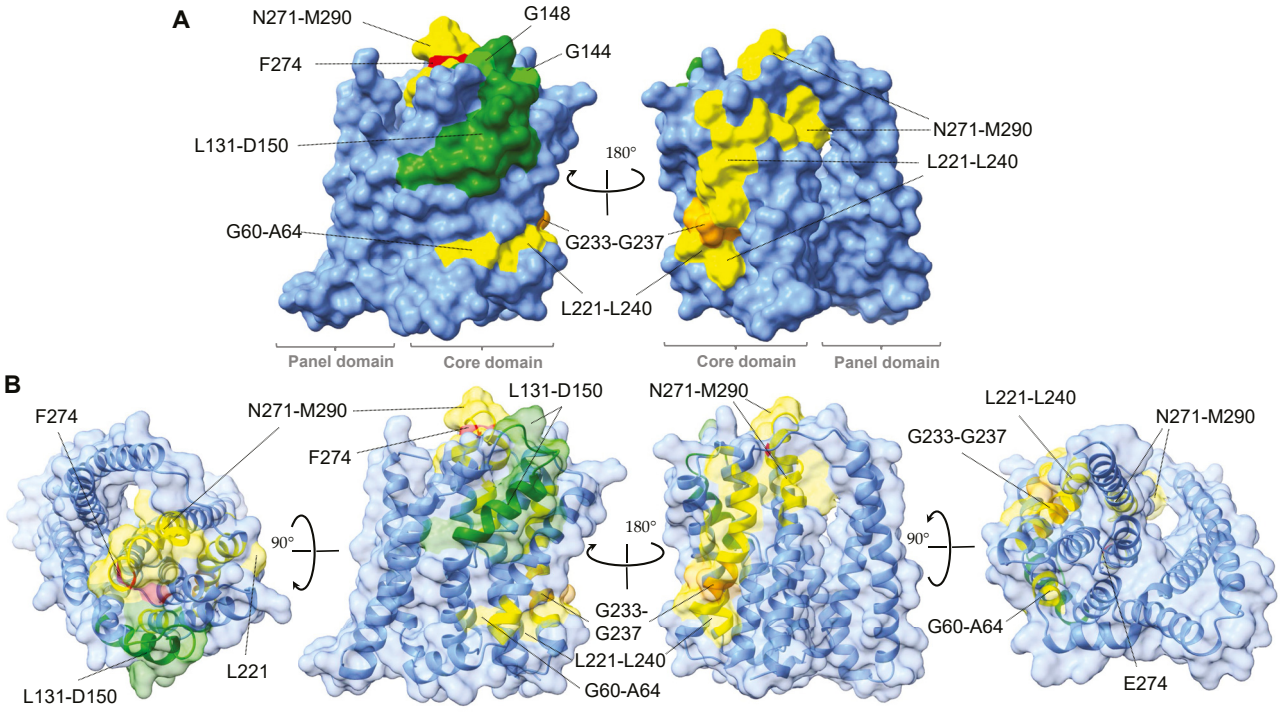

Figure 3: Amino acids and binding motifs required for NTCP PPIs, which are essential for the NTCP-driven HBV/HDV internalization. (A) Protein surface-exposed regions within NTCP, which are essential for NTCP oligomerization [G60-A64 (yellow), L221-L240 (yellow), G233-G237 (orange), N271-M290 (yellow), and F274 (red)] and NTCP-EGFR binding [L131-D150 (green), and G144/G148 (light green)]. (B) Protein core-localized amino acids and motifs, which are required for the respective NTCP PPI.

3.2 NTCP cofactors involved in HBV/HDV entry

Until now, the best characterized PPI is the interaction of NTCP with EGFR. The EGFR is a plasma membrane protein with a kinase activity that triggers a cascade of downstream protein phosphorylation and signaling pathways when it is engaged by its ligand EGF (Sabbah et al. 2020). Regardless of its role in the initiation of signal transduction, EGFR has been well-documented to drive protein internalization and post-endocytic protein sorting (Caldieri et al. 2018). Due to this function, it has been implicated in the entry mechanism of different virus classes, such as human cytomegalovirus, hepatitis C virus (HCV), and, more recently, HBV (Iwamoto et al. 2019). It was shown that after the binding of HBV to NTCP, ligand-activated EGFR associates with HBV-bound NTCP and drives the complex to early/late endosomes via clathrin-mediated endocytosis (Figure 2) (Herrscher et al. 2020a; Iwamoto et al. 2019, 2020). This process is dependent on the EGFR activation, since the inhibition of the receptor with gefitinib, a specific inhibitor of the EGFR tyrosine kinase, significantly impeded HBV entry (Fukano et al. 2021, Iwamoto et al. 2019). However, it has been suggested that EGFR downstream signaling has a minor role in HBV infection, since inhibition of the Ras-MAPK, PI3K-Akt, or JAK-STAT pathways had no drastic effects on the HBV infection of HepG2-NTCP cells or primary human hepatocytes, with only a marginal reduction of the HBV infection upon treatment with the PI3K inhibitor wortmannin in both cell types (Iwamoto et al. 2019). This implies that the NTCP interaction with activated EGFR itself but not EGFR downstream signaling cascades are essential for supporting HBV internalization. Thereby, conformational changes of the EGFR after EGF-induced autophosphorylation seem to play the key role for the interaction with NTCP and internalization of the virus-NTCP receptor complex (Kaplan et al. 2016; Srinivasan et al. 2022). This internalization process involves EGFR-sorting adaptor proteins, such as EPS15 and AP2A1 (see above).

Increasing evidence suggests that EGFR assembly with NTCP involves a short NTCP region localized between L131 and D150 (Figure 3), since the introduction of a decoy peptide corresponding to the aforementioned protein fragment attenuated the EGF-induced internalization of preS1-bound NTCP (Table 1) (Fukano et al. 2021). Moreover, glycine to alanine mutations within the $_{144}$GXXXG$_{148}$ NTCP motif abolished its interaction with EGFR, indicating that this motif is important for EGFR-NTCP heterodimerization and HBV/HDV

internalization into host cells (Table 1) (Fukano et al. 2021). Based on the recent cryo-EM structures of NTCP, the $_{144}$GXXXG$_{148}$ NTCP motif is localized near the second extracellular loop bridging TMDs 4 and 5 (Figure 3) (Asami et al. 2022, Goutam et al. 2022, Liu et al. 2022). In the same region, NTCP bears a highly conserved tyrosine-rich motif, $_{139}$YIYSRGIY$_{146}$. Within this motif, Y146 was identified to be essential for preS1-peptide binding to NTCP and HBV infection of NTCP-expressing hepatoma cells (Zakrzewicz et al. 2022). Based on this, it can be hypothesized that virus-binding to NTCP directly interferes with EGFR recruitment and NTCP/EGFR heterodimerization (Figure 3), making pharmacological inhibitors targeting this 139Y-G148 domain of NTCP an attractive tool to block virus binding to NTCP and NTCP/EGFR heterodimerization in parallel. Such inhibitors could be identified based on the recent cryo-EM structures of NTCP by *in silico* docking with large compounds libraries, such as ZINC (Irwin et al. 2012).

The EGFR-mediated endocytosis results in the HBV/HDV transport via a clathrin-mediated pathway toward early/late endosomes and lysosomes, which are dynamic organelles that receive and degrade macromolecules from the secretory, endocytic, and phagocytic pathways (Iwamoto et al. 2020; Naslavsky and Caplan 2018). Moreover, endosomes play a key role in virus trafficking along the endocytic pathway (Gruenberg 2009; Iwamoto et al. 2020; Kaksonen and Roux 2018). Their establishment and membrane trafficking routes are dependent on a complex machinery that involves membrane structures, macromolecules, and a number of cellular proteins (Naslavsky and Caplan 2018). Among them, interferon-induced transmembrane protein 3 (IFITM3) seems to be involved in endosome formation and endosomal protein sorting during viral infection (Amini-Bavil-Olyaee et al. 2013; Spence et al. 2019). The IFITM3 protein expression is positively correlated with endosomal levels of cholesterol, a lipid known to control membrane sorting and dynamics in this compartment (Amini-Bavil-Olyaee et al. 2013). Given the general upregulation of interferon-induced transmembrane proteins, IFITM3 is a putative host cell factor for HBV infection of hepatocytes. Interestingly, a membrane yeast two-hybrid screen for NTCP interacting partners revealed IFITM3 as the prominent hit, at least in yeast cells (Palatini et al. 2022). Moreover, co-immunoprecipitation experiments confirmed direct NTCP-IFITM3 PPI in HepG2 and HuH7 hepatoma cells (Table 1). Interestingly, knockdown of IFITM3 reduced HBV infection rates of primary human hepatocytes. In addition, NTCP-expressing HuH7 cells showed significantly lower HDV infection rates under IFITM3 knockdown, while myr-preS1 peptide binding remained intact (Palatini et al. 2022). This indicates that IFITM3-mediated HBV/HDV infection enhancement occurs in a step downstream of the virus attachment to NTCP. However, domains within NTCP that are important for PPI with IFITM3 have not been identified so far.

Another interacting partner of NTCP in hepatocytes, which closely cooperates with EGFR, is E-cadherin (Table 1) (Ramirez Moreno and Bulgakova 2021). E-cadherin is a cell adhesion molecule that regulates EGFR localization and activity by controlling junctional tissue polarization, tight junction positioning, and, consequently, the distribution of cell-surface proteins, such as EGFR (Ramirez Moreno and Bulgakova 2021; Rubsam et al. 2017). This consequently modulates the HBV infection process and virus entry into the host cells (Schulze et al. 2012). It has been reported that E-cadherin interacts with glycosylated NTCP, facilitates its plasma membrane distribution, and, subsequently, influences the NTCP-dependent HBV entry into hepatocytes (Table 1 and Figure 2) (Hu et al. 2020). Hence, E-cadherin may directly interact with the cell surface virus-NTCP complex. Alternatively, E-cadherin may regulate the hepatocyte polarization that is mandatory for the HBV entry (Hu et al. 2020). Although a direct crosstalk between surface-associated EGFR, E-cadherin, and NTCP upon virus infection was suggested, the direct interaction of E-cadherin and NTCP needs more detailed analysis to decipher the structural regions critical for NTCP/E-cadherin PPI.

3.3 Cofactors relevant for NTCP trafficking to the cell surface

The functional outcome of membrane proteins, such as ligand-receptor binding, substrate transport, or intracellular signaling, can be regulated in many ways. Among them, the regulation of plasma membrane expression is of particular relevance (Appelman et al. 2021; Magalhaes et al. 2012). This involves endogenous processes of co-translational translocation into the plasma membrane, balance between *de novo* protein synthesis and degradation, and, finally, protein sorting and trafficking to the plasma membrane (Hanyaloglu 2018; Zakrzewicz et al. 2014, 2018). Membrane proteins reach the plasma membrane by a constant exocytosis from the trans-Golgi network (Hanyaloglu 2018; Naslavsky and Caplan 2018). Once they have reached the plasma membrane, their surface residence is determined by, for example, clathrin-dependent endocytosis. Thereby, clathrin-coated vesicles are formed, which transport membrane proteins to early endosomes, from which they are either recycled back to the plasma membrane or degraded via late endosomes and lysosomes (Kaksonen and Roux 2018;

Naslavsky and Caplan 2018). Based on experimental data, it is likely that NTCP uses the classical trans-Golgi trafficking pathway mentioned above to reach the plasma membrane and undergoes endocytosis to early endosomes via the clathrin-dependent pathway, at least, during entry of the virus-NTCP complex (Herrscher et al. 2020a; Iwamoto et al. 2020). However, it remains unclear whether NTCP undergoes recycling or degradation in lysosomes afterwards. The plasma membrane sorting motif of NTCP seems to be located at the C-terminus starting from Y307 (Bijsmans et al. 2012; Sun et al. 2001a, b; Zakrzewicz and Geyer 2022). However, more detailed analysis of single amino acid positions have only been done for rat Ntcp (rNtcp). One study revealed that Y307 and Y321, both localized at the cytoplasmic C-terminus of rNtcp, are critical for the basolateral membrane sorting (Sun et al. 2001a). Another amino acid that is important for the translocation of rNtcp to the plasma membrane is S226, which can be phosphorylated (Anwer et al. 2005). Mutation of this residue resulted in a 30% decrease in the total rNtcp phosphorylation and a significant increase of rNtcp retention in the plasma membrane and rNtcp-mediated taurocholate uptake (Anwer et al. 2005). This suggests, at least for rNtcp, that phosphorylation has a significant importance for protein trafficking and overall bile salt transport in the liver. According to the newest cryo-EM structure of rNtcp (PDB: 7VAF), S226 is localized in TM7 and oriented to the inside of the core domain. There, S226 does not seem to be accessible for post-translational phosphorylation (Asami et al. 2022). This may suggest that the phosphorylation of S226 within rNtcp occurs co-translationally, probably to achieve a stable and functionally active protein conformation.

Regardless of this fact, the question remains whether human NTCP might be phosphorylated by serine/tyrosine kinases at all and at which positions, and if this plays a role in the sorting of the human NTCP toward the plasma membrane. Adenosine 3′,5′-cyclic monophosphate (cAMP) is an important intracellular second messenger that activates the phosphoinositide 3-kinase (PI3K) pathway, consisting of three groups of downstream targets: protein kinase B (PKB/AKT), P70 S6 kinase (S6K), and protein kinase C (PKC) (Castel et al. 2021). It has been demonstrated that the intracellular shuttling of rNtcp and NTCP between plasma membrane and endosomes is dependent on cellular cAMP levels, and facilitated by PI3K/PKB/PKC isoforms (McConkey et al. 2004; Park et al. 2012; Sarkar et al. 2006; Webster et al. 2002). The pharmacological activation of PKB resulted in an increased translocation of NTCP toward the plasma membrane, while the inhibition of PKB and PKCδ using the compounds SB203580, rottlerin, and LY294002, respectively, blocked the cAMP-mediated increase in taurocholate uptake and NTCP translocation (Park et al. 2012; Webster et al. 2002) (Figure 2). Since the inhibition of cAMP levels and cAMP-induced PKB activity may markedly reduce the expression of cell surface-associated NTCP, PKB/C kinase inhibitors may be applied to dampen the HBV internalization, a process which depends strongly on the NTCP presence in the plasma membrane.

It is probable that numerous cytosolic proteins participate in the trafficking of NTCP across intracellular compartments toward the plasma membrane. Recent studies by Gad et al. (Gad et al. 2022) identified KIF4 as a critical protein that contributes to the delivery of NTCP to the cell surface. It was demonstrated that KIF4 interacts directly with NTCP and its cellular expression is positively correlated with the cell-surface levels of NTCP (Gad et al. 2022). Since the expression level of KIF4 can be inhibited by RAR and RXR agonists, such as bexarotene in a FOXM1-dependent manner, it is not surprising that bexarotene treatment suppressed the levels of NTCP expressed at the plasma membrane. Accordingly, the NTCP-dependent preS1-peptide binding and susceptibility of primary human hepatocytes to HBV infection were significantly reduced by bexarotene treatment (Gad et al. 2022). This demonstrates that bexarotene, similar to other RXR agonists such as epigallocatechin-3-gallate (EGCG) (Zhong et al. 2015) and Ro41-5253 (Tsukuda et al. 2015), are potent anti-HBV agents by inhibiting NTCP trafficking to the plasma membrane (Figure 2). However, as the pharmacological inhibition of both the transcription factors FOXM1 and KIF4 via RXR agonists may result in severe cellular side effects, including FOXM1-dependent gene regulation, and the movement of filaments and signaling molecules along microtubules, a more specific strategy could be a blockage of the PPI between NTCP and KIF4. This would include the identification of binding sites of both proteins and mutational analysis of the amino acid residues mentioned above to corroborate their involvement in KIF4-driven NTCP trafficking.

4 Conclusions and future directions

There is a growing body of evidence that physical interactions between NTCP and other membrane and cytosolic proteins are critical not only for maintaining the transport of bile salts through the plasma membrane but also for HBV/HDV infection in the liver. This process involves virus attachment and entry into hepatocytes, which are both highly dependent on plasma membrane-associated NTCP (Yan et al. 2012; Yuen et al. 2018). Several novel NTCP interacting partners have been identified over the past decade, and their significant role in the NTCP-driven HBV/HDV

infection process has been corroborated (Table 1). Potential binding sites have already been identified within the NTCP protein for some of the interacting partners (e.g. for EGFR and NTCP oligomerization), however, for some others, the relevant domains for PPI are largely unknown (e.g. IFITM3, E-cadherin, KIF4). Nevertheless, these NTCP/cofactor interaction sites represent attractive novel target sites of NTCP for the development of specific anti-HBV/HDV drugs. Unfortunately, most studies on NTCP interactions, except NTCP oligomerization and EGFR-based research, have missed detailed analyses of the NTCP-involved protein complex assembly during the process of virus entry. Hence, based on the experimental data available, it is still unclear whether the effects of IFITM3, E-cadherin, or KIF4 proteins on HBV/HDV infection observed result completely and directly from PPIs with NTCP. At least, in part, these effects may result indirectly from the dysregulation of other cellular factors, such as endosome formation (IFITM3), hepatocyte polarization (E-cadherin), or the transfer of cargos along microtubules (KIF4) in the cell (Amini-Bavil-Olyaee et al. 2013; Rubsam et al. 2017; Sheng et al. 2018; Spence et al. 2019). Regardless of this, NTCP, with its interaction domains for all these cofactors, remains the most promising drug target for the discovery of novel HBV/HDV entry inhibitors.

The most relevant NTCP protein domains and single amino acid residues important for NTCP oligomerization seem to be $_{60}$GXXXA$_{64}$, L221-L240, $_{233}$GXXXG$_{237}$, N271-M290, and F274. Most of these sites have also been successfully assessed regarding their relevance in suppressing the virus preS1-peptide binding and, consequently, HBV internalization (Table 1 and Figure 3). Therefore, drugs that can enhance or disrupt NTCP oligomer formation might also regulate NTCP oligomerization-dependent HBV infection. According to recently published NTCP structures, these sites are all localized in the core domain of NTCP, namely, in TMD 2 ($_{60}$GXXXA$_{64}$), TMD 7 (L221-L240 and $_{233}$GXXXG$_{237}$), TMDs 8b and 9 (N271-M290), and TMD 8b (F274). In addition, most of them lie in direct proximity to each other, suggesting an orchestrated process of self-assembly of two or more NTCP molecules that involves several domains of NTCP (Figure 3). A similar process of oligomerization has already been proposed for G-protein-coupled receptors (George et al. 2002; Sleno and Hebert 2018; Takenouchi et al. 2018).

In conclusion, PPI sites of the HBV/HDV entry receptor NTCP have an immense potential as targets for new antiviral drugs that inhibit virus entry by preventing NTCP oligomerization or cofactor binding. Such drugs would be very specific and not hamper the physiological bile salt transport function of NTCP. Small chemical compounds designed using NTCP structure-based computational methods as well as short peptides constructed to mimic particular interaction domains of NTCP would be very effective approaches. Peptidomimetics or cyclic peptides possess conformational flexibility, increased selectivity, and, most importantly, they can be produced at low costs by chemical synthesis or biological expression (Rhodes and Pei 2017; Stone and Deber 2017). Peptides that interrupt PPIs have successfully been used as therapeutic agents to regulate a variety of biochemical events, including signal transduction, protein degradation, immune responses, and viral infections (Hall et al. 2007; Lalonde et al. 2011). Therefore, even more detailed analyses of NTCP PPIs relevant for HBV/HDV infections are promising to produce new therapeutic approaches against virus entry and infection.

Acknowledgements: Molecular graphics were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (Pettersen et al. 2004).

Author contributions: All authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), Project-ID 197785619-SFB1021 to J.G., and by the Tenure-Track Program of the Justus Liebig University Giessen (Germany), Project number 60001166 to D.Z.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

References

Alhadeff, R., Ganoth, A., and Arkin, I.T. (2015). Mechanistic studies of the apical sodium-dependent bile acid transporter. Proteins 83: 1107–1117.

Aljada, A., Garg, R., Ghanim, H., Mohanty, P., Hamouda, W., Assian, E., and Dandona, P. (2001a). Nuclear factor-kappaB suppressive and inhibitor-kappaB stimulatory effects of troglitazone in obese patients with type 2 diabetes: evidence of an antiinflammatory action? J. Clin. Endocrinol. Metab. 86: 3250–3256.

Aljada, A., Ghanim, H., Friedman, J., Garg, R., Mohanty, P., and Dandona, P. (2001b). Troglitazone reduces the expression of PPARγ while stimulating that of PPARα in mononuclear cells in obese subjects. J. Clin. Endocrinol. Metab. 86: 3130–3133.

Amini-Bavil-Olyaee, S., Choi, Y.J., Lee, J.H., Shi, M., Huang, I.C., Farzan, M., and Jung, J.U. (2013). The antiviral effector IFITM3 disrupts intracellular cholesterol homeostasis to block viral entry. Cell Host Microbe 13: 452–464.

Anwer, M.S., Gillin, H., Mukhopadhyay, S., Balasubramaniyan, N., Suchy, F.J., and Ananthanarayanan, M. (2005). Dephosphorylation of Ser-226 facilitates plasma membrane retention of Ntcp. J. Biol. Chem. 280: 33687–33692.

Anwer, M.S. and Stieger, B. (2014). Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters. Pflugers Arch. 466: 77–89.

Appelman, M.D., Robin, M.J.D., Vogels, E.W.M., Wolzak, C., Vos, W.G., Vos, H.R., Van Es, R.M., Burgering, B.M.T., and Van De Graaf, S.F.J. (2020). The lipid raft component stomatin interacts with the Na^+ taurocholate cotransporting polypeptide (NTCP) and modulates bile salt uptake. Cells 9: 986.

Appelman, M.D., Wettengel, J.M., Protzer, U., Oude Elferink, R.P.J., and Van De Graaf, S.F.J. (2021). Molecular regulation of the hepatic bile acid uptake transporter and HBV entry receptor NTCP. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1866: 158960.

Asami, J., Kimura, K.T., Fujita-Fujiharu, Y., Ishida, H., Zhang, Z., Nomura, Y., Liu, K., Uemura, T., Sato, Y., Ono, M., et al. (2022). Structure of the bile acid transporter and HBV receptor NTCP. Nature 606: 1021–1026.

Bi, Y.A., Qiu, X., Rotter, C.J., Kimoto, E., Piotrowski, M., Varma, M.V., Ei-Kattan, A.F., and Lai, Y. (2013). Quantitative assessment of the contribution of sodium-dependent taurocholate co-transporting polypeptide (NTCP) to the hepatic uptake of rosuvastatin, pitavastatin and fluvastatin. Biopharm. Drug Dispos. 34: 452–461.

Bijsmans, I.T., Bouwmeester, R.A., Geyer, J., Faber, K.N., and Van De Graaf, S.F. (2012). Homo- and hetero-dimeric architecture of the human liver Na^+-dependent taurocholate co-transporting protein. Biochem. J. 441: 1007–1015.

Binh, M.T., Hoan, N.X., Van Tong, H., Sy, B.T., Trung, N.T., Bock, C.T., Toan, N.L., Song, L.H., Bang, M.H., Meyer, C.G., et al. (2019). NTCP S267F variant associates with decreased susceptibility to HBV and HDV infection and decelerated progression of related liver diseases. Int. J. Infect. Dis. 80: 147–152.

Bogomolov, P., Alexandrov, A., Voronkova, N., Macievich, M., Kokina, K., Petrachenkova, M., Lehr, T., Lempp, F.A., Wedemeyer, H., Haag, M., et al. (2016). Treatment of chronic hepatitis D with the entry inhibitor myrcludex B: first results of a phase Ib/IIa study. J. Hepatol. 65: 490–498.

Bonvicino, C.R., Moreira, M.A., and Soares, M.A. (2014). Hepatitis B virus lineages in mammalian hosts: potential for bidirectional cross-species transmission. World J. Gastroenterol. 20: 7665–7674.

Caldieri, G., Malabarba, M.G., Di Fiore, P.P., and Sigismund, S. (2018). EGFR trafficking in physiology and cancer. Prog. Mol. Subcell Biol. 57: 235–272.

Castel, P., Toska, E., Engelman, J.A., and Scaltriti, M. (2021). The present and future of PI3K inhibitors for cancer therapy. Nat. Cancer 2: 587–597.

Chen, S., Zhang, L., Chen, Y., and Fu, L. (2022). Inhibiting Sodium taurocholate cotransporting polypeptide in HBV-related diseases: from biological function to therapeutic potential. J. Med. Chem. 65: 12546–12561.

Devchand, P.R., Liu, T., Altman, R.B., Fitzgerald, G.A., and Schadt, E.E. (2018). The pioglitazone trek via human PPARγ: from discovery to a medicine at the FDA and beyond. Front. Pharmacol. 9: 1093.

Döring, B., Lütteke, T., Geyer, J., and Petzinger, E. (2012). The SLC10 carrier family: transport functions and molecular structure. Curr. Top Membr. 70: 105–168.

Drew, D. and Boudker, O. (2016). Shared molecular mechanisms of membrane transporters. Annu. Rev. Biochem. 85: 543–572.

Drexler, J.F., Geipel, A., König, A., Corman, V.M., Van Riel, D., Leijten, L.M., Bremer, C.M., Rasche, A., Cottontail, V.M., Maganga, G.D., et al. (2013). Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. Proc. Natl. Acad. Sci. U. S. A. 110: 16151–16156.

Fu, L., Hu, H., Liu, Y., Jing, Z., and Li, W. (2017). Woodchuck sodium taurocholate cotransporting polypeptide supports low-level hepatitis B and D virus entry. Virology 505: 1–11.

Fujino, H., Saito, T., Ogawa, S., and Kojima, J. (2005). Transporter-mediated influx and efflux mechanisms of pitavastatin, a new inhibitor of HMG-CoA reductase. J. Pharm. Pharmacol. 57: 1305–1311.

Fukano, K., Oshima, M., Tsukuda, S., Aizaki, H., Ohki, M., Park, S.Y., Wakita, T., Wakae, K., Watashi, K., and Muramatsu, M. (2021). NTCP oligomerization occurs downstream of the NTCP-EGFR interaction during hepatitis B virus internalization. J. Virol. 95: e0093821.

Fukano, K., Tsukuda, S., Oshima, M., Suzuki, R., Aizaki, H., Ohki, M., Park, S.Y., Muramatsu, M., Wakita, T., Sureau, C., et al. (2018). Troglitazone impedes the oligomerization of sodium taurocholate cotransporting polypeptide and entry of hepatitis B virus into hepatocytes. Front. Microbiol. 9: 3257.

Gad, S.A., Sugiyama, M., Tsuge, M., Wakae, K., Fukano, K., Oshima, M., Sureau, C., Watanabe, N., Kato, T., Murayama, A., et al. (2022). The kinesin KIF4 mediates HBV/HDV entry through the regulation of surface NTCP localization and can be targeted by RXR agonists *in vitro*. PLoS Pathog. 18: e1009983.

Garaeva, A.A. and Slotboom, D.J. (2020). Elevator-type mechanisms of membrane transport. Biochem. Soc. Trans. 48: 1227–1241.

George, S.R., O'dowd, B.F., and Lee, S.P. (2002). G-protein-coupled receptor oligomerization and its potential for drug discovery. Nat. Rev. Drug Discov. 1: 808–820.

Goutam, K., Ielasi, F.S., Pardon, E., Steyaert, J., and Reyes, N. (2022). Structural basis of sodium-dependent bile salt uptake into the liver. Nature 606: 1015–1020.

Greupink, R., Dillen, L., Monshouwer, M., Huisman, M.T., and Russel, F.G. (2011). Interaction of fluvastatin with the liver-specific Na^+-dependent taurocholate cotransporting polypeptide (NTCP). Eur. J. Pharm. Sci. 44: 487–496.

Grosser, G., Bennien, J., Sanchez-Guijo, A., Bakhaus, K., Döring, B., Hartmann, M., Wudy, S.A., and Geyer, J. (2018). Transport of steroid 3-sulfates and steroid 17-sulfates by the sodium-dependent organic anion transporter SOAT (SLC10A6). J. Steroid Biochem. Mol. Biol. 179: 20–25.

Grosser, G., Müller, S.F., Kirstgen, M., Döring, B., and Geyer, J. (2021). Substrate specificities and inhibition pattern of the solute carrier family 10 members NTCP, ASBT and SOAT. Front. Mol. Biosci. 8: 689757.

Gruenberg, J. (2009). Viruses and endosome membrane dynamics. Curr. Opin. Cell Biol. 21: 582–588.

Hagenbuch, B. and Meier, P.J. (1996). Sinusoidal (basolateral) bile salt uptake systems of hepatocytes. Semin. Liver Dis. 16: 129–136.

Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H., and Meier, P.J. (1991). Functional expression cloning and characterization of the hepatocyte Na+/bile acid cotransport system. Proc. Natl. Acad. Sci. U. S. A. 88: 10629–10633.

Hall, P.R., Malone, L., Sillerud, L.O., Ye, C., Hjelle, B.L., and Larson, R.S. (2007). Characterization and NMR solution structure of a novel cyclic pentapeptide inhibitor of pathogenic hantaviruses. Chem. Biol. Drug Des. 69: 180–190.

Hanyaloglu, A.C. (2018). Advances in membrane trafficking and endosomal signaling of G protein-coupled receptors. Int. Rev. Cell. Mol. Biol. 339: 93–131.

Herrscher, C., Pastor, F., Burlaud-Gaillard, J., Dumans, A., Seigneuret, F., Moreau, A., Patient, R., Eymieux, S., De Rocquigny, H., Hourioux, C., et al. (2020a). Hepatitis B virus entry into HepG2-NTCP cells requires clathrin-mediated endocytosis. Cell. Microbiol. 22: e13205.

Herrscher, C., Roingeard, P., and Blanchard, E. (2020b). Hepatitis B virus entry into cells. Cells 9: 1486.

Ho, R.H., Leake, B.F., Roberts, R.L., Lee, W., and Kim, R.B. (2004). Ethnicity-dependent polymorphism in Na^+-taurocholate cotransporting polypeptide (SLC10A1) reveals a domain critical for bile acid substrate recognition. J. Biol. Chem. 279: 7213–7222.

Ho, R.H., Tirona, R.G., Leake, B.F., Glaeser, H., Lee, W., Lemke, C.J., Wang, Y., and Kim, R.B. (2006). Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. Gastroenterology 130: 1793–1806.

Hu, H.H., Liu, J., Lin, Y.L., Luo, W.S., Chu, Y.J., Chang, C.L., Jen, C.L., Lee, M.H., Lu, S.N., Wang, L.Y., et al. (2016). The rs2296651 (S267F) variant on NTCP (SLC10A1) is inversely associated with chronic hepatitis B and progression to cirrhosis and hepatocellular carcinoma in patients with chronic hepatitis B. Gut 65: 1514–1521.

Hu, N.J., Iwata, S., Cameron, A.D., and Drew, D. (2011). Crystal structure of a bacterial homologue of the bile acid sodium symporter ASBT. Nature 478: 408–411.

Hu, Q., Zhang, F., Duan, L., Wang, B., Ye, Y., Li, P., Li, D., Yang, S., Zhou, L., and Chen, W. (2020). E-Cadherin plays a role in hepatitis B virus entry through affecting glycosylated sodium-taurocholate cotransporting polypeptide distribution. Front. Cell Infect. Microbiol. 10: 74.

Hughes, S.A., Wedemeyer, H., and Harrison, P.M. (2011). Hepatitis delta virus. Lancet 378: 73–85.

Iannacone, M. and Guidotti, L.G. (2022). Immunobiology and pathogenesis of hepatitis B virus infection. Nat. Rev. Immunol. 22: 19–32.

Irwin, J.J., Sterling, T., Mysinger, M.M., Bolstad, E.S., and Coleman, R.G. (2012). ZINC: a free tool to discover chemistry for biology. J. Chem. Inf. Model. 52: 1757–1768.

Iwamoto, M., Saso, W., Nishioka, K., Ohashi, H., Sugiyama, R., Ryo, A., Ohki, M., Yun, J.H., Park, S.Y., Ohshima, T., et al. (2020). The machinery for endocytosis of epidermal growth factor receptor coordinates the transport of incoming hepatitis B virus to the endosomal network. J. Biol. Chem. 295: 800–807.

Iwamoto, M., Saso, W., Sugiyama, R., Ishii, K., Ohki, M., Nagamori, S., Suzuki, R., Aizaki, H., Ryo, A., Yun, J.H., et al. (2019). Epidermal growth factor receptor is a host-entry cofactor triggering hepatitis B virus internalization. Proc. Natl. Acad. Sci. U. S. A. 116: 8487–8492.

Jardetzky, O. (1966). Simple allosteric model for membrane pumps. Nature 211: 969–970.

Jetter, A. and Kullak-Ublick, G.A. (2020). Drugs and hepatic transporters: a review. Pharmacol. Res. 154: 104234.

Kaksonen, M. and Roux, A. (2018). Mechanisms of clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. 19: 313–326.

Kaplan, M., Narasimhan, S., De Heus, C., Mance, D., Van Doorn, S., Houben, K., Popov-Celeketic, D., Damman, R., Katrukha, E.A., Jain, P., et al. (2016). EGFR dynamics change during activation in native membranes as revealed by NMR. Cell 167: 1241–1251 e11.

Kersseboom, S., Van Gucht, A.L.M., Van Mullem, A., Brigante, G., Farina, S., Carlsson, B., Donkers, J.M., Van De Graaf, S.F.J., Peeters, R.P., and Visser, T.J. (2017). Role of the bile acid transporter SLC10A1 in liver targeting of the lipid-lowering thyroid hormone analog eprotirome. Endocrinology 158: 3307–3318.

Killer, M., Wald, J., Pieprzyk, J., Marlovits, T.C., and Low, C. (2021). Structural snapshots of human PepT1 and PepT2 reveal mechanistic insights into substrate and drug transport across epithelial membranes. Sci. Adv. 7: eabk3259.

Kim, S.W., Yoon, J.S., Lee, M., and Cho, Y. (2022). Toward a complete cure for chronic hepatitis B: novel therapeutic targets for hepatitis B virus. Clin. Mol. Hepatol. 28: 17–30.

Kirstgen, M., Lowjaga, K., Müller, S.F., Goldmann, N., Lehmann, F., Alakurtti, S., Yli-Kauhaluoma, J., Glebe, D., and Geyer, J. (2020). Selective hepatitis B and D virus entry inhibitors from the group of pentacyclic lupane-type betulin-derived triterpenoids. Sci. Rep. 10: 21772.

Kirstgen, M., Lowjaga, K., Müller, S.F., Goldmann, N., Lehmann, F., Glebe, D., Baringhaus, K.H., and Geyer, J. (2021a). Hepatitis D virus entry inhibitors based on repurposing intestinal bile acid reabsorption inhibitors. Viruses 13: 666.

Kirstgen, M., Müller, S.F., Lowjaga, K., Goldmann, N., Lehmann, F., Alakurtti, S., Yli-Kauhaluoma, J., Baringhaus, K.H., Krieg, R., Glebe, D., et al. (2021b). Identification of novel HBV/HDV entry inhibitors by pharmacophore- and QSAR-guided virtual screening. Viruses 13: 1489.

Komatsu, Y., Yoshino, T., Yamazaki, K., Yuki, S., Machida, N., Sasaki, T., Hyodo, I., Yachi, Y., Onuma, H., and Ohtsu, A. (2014). Phase 1 study of efatutazone, a novel oral peroxisome proliferator-activated receptor gamma agonist, in combination with FOLFIRI as second-line therapy in patients with metastatic colorectal cancer. Invest. New Drugs 32: 473–480.

Kullak-Ublick, G.A., Beuers, U., and Paumgartner, G. (2000). Hepatobiliary transport. J. Hepatol. 32: 3–18.

Kullak-Ublick, G.A., Glasa, J., Boker, C., Oswald, M., Grutzner, U., Hagenbuch, B., Stieger, B., Meier, P.J., Beuers, U., Kramer, W., et al. (1997). Chlorambucil-taurocholate is transported by bile acid carriers expressed in human hepatocellular carcinomas. Gastroenterology 113: 1295–1305.

Kumari, N. and Yadav, S. (2019). Modulation of protein oligomerization: an overview. Prog. Biophys. Mol. Biol. 149: 99–113.

Lalonde, M.S., Lobritz, M.A., Ratcliff, A., Chamanian, M., Athanassiou, Z., Tyagi, M., Wong, J., Robinson, J.A., Karn, J., Varani, G., et al. (2011). Inhibition of both HIV-1 reverse transcription and gene expression by a cyclic peptide that binds the Tat-transactivating response element (TAR) RNA. PLoS Pathog. 7: e1002038.

Liu, C., Xu, G., Gao, Z., Zhou, Z., Guo, G., Li, D., Jing, Z., Sui, J., and Li, W. (2018). The p.Ser267Phe variant of sodium taurocholate cotransporting polypeptide (NTCP) supports HBV infection with a low efficiency. Virology 522: 168–176.

Liu, H., Irobalieva, R.N., Bang-Sorensen, R., Nosol, K., Mukherjee, S., Agrawal, P., Stieger, B., Kossiakoff, A.A., and Locher, K.P. (2022). Structure of human NTCP reveals the basis of recognition and sodium-driven transport of bile salts into the liver. Cell Res. 32: 773–776.

Liu, R., Chen, C., Xia, X., Liao, Q., Wang, Q., Newcombe, P.J., Xu, S., Chen, M., Ding, Y., Li, X., et al. (2017). Homozygous p.Ser267Phe in SLC10A1 is associated with a new type of hypercholanemia and implications for personalized medicine. Sci. Rep. 7: 9214.

Lu, P.H., Li, C.C., Chiang, Y.W., Liu, J.H., Chiang, W.T., Chao, Y.H., Li, G.S., Weng, S.E., Lin, S.Y., and Hu, N.J. (2021). Dissecting the conformational dynamics of the bile acid transporter homologue ASBT(NM). J. Mol. Biol. 433: 166764.

Lucifora, J., Esser, K., and Protzer, U. (2013). Ezetimibe blocks hepatitis B virus infection after virus uptake into hepatocytes. Antiviral Res. 97: 195–197.

Magalhaes, A.C., Dunn, H., and Ferguson, S.S. (2012). Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. Br. J. Pharmacol. 165: 1717–1736.

Mcconkey, M., Gillin, H., Webster, C.R., and Anwer, M.S. (2004). Cross-talk between protein kinases Czeta and B in cyclic AMP-mediated sodium

taurocholate co-transporting polypeptide translocation in hepatocytes. J. Biol. Chem. 279: 20882–20888.

Meier, A., Mehrle, S., Weiss, T.S., Mier, W., and Urban, S. (2013). Myristoylated PreS1-domain of the hepatitis B virus L-protein mediates specific binding to differentiated hepatocytes. Hepatology 58: 31–42.

Mokaya, J., Mcnaughton, A.L., Hadley, M.J., Beloukas, A., Geretti, A.M., Goedhals, D., and Matthews, P.C. (2018). A systematic review of hepatitis B virus (HBV) drug and vaccine escape mutations in Africa: a call for urgent action. PLoS Negl. Trop. Dis. 12: e0006629.

Müller, S.F., Konig, A., Döring, B., Glebe, D., and Geyer, J. (2018). Characterisation of the hepatitis B virus cross-species transmission pattern via Na^+/taurocholate co-transporting polypeptides from 11 New World and Old World primate species. PLoS One 13: e0199200.

Murakami, H., Ono, A., Takahashi, T., Onozawa, Y., Tsushima, T., Yamazaki, K., Jikoh, T., Boku, N., and Yamamoto, N. (2014). Phase I study of Efatutazone, an oral PPARγ agonist, in patients with metastatic solid tumors. Anticancer Res. 34: 5133–5141.

Naslavsky, N. and Caplan, S. (2018). The enigmatic endosome - sorting the ins and outs of endocytic trafficking. J. Cell Sci. 131: 216499.

Nkongolo, S., Ni, Y., Lempp, F.A., Kaufman, C., Lindner, T., Esser-Nobis, K., Lohmann, V., Mier, W., Mehrle, S., and Urban, S. (2014). Cyclosporin A inhibits hepatitis B and hepatitis D virus entry by cyclophilin-independent interference with the NTCP receptor. J. Hepatol. 60: 723–731.

Noppes, S., Müller, S.F., Bennien, J., Holtemeyer, M., Palatini, M., Leidolf, R., Alber, J., and Geyer, J. (2019). Homo- and heterodimerization is a common feature of the solute carrier family SLC10 members. Biol. Chem. 400: 1371–1384.

Palatini, M., Müller, S.F., Kirstgen, M., Leiting, S., Lehmann, F., Soppa, L., Goldmann, N., Muller, C., Lowjaga, K., Alber, J., et al. (2022). IFITM3 interacts with the HBV/HDV receptor NTCP and modulates virus entry and infection. Viruses 14: 727.

Palatini, M., Müller, S.F., Lowjaga, K., Noppes, S., Alber, J., Lehmann, F., Goldmann, N., Glebe, D., and Geyer, J. (2021). Mutational analysis of the GXXXG/A motifs in the human Na^+/Taurocholate Co-transporting polypeptide NTCP on its bile acid transport function and hepatitis B/D virus receptor function. Front. Mol. Biosci. 8: 699443.

Park, J.H., Iwamoto, M., Yun, J.H., Uchikubo-Kamo, T., Son, D., Jin, Z., Yoshida, H., Ohki, M., Ishimoto, N., Mizutani, K., et al. (2022). Structural insights into the HBV receptor and bile acid transporter NTCP. Nature 606: 1027–1031.

Park, S.W., Schonhoff, C.M., Webster, C.R., and Anwer, M.S. (2012). Protein kinase Cdelta differentially regulates cAMP-dependent translocation of NTCP and MRP2 to the plasma membrane. Am. J. Physiol. Gastrointest. Liver Physiol. 303: G657–G665.

Passioura, T., Watashi, K., Fukano, K., Shimura, S., Saso, W., Morishita, R., Ogasawara, Y., Tanaka, Y., Mizokami, M., Sureau, C., et al. (2018). De novo macrocyclic peptide inhibitors of hepatitis B virus cellular entry. Cell Chem. Biol. 25: 906–915 e5.

Petersen, J., Dandri, M., Mier, W., Lutgehetmann, M., Volz, T., Von Weizsacker, F., Haberkorn, U., Fischer, L., Pollok, J.M., Erbes, B., et al. (2008). Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat. Biotechnol. 26: 335–341.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera – a visualization system for exploratory research and analysis. J. Comput. Chem. 25: 1605–1612.

Polaris Observatory, C. (2018). Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. Lancet Gastroenterol. Hepatol. 3: 383–403.

Qin, T., Wang, Y., Nie, J., Yu, L., and Zeng, S. (2022). Oligomerization of the HBV/HDV functional receptor NTCP expressed in Sf9 insect cell. Biochim. Biophys. Acta Gen. Subj. 1866: 130224.

Quistgaard, E.M., Low, C., Guettou, F., and Nordlund, P. (2016). Understanding transport by the major facilitator superfamily (MFS): structures pave the way. Nat. Rev. Mol. Cell Biol. 17: 123–132.

Ramirez Moreno, M. and Bulgakova, N.A. (2021). The cross-talk between EGFR and E-cadherin. Front. Cell Dev. Biol. 9: 828673.

Rhodes, C.A. and Pei, D. (2017). Bicyclic peptides as next-generation therapeutics. Chemistry 23: 12690–12703.

Robin, M.J.D., Appelman, M.D., Vos, H.R., Van Es, R.M., Paton, J.C., Paton, A.W., Burgering, B., Fickert, P., Heijmans, J., and Van De Graaf, S.F.J. (2018). Calnexin depletion by endoplasmic reticulum stress during cholestasis inhibits the Na^+-taurocholate cotransporting polypeptide. Hepatol. Commun. 2: 1550–1566.

Rubsam, M., Mertz, A.F., Kubo, A., Marg, S., Jungst, C., Goranci-Buzhala, G., Schauss, A.C., Horsley, V., Dufresne, E.R., Moser, M., et al. (2017). E-cadherin integrates mechanotransduction and EGFR signaling to control junctional tissue polarization and tight junction positioning. Nat. Commun. 8: 1250.

Ruggiero, M.J., Malhotra, S., Fenton, A.W., Swint-Kruse, L., Karanicolas, J., and Hagenbuch, B. (2021). A clinically relevant polymorphism in the Na^+/taurocholate cotransporting polypeptide (NTCP) occurs at a rheostat position. J. Biol. Chem. 296: 100047.

Sabbah, D.A., Hajjo, R., and Sweidan, K. (2020). Review on epidermal growth factor receptor (EGFR) structure, signaling pathways, interactions, and recent updates of EGFR inhibitors. Curr. Top. Med. Chem. 20: 815–834.

Saeki, T., Takahashi, N., Kanamoto, R., and Iwami, K. (2002). Characterization of cloned mouse Na+/taurocholate cotransporting polypeptide by transient expression in COS-7 cells. Biosci. Biotechnol. Biochem. 66: 1116–1118.

Salhab, A., Amer, J., Lu, Y., and Safadi, R. (2022). Sodium(+)/taurocholate cotransporting polypeptide as target therapy for liver fibrosis. Gut 71: 1373–1385.

Sarkar, S., Bananis, E., Nath, S., Anwer, M.S., Wolkoff, A.W., and Murray, J.W. (2006). PKCzeta is required for microtubule-based motility of vesicles containing the NTCP transporter. Traffic 7: 1078–1091.

Schroeder, A., Eckhardt, U., Stieger, B., Tynes, R., Schteingart, C.D., Hofmann, A.F., Meier, P.J., and Hagenbuch, B. (1998). Substrate specificity of the rat liver Na^+-bile salt cotransporter in *Xenopus laevis* oocytes and in CHO cells. Am. J. Physiol. 274: G370–G375.

Schulze, A., Gripon, P., and Urban, S. (2007). Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology 46: 1759–1768.

Schulze, A., Mills, K., Weiss, T.S., and Urban, S. (2012). Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. Hepatology 55: 373–383.

Schulze, A., Schieck, A., Ni, Y., Mier, W., and Urban, S. (2010). Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction. J. Virol. 84: 1989–2000.

Sheng, L., Hao, S.L., Yang, W.X., and Sun, Y. (2018). The multiple functions of kinesin-4 family motor protein KIF4 and its clinical potential. Gene 678: 90–99.

Sleno, R. and Hebert, T.E. (2018). The dynamics of GPCR oligomerization and their functional consequences. Int. Rev. Cell Mol. Biol. 338: 141–171.

Spence, J.S., He, R., Hoffmann, H.H., Das, T., Thinon, E., Rice, C.M., Peng, T., Chandran, K., and Hang, H.C. (2019). IFITM3 directly engages and

shuttles incoming virus particles to lysosomes. Nat. Chem. Biol. 15: 259–268.

Srinivasan, S., Regmi, R., Lin, X., Dreyer, C.A., Chen, X., Quinn, S.D., He, W., Coleman, M.A., Carraway, K.L., 3rd., Zhang, B., et al. (2022). Ligand-induced transmembrane conformational coupling in monomeric EGFR. Nat. Commun. 13: 3709.

St-Pierre, M.V., Kullak-Ublick, G.A., Hagenbuch, B., and Meier, P.J. (2001). Transport of bile acids in hepatic and non-hepatic tissues. J. Exp. Biol. 204: 1673–1686.

Stone, T.A. and Deber, C.M. (2017). Therapeutic design of peptide modulators of protein-protein interactions in membranes. Biochim. Biophys. Acta Biomembr. 1859: 577–585.

Sun, A.Q., Arrese, M.A., Zeng, L., Swaby, I., Zhou, M.M., and Suchy, F.J. (2001a). The rat liver Na^+/bile acid cotransporter. Importance of the cytoplasmic tail to function and plasma membrane targeting. J. Biol. Chem. 276: 6825–6833.

Sun, A.Q., Swaby, I., Xu, S., and Suchy, F.J. (2001b). Cell-specific basolateral membrane sorting of the human liver Na^+-dependent bile acid cotransporter. Am. J. Physiol. Gastrointest. Liver Physiol. 280: G1305–G1313.

Takenouchi, O., Yoshimura, H., and Ozawa, T. (2018). Unique roles of β-arrestin in GPCR trafficking revealed by photoinducible dimerizers. Sci. Rep. 8: 677.

Teese, M.G. and Langosch, D. (2015). Role of GxxxG motifs in transmembrane domain interactions. Biochemistry 54: 5125–5135.

Tsounis, E.P., Tourkochristou, E., Mouzaki, A., and Triantos, C. (2021). Toward a new era of hepatitis B virus therapeutics: the pursuit of a functional cure. World J. Gastroenterol. 27: 2727–2757.

Tsukuda, S. and Watashi, K. (2020). Hepatitis B virus biology and life cycle. Antiviral Res. 182: 104925.

Tsukuda, S., Watashi, K., Iwamoto, M., Suzuki, R., Aizaki, H., Okada, M., Sugiyama, M., Kojima, S., Tanaka, Y., Mizokami, M., et al. (2015). Dysregulation of retinoic acid receptor diminishes hepatocyte permissiveness to hepatitis B virus infection through modulation of sodium taurocholate cotransporting polypeptide (NTCP) expression. J. Biol. Chem. 290: 5673–5684.

Uchida, T., Park, S.B., Inuzuka, T., Zhang, M., Allen, J.N., Chayama, K., and Liang, T.J. (2021). Genetically edited hepatic cells expressing the NTCP-S267F variant are resistant to hepatitis B virus infection. Mol. Ther. Methods Clin. Dev. 23: 597–605.

Varadi, M., Anyango, S., Deshpande, M., Nair, S., Natassia, C., Yordanova, G., Yuan, D., Stroe, O., Wood, G., Laydon, A., et al. (2022). AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. Nucleic Acids Res. 50: D439–D444.

Vaz, F.M., Paulusma, C.C., Huidekoper, H., De Ru, M., Lim, C., Koster, J., Ho-Mok, K., Bootsma, A.H., Groen, A.K., Schaap, F.G., et al. (2015). Sodium taurocholate cotransporting polypeptide (SLC10A1) deficiency: conjugated hypercholanemia without a clear clinical phenotype. Hepatology 61: 260–267.

Visser, W.E., Wong, W.S., Van Mullem, A.A., Friesema, E.C., Geyer, J., and Visser, T.J. (2010). Study of the transport of thyroid hormone by transporters of the SLC10 family. Mol. Cell. Endocrinol. 315: 138–145.

Wang, X., Lyu, Y., Ji, Y., Sun, Z., and Zhou, X. (2021a). An engineered disulfide bridge traps and validates an outward-facing conformation in a bile acid transporter. Acta Crystallogr., Sect. D: Struct. Biol. 77: 108–116.

Wang, X., Lyu, Y., Ji, Y., Sun, Z., and Zhou, X. (2021b). Substrate binding in the bile acid transporter ASBTYf from *Yersinia frederiksenii*. Acta Crystallogr., Sect. D: Struct. Biol. 77: 117–125.

Watashi, K., Sluder, A., Daito, T., Matsunaga, S., Ryo, A., Nagamori, S., Iwamoto, M., Nakajima, S., Tsukuda, S., Borroto-Esoda, K., et al. (2014). Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter, sodium taurocholate cotransporting polypeptide (NTCP). Hepatology 59: 1726–1737.

Webster, C.R., Srinivasulu, U., Ananthanarayanan, M., Suchy, F.J., and Anwer, M.S. (2002). Protein kinase B/Akt mediates cAMP- and cell swelling-stimulated Na^+/taurocholate cotransport and NTCP translocation. J. Biol. Chem. 277: 28578–28583.

Weinman, S.A., Carruth, M.W., and Dawson, P.A. (1998). Bile acid uptake via the human apical sodium-bile acid cotransporter is electrogenic. J. Biol. Chem. 273: 34691–34695.

Yan, H., Peng, B., He, W., Zhong, G., Qi, Y., Ren, B., Gao, Z., Jing, Z., Song, M., Xu, G., et al. (2013). Molecular determinants of hepatitis B and D virus entry restriction in mouse sodium taurocholate cotransporting polypeptide. J. Virol. 87: 7977–7991.

Yan, H., Peng, B., Liu, Y., Xu, G., He, W., Ren, B., Jing, Z., Sui, J., and Li, W. (2014). Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. J. Virol. 88: 3273–3284.

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., et al. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife 1: e00049.

Yuen, M.F., Chen, D.S., Dusheiko, G.M., Janssen, H.L.A., Lau, D.T.Y., Locarnini, S.A., Peters, M.G., and Lai, C.L. (2018). Hepatitis B virus infection. Nat. Rev. Dis. Primers 4: 18035.

Zahner, D., Eckhardt, U., and Petzinger, E. (2003). Transport of taurocholate by mutants of negatively charged amino acids, cysteines, and threonines of the rat liver sodium-dependent taurocholate cotransporting polypeptide NTCP. Eur. J. Biochem. 270: 1117–1127.

Zakrzewicz, D., Didiasova, M., Kruger, M., Giaimo, B.D., Borggrefe, T., Mieth, M., Hocke, A.C., Zakrzewicz, A., Schaefer, L., Preissner, K.T., et al. (2018). Protein arginine methyltransferase 5 mediates enolase-1 cell surface trafficking in human lung adenocarcinoma cells. Biochim. Biophys. Acta Mol. Basis Dis. 1864: 1816–1827.

Zakrzewicz, D., Didiasova, M., Zakrzewicz, A., Hocke, A.C., Uhle, F., Markart, P., Preissner, K.T., and Wygrecka, M. (2014). The interaction of enolase-1 with caveolae-associated proteins regulates its subcellular localization. Biochem. J. 460: 295–307.

Zakrzewicz, D. and Geyer, J. (2022). Multitasking Na^+/Taurocholate cotransporting polypeptide (NTCP) as a drug target for HBV infection: from protein engineering to drug discovery. Biomedicines 10: 196.

Zakrzewicz, D., Leidolf, R., Kunz, S., Müller, S.F., Neubauer, A., Leiting, S., Goldmann, N., Lehmann, F., Glebe, D., and Geyer, J. (2022). Tyrosine 146 of the human Na^+/Taurocholate cotransporting polypeptide (NTCP) is essential for its hepatitis B virus (HBV) receptor function and HBV entry into hepatocytes. Viruses 14: 1259.

Zhong, L., Hu, J., Shu, W., Gao, B., and Xiong, S. (2015). Epigallocatechin-3-gallate opposes HBV-induced incomplete autophagy by enhancing lysosomal acidification, which is unfavorable for HBV replication. Cell Death Dis. 6: e1770.

Zhou, X., Levin, E.J., Pan, Y., Mccoy, J.G., Sharma, R., Kloss, B., Bruni, R., Quick, M., and Zhou, M. (2014). Structural basis of the alternating-access mechanism in a bile acid transporter. Nature 505: 569–573.

Bionotes

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