

REVIEWS IN BASIC AND CLINICAL GASTROENTEROLOGY AND HEPATOLOGY

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Strategies to Inhibit Entry of HBV and HDV Into Hepatocytes

Stephan Urban,^{1,2} Ralf Bartenschlager,^{1,2} Ralf Kubitz,³ and Fabien Zoulim⁴

¹Department of Infectious Diseases, Molecular Virology, University Hospital Heidelberg, Heidelberg, Germany; ²German Center for Infection Research, Heidelberg University, Heidelberg, Germany; ³Department of Gastroenterology, Hepatology and Infectious Diseases, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany; and ⁴INSERM Unité 1052, Cancer Research Center of Lyon, Lyon University, Lyon, France

Although there has been much research into the pathogenesis and treatment of hepatitis B virus (HBV) and hepatitis D virus (HDV) infections, we still do not completely understand how these pathogens enter hepatocytes. This is because *in vitro* infection studies have only been performed in primary human hepatocytes. Development of a polarizable, HBV-susceptible human hepatoma cell line and studies of primary hepatocytes from *Tupaia belangeri* have provided important insights into the viral and cellular factors involved in virus binding and infection. The large envelope (L) protein on the surface of HBV and HDV particles has many different functions and is required for virus entry. The L protein mediates attachment of virions to heparan sulfate proteoglycans on the surface of hepatocytes. The myristoylated N-terminal preS1 domain of the L protein subsequently binds to the sodium taurocholate cotransporting polypeptide (NTCP, encoded by *SLC10A1*), the recently identified *bona fide* receptor for HBV and HDV. The receptor functions of NTCP and virus entry are blocked, *in vitro* and *in vivo*, by Myrcludex B, a synthetic *N*-acylated preS1 lipopeptide. Currently, the only agents available to treat chronic HBV infection target the viral polymerase, and no selective therapies are available for HDV infection. It is therefore important to study the therapeutic potential of virus entry inhibitors, especially when combined with strategies to induce immune-mediated killing of infected hepatocytes.

Keywords: Hepatitis B Virus Receptor; Sodium Taurocholate Cotransporting Polypeptide; *SLC10A1*.

Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. Persistently infected people are at high risk for development of cirrhosis and hepatocellular carcinoma.¹ Coinfection or superinfection with hepatitis D virus (HDV), a satellite virus that requires the HBV envelope proteins for dissemination, accelerates and worsens the disease.² Chronic HBV infection is treated with interferon (IFN)- α (or pegylated form) and/or nucleos(t)ide analogues.³ IFN drugs induce a sustained virologic response, in which serum levels of HBV DNA remain <2000 IU/mL after cessation of treatment, in approximately one-third of patients. Nucleos(t)ide analogues suppress viral replication

in most patients. However, long-term therapy is required; even then, virus elimination is rare.⁴

Eradication of HBV and HDV is a challenge. New drugs that target different steps of the viral replication process are required.⁵ Patients coinfecting with HBV and HDV are treated with IFN- α ; however, as for HBV monoinfection, sustained virologic response is rarely achieved. Patients have been treated with a combination of IFN- α and a nucleos(t)ide analogue, but rates of success are disappointing²; the addition of a potent nucleos(t)ide analogue to pegylated IFN- α therapy did not increase the rate of HDV clearance.⁶

Cell culture and animal models of HBV and HDV infection are required to study the replication cycles of these viruses, disease pathogenesis, and the mechanisms of antiviral agents.^{7,8} The recent identification of sodium taurocholate cotransporting polypeptide (NTCP, encoded by *SLC10A1*) as a receptor for HBV infection⁹ should help researchers overcome their dependence on primary hepatocytes and differentiated HepaRG cells and facilitate studies of viral pathogenesis and new antiviral agents. We summarize our knowledge of HBV and HDV entry into hepatocytes and present current *in vitro* and *in vivo* infection systems. We also discuss potential directions of research following the discovery of the HBV and HDV receptor as well as the concept of entry inhibition for the treatment of HBV and HDV infections.

Cell Culture Systems

Systems for culturing primary human hepatocytes (PHH)¹⁰ and hepatocytes from *Tupaia belangeri* (PTH),^{11,12}

Abbreviations used in this paper: aa, amino acids; AGL, antigenic loop; cccDNA, covalently closed circular DNA; cyNTCP, cynomolgus monkey sodium taurocholate cotransporting polypeptide; DMSO, dimethyl sulfoxide; HBIG, hepatitis B immune globulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDV, hepatitis D virus; hNTCP, human sodium taurocholate cotransporting polypeptide; HSPG, heparin sulfate proteoglycan; IFN, interferon; L, large; M, middle; mRNA, messenger RNA; NTCP, sodium taurocholate cotransporting polypeptide; PHH, primary human hepatocytes; PKC, protein kinase C; PTH, primary *Tupaia* hepatocytes; RNP, ribonucleoprotein complex; S, small; SVP, subviral particle; TM, transmembrane; TNF, tumor necrosis factor; uPA, urokinase plasminogen activator.

as well as the hepatic cell line HepaRG,¹³ are used to study infection. These systems support the complete replication cycles of HBV and HDV under conditions that induce and maintain a differentiated state of the cells. The hepatoma cell lines HepG2 and HuH7 are not susceptible to HBV infection but support replication of the viral genome and assembly of infectious virions after transfection of more-than-genome-length plasmids.¹³ Moreover, cell lines with stable integration of HBV (HepG2.2.15 or HepAd38) are useful for studying the late replication steps and in drug screening.^{14,15}

Early attempts to infect PHH revealed donor-dependent differences in infection efficiency.⁹ The medium additives dimethyl sulfoxide (DMSO), hydrocortisone, and polyethylene glycol increase the efficacy and reproducibility of infection.^{9,16} Infection of HepaRG cells, which occurs only when these cells are differentiated, also requires these substances. Incubation of cells with 2% DMSO for 2 weeks renders a subpopulation susceptible to infection with HBV and HDV.^{12,17} It is unclear how DMSO stimulates infection. One effect is induction and sustained expression of NTCP during HepaRG cell differentiation¹⁸ and PHH cultivation.¹² DMSO also increases HBV replication when added to HepG2 cells stably transfected with HBV,¹⁹ indicating that multiple mechanisms are involved. Polyethylene glycol increases binding of HBV and HDV to cells, which is mediated by increased association of virus particles with glycosaminoglycans.²⁰ Constitutive expression of human NTCP (hNTCP) in HepG2 or HuH7 cells makes them susceptible to HBV or HDV infection, extending the repertoire of cell culture systems,²¹ and will allow for high-content screening for antiviral agents or host cell factors that promote or restrict viral replication. However, as for PHH, PTH, and HepaRG cells, efficient infection is only achieved in the presence of DMSO and polyethylene glycol.²²

Animal Models

Chimpanzees are the only immune-competent animal model for HBV infection or HBV and HDV coinfection. They have been used to study viral infectivity and the natural course of HBV infections, especially the immune processes of viral clearance.²³ These studies, along with those performed in woodchucks using woodchuck HBV, have led to complementary concepts of how infected cells are eliminated during acute infection. One pathway involves cytolytic T cell-mediated killing of hepatocytes,²⁴ whereas the other, which is less understood, involves clearance by noncytolytic cells, tumor necrosis factor (TNF)- α , and IFN- γ .²³ Studies of infected chimpanzees were essential for understanding virus-induced immune response and pathogenesis. However, ethical considerations limit detailed analyses. Therefore, immune-competent small animal models are urgently needed.

Although attempts to infect mice and rats with HBV and HDV failed, researchers made the surprising observation that HBV infects tree shrews (*Tupaia belangeri*), resulting in transient viremia, virus clearance, and hepatitis B surface antigen (HBsAg) seroconversion.¹⁰ HBV infection of *Tupaia belangeri* persists when the animals are inoculated soon

after birth²⁵ and progresses via a similar course as in humans. Although *Tupaia belangeri* can be bred in captivity, the lack of immunologic tools for their study is a serious limitation.

It has been possible to study virus replication in an immune-deficient host using chimeric mice. Several models have been developed.²⁶ The most frequently used system takes advantage of immune-deficient urokinase plasminogen activator (uPA-SCID) transgenic mice with xenografts of PHH or PTH cells.^{27,28} Another model takes advantage of mice lacking the gene encoding fumaryl acetoacetate hydrolase (*Fah*), the recombination activating gene 2 (*Rag2*), and the gene encoding the γ chain of interleukin 2 receptor (*Il2r γ*).²⁹ In mice with PHH or PTH cell xenografts, >50% of PHH form clonal islands with accurate cell architecture. HBV infection kinetics in these animals is slow but finally results in up to 100% of infected transplanted cells. HBV titers, as well as HBsAg and hepatitis B e antigen (HBeAg) levels in serum, compare with those of infected patients. uPA-SCID mice with PHH xenografts have been used to study the dynamics of hepatocyte turnover, including the stability of covalently closed circular DNA (cccDNA),³⁰ and the mechanisms of action of drugs.³¹ These mice were used to show the ability of the NTCP inhibitor Myrcludex B to block HBV and HDV entry.³²⁻³⁴

Transfection of hepatoma cells with HBV-encoding plasmids resulted in transcription of viral RNAs, HBV genome replication, and virus production. Likewise, HBV transgenic mice produce virions and have been used to study the immune response to and pathogenesis of HBV infection. However, these mice are immune tolerant and are therefore not an ideal model of infected patients. Moreover, hepatocytes of these mice do not support de novo entry of virions or produce detectable amounts of cccDNA, the template for transcription of viral messenger RNA (mRNA).³⁵ These animals are therefore inappropriate for studying early infection events, the dynamics of virus spread, or mechanisms that affect the regulation and transcriptional activity of cccDNA. One alternative to overcome these limitations is hydrodynamic injection of HBV DNA into mice.³⁶ This results in long-term in vivo transfection of hepatocytes, resulting in viral gene expression and replication. The technique is used to simulate acute infection for investigation of immune responses and evaluation of antiviral drugs.^{36,37} An alternative to hydrodynamic transfer of HBV-encoding plasmids involves transfer of HBV genomes via adenoviral vectors. This simulates acute infection and, when low levels of adenovirus vector are transferred, persistent HBV infection.^{38,39}

Host Factors and Methods to Determine Viral Entry

No high-throughput approaches have been undertaken to identify host factors required for HBV or HDV infections. Host pattern recognition factors recognize HBV, as described in a recent review.⁴⁰ Factors that regulate transcription have been identified and characterized through analyses of specific DNA motifs within the viral cccDNA.⁴¹ Additionally, cellular factors have been identified that

participate in the maturation, assembly, and egress of virions and subviral particles (SVPs).⁴² Until recently, almost nothing was known about host factors involved in early infection events, including the specific receptor(s) or proteins involved in transport of nucleocapsids to the nucleus or enzymes that convert (repair) relaxed circular DNA to cccDNA (for a comprehensive illustration of the HBV replication cycle, see Urban et al⁴³). Identification of these factors is crucial for our understanding of viral replication, the development of small animal models, and the identification of drug targets. Recently developed cell culture systems (notably HepG2^{hNTCP} cells) might be used to identify these factors.²²

The identical envelope protein composition of the genetically dissimilar HBV and HDV particles allows for discrimination between host factors involved in prefusion

and postfusion events of each virus (Figure 1). It is assumed that both viruses enter hepatocytes by similar or even identical mechanisms.⁴⁴ This assumption is based on the finding that HBV and HDV use the same receptor^{21,22} and are sensitive to peptide entry inhibitors, suramin, or neutralizing antibodies.⁴⁵ However, on release into the cytoplasm, HBV nucleocapsids or the HDV-ribonucleoprotein complex (RNP) follow different paths and require different host factors. HDV is less restricted by liver-specific postentry factors than HBV and even replicates in nonhepatoma cells from nonhuman species.⁴⁶ Therefore, during early stages of infection, both viruses use the same host factors,^{20,47-49} whereas in later events, viruses use different host factors.

Viral Determinants of Cell Entry

HBV is a small enveloped virus. It contains a partially double-stranded DNA genome of approximately 3.2 kilobases, which is packaged into an icosahedral nucleocapsid.⁵⁰ In contrast to large enveloped viruses, the mature nucleocapsid of HBV induces an ordered arrangement of the envelope proteins through defined interactions among proteins during envelopment (Figure 1A).⁵¹ The in-frame coded proteins incorporated into the virus shell are called the large (L), middle (M), and small (S) envelope proteins (Figure 2B).

One peculiarity of HBV is the ability of the envelope proteins to form noninfectious, spherical and filamentous SVPs with a diameter of 22 nm; these are the major constituents of the clinically relevant HBsAg (Figure 1B and C). In the presence of nucleocapsids, specific interaction with the L protein results in envelopment and release of mature virions.⁵² Virions and SVPs contain different ratios of L, M, and S proteins, with the highest L protein content found in the virus particle. L, M, and S proteins share the C-terminal S domain, which contains 4 putative membrane-spanning helices. The 2 N-terminal extensions (preS2 and preS1/2) of M and L proteins have diverse functions, most importantly binding of the nucleocapsid during envelopment⁵² and receptor binding during entry.⁵³⁻⁵⁹ The L protein is not synthesized with a signal sequence but is inserted into the membrane via the transmembrane (TM)-1 domain. Thus, the preS1 domain initially faces the cytoplasm and resides in the interior of the virus particle. The very N-terminus of the preS1 domain is modified by myristic acid.⁶⁰ Subsequently, the preS1 domain undergoes a complex posttranslational translocation process,⁶¹ resulting in its exposure on the virion surface.⁵²

The envelope of HDV is structurally related to that of HBV, as HDV exploits the HBV surface proteins for RNP envelopment and particle release.⁴⁶ In contrast to HBV, the RNP complex of HDV contacts the cytosolic loop of the S domain⁶² and not the capsid binding site within preS (Figure 2). This results in weaker contacts between the envelope and the RNP and can allow for the formation of infectious (L protein containing) and noninfectious (only S protein containing) HDV particles (Figure 1D and E). Productive HDV propagation therefore requires synthesis

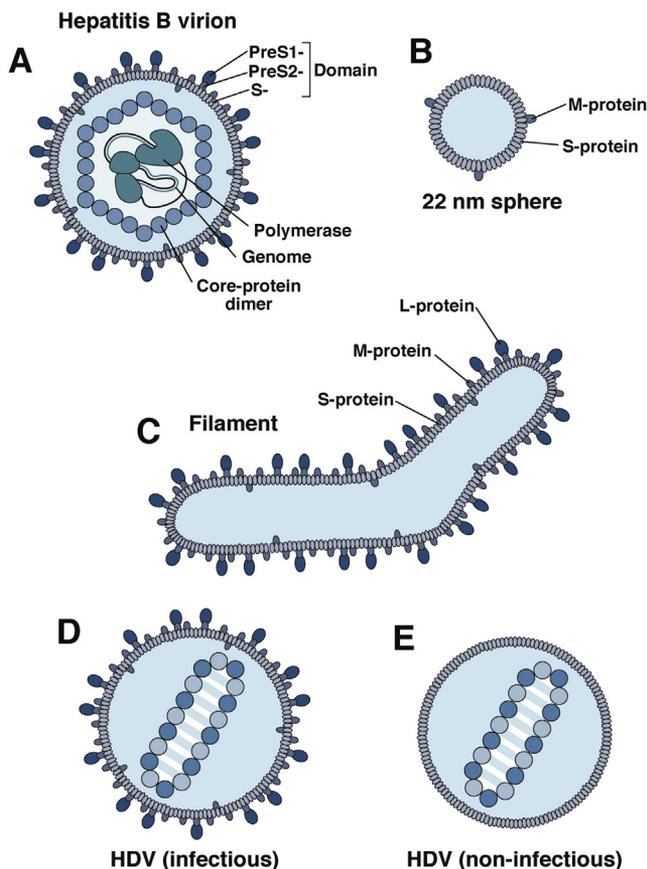


Figure 1. HBV, HDV, and empty SVPs. (A) HBV virion with a DNA-containing nucleocapsid, which is enclosed in the compactly packed protein envelope comprising the L, M, and S surface protein. The preS1, preS2, and S domains are indicated. The nucleocapsid incorporates the viral polymerase which is covalently attached to the partially double-stranded DNA genome. The capsid is built of 240 core protein dimers in an icosahedral arrangement. (B) Spherical 22 nm SVP consisting of mostly S and M protein. (C) Filamentous SVP comprising L, M, and S proteins. SVPs are produced in 1000-fold to 10,000-fold excess during viral replication. (D) Infectious HDV particle that includes the L, M, and S proteins of the HBV envelope, which enclose the HDV RNP. (E) A noninfectious HDV particle that contains only the S protein in its envelope.

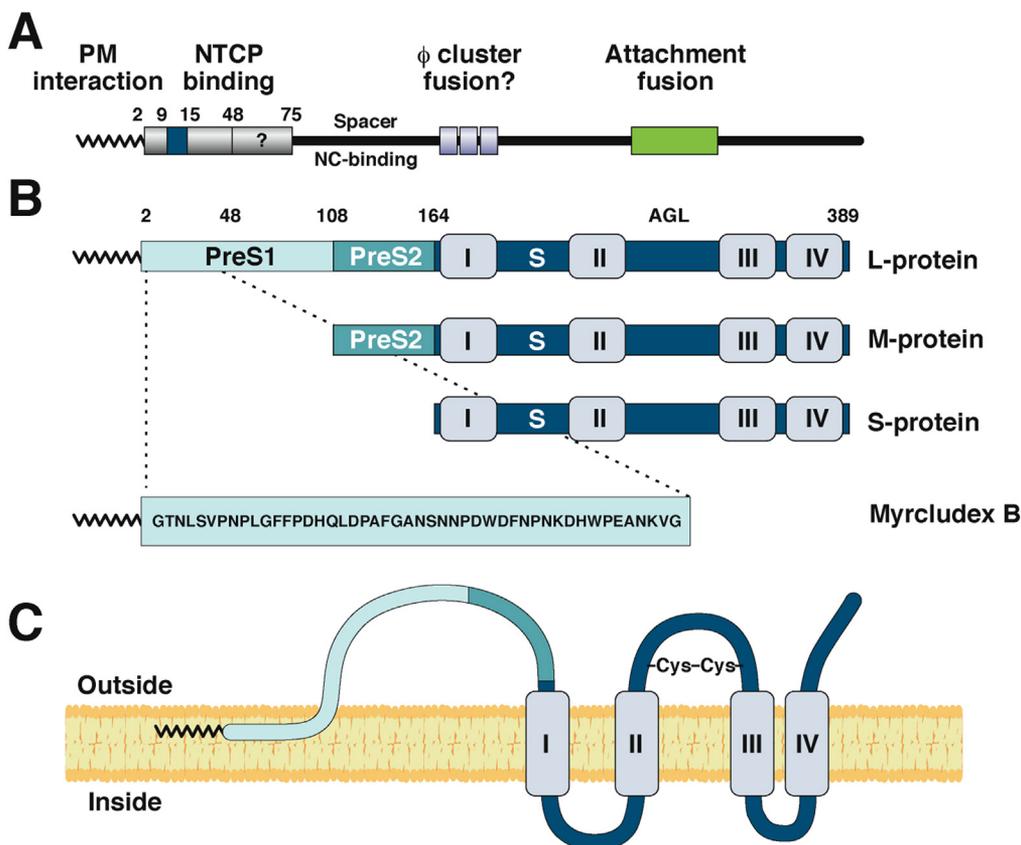


Figure 2. Determinants of infectivity and domain structure of HBV surface proteins. (A) Localization of the infectivity determinants in the HBV envelope proteins. N-terminal myristoylation of L protein is required for plasma membrane association (PM-interaction). The preS1 sequence (2–48) specifically interacts with NTCP. Within this sequence, a highly conserved motif (9-NPLGF(F/L)P-15) is crucial for binding. Furthermore, aa 49–75 are also required for infection. The precise function of this sequence is not known but may also target NTCP. C-terminal to the NTCP binding site is a spacer region that contains the nucleocapsid binding sequence required for envelopment of HBV. The first TM helix of the S domain contains 3 short hydrophobic (ϕ) clusters, which are presumably involved in membrane fusion. A complex disulfide-bridged conformation in the AGL of the S domain is required for HBV and HDV infection. This part probably contributes to HSPG binding and membrane fusion. (B) The 3 HBV envelope proteins (L, M, and S protein) share the S domain with 4 putative TM domains (I–IV). The M protein contains, in addition to -domain S, a hydrophilic N-terminal extension of 55 aa called the preS2 domain. The L protein contains an additional 107 aa (preS1) that becomes myristoylated at the N-terminus, at glycine 2. A peptide that mimics the myristoylated N-terminal 47 aa of the HBV L protein (Myrcludex B) inhibits HBV by inactivating the receptor function of NTCP. (C) Proposed topology of the HBV L protein in infectious HBV and HDV particles with the preS domain facing outside the virion. Note that the myristoylated receptor binding site within the preS1 domain is presumably buried in the viral membrane. The disulfide-linked cysteine moieties, as a part of the AGL, are required for infectivity.

of HBV surface protein in the same cell. HDV can enter hepatocytes *de novo* in the absence of HBV and initiate efficient HDV RNA replication and expression of δ Ag. However, no secretion of infectious particles occurs. The self-complementary rod-like HDV genome replicates via a rolling circle mechanism using the host DNA-dependent RNA polymerase II.⁴⁶ Accordingly, none of the clinically approved polymerase inhibitors interfere with HDV genome replication *in vitro*, which is reflected in the ineffectiveness of treating HDV-infected patients with nucleoside analogues.^{6,63,64} Considering the similarities and differences between the 2 viruses, therapeutic strategies for HDV and HBV coinfecting patients should aim at common early infection events, such as receptor interference.

Determinants of Infectivity in Envelope Proteins

Using PHHs, 2 groups showed that N-terminal myristoylation of the HBV L protein is essential for virus infectivity.^{65,66} Because myristoylation also occurs in the L proteins of woodchuck HBV and duck HBV,⁶⁷ this modification has a general role in hepadnaviral infection, presumably by increasing receptor binding.⁶⁸ In a systematic approach, LeSeyec et al⁶⁹ mapped functionally important sites in the preS1 and preS2 domains of the L protein using HBV genotype D. Consecutive deletions of 5 amino acids (aa) within the N-terminal 77 aa of the L protein (genotype D) blocked infectivity. Conversely, deletions within the C-terminal of this region (aa 78–87)⁵⁸ and within the preS2 domain (aa 114–163) did not affect infectivity.⁶⁹ Mutations affecting aa 88–108 of preS1 and the first 5 aa of preS2 are

involved in capsid binding (Figure 2) and interfere with HBV assembly, so functional characterization of this sequence for HBV infectivity is impossible. The use of HDV as a surrogate model verified the importance of the N-terminal 75 aa of preS1 and ruled out a contribution of the nucleocapsid binding site and a proposed translocation motif⁷⁰ within the preS2 domain for infection.⁷¹⁻⁷³ Finally, it was shown that M protein-deficient HBV with a randomized preS2 sequence within the L protein is infectious.⁷⁴ The failure to functionally separate myristoylation from the required preS1 sequences provides evidence that that these infectivity determinants act in a concerted manner.⁷⁵

A second determinant of infectivity is located in the antigenic loop (AGL) of the S domain between TM-II and TM-III. Mutation analysis revealed that Gly-119, Pro-120, Cys-121, Arg-122, and Cys-124 are required for infectivity.⁷⁶ Moreover, Cys-121 and Cys-149 are involved in the formation of a disulfide network, which upholds a distinct AGL conformation.⁷⁷ Interestingly, incubation of HDV particles with membrane-impermeable reducing agents resulted in a loss of infectivity. This indicates that reduction of disulfide bridges during viral entry might be important for triggering a postbinding step. A plausible hypothesis is that the disulfide bridge keeps the S domain in a spring-loaded, pre-fusion state. Such a mechanism has been proposed for duck HBV.⁷⁸ Within the AGL, a second region (aa 137-148) is crucial for protective neutralization by vaccine-induced antibodies. Recent reports indicate vaccine escape mutation within that part of the S domain.⁷⁹⁻⁸¹ A third region comprising 3 hydrophobic (φ) clusters located in the TM1 domain of the L protein has been identified.⁸² Its role for infectivity is restricted to the L protein (Figure 2A). The same domain is essential for duck HBV, which is involved in membrane fusion.⁸³ It is not clear how fusion of the viral and cellular membranes occurs.

Besides these envelope protein determinants, the lipid composition of the viral membrane is crucial for infectivity. Although depletion of cholesterol from cellular membranes with methyl- β -cyclodextrin has little effect on infection, depletion from the virus membrane strongly reduces infectivity in a reversible manner.⁸⁴ This indicates a raft-independent localization of the virus receptor and suggests that a defined membrane association of HBV envelope proteins is required for productive entry.

The importance of the preS1 domain and the AGL for virus entry into hepatocytes is also reflected by the neutralizing activity of antibodies against epitopes within the preS1 domain and the AGL of the S domain.^{55,85,86} In contrast, most antibodies directed against the preS2 domain are not neutralizing. Consistent with the requirement of a highly structured arrangement of the AGL, only conformational S antibodies have neutralizing activity.⁸⁵ Antibodies against epitopes within the preS1 domain also display strong neutralizing activity because they bind the virion-enriched L protein, thereby preventing receptor interaction. AGL-specific antibodies are used in hepatitis B immune globulin (HBIG) to prevent HBV infection in scenarios such as during liver transplantation.

HBV preS-Derived Peptides Block Infection by Receptor Interference

Evidence that the preS1 domain of the L protein is involved in recognition of a hepatocyte-specific receptor came from the observation that a myristoylated peptide, the preS1 infectivity determinant, comprising aa 2-78 (LeSeyec et al⁵⁸), inhibited HBV infection of HepaRG cells and PHH.¹² Consistent with this observation, L protein-containing SVP bound specifically to PTH.⁵⁵ Because the synthetic lipopeptides inhibit HBV and HDV infection when administered to cells before virus inoculation, direct inactivation of a receptor was proposed.⁵⁷ Fatty acid moiety analyses of PHH and HepaRG cells revealed that myristic acid at the N-terminus of the HBV preS1 peptide can be replaced by other fatty acids or even cholesterol.^{56,57}

Mutational sequence analyses revealed the requirement for aa 9-NPLGF(F/L)P-15.^{53,54,56,87} This motif, located at slightly variable positions within the N-terminus of the preS1 domain of the 7 HBV genotypes (Glebe et al⁵⁶), is conserved among human and primate hepadnaviruses, which infect woolly monkeys, orangutans, gorillas, chimpanzees,^{88,89} and even bats,⁹⁰ the only variation being a Phe-Leu polymorphism at position 14. Accordingly, preS lipopeptides derived from woolly monkey HBV⁵⁷ and bat HBV L protein⁹⁰ interfere with infection by HBV and HDV, so hepadnaviruses that encode this motif must target homologous receptors. Remarkably, the aa that are C-terminal and N-terminal to this essential receptor binding site contribute, in a complex manner, to the inhibitory activity, with 2 parts increasing (aa 2-8 and 18-48) and 1 part decreasing (aa 49-78) inhibitory activities.^{56,57} The most powerful inhibitor (aa 2-48) was derived from a consensus sequence (Figure 2B) and has a median inhibitory concentration of ~80 pmol/L. This peptide, called Myrcludex B, is being tested in phase 2 trials.

Preclinical Studies

Interference with virus entry protects naive hepatocytes against the initiation of infection and the spreading virus during infection. In persistent infections, long-term inhibition of viral entry may result in clearance of virus during natural or immune-mediated turnover of infected cells. Entry inhibition is clinically important for the prevention of infection in the course of postexposure prophylaxis, organ transplantation, reactivation after therapeutic immunosuppression, or perinatal transfer of virus from infected mothers to children.

HBIGs have been shown to prevent infection by HBV and HDV. In patients undergoing liver transplantation, administration of HBIGs during and after organ transplantation reduces reinfection of grafts from 100% to 20%-30%.⁹¹ HBIGs are also effective in patients with HBV and HDV coinfection.⁹² Combined administration with nucleos(t)ide analogues in HBV-infected patients reduces the proportion of reinfected organs to <5%. Moreover, the use of nucleos(t)ide analogues with a high barrier to resistance allows for a reduced duration of HBIG treatment after transplantation for HBV-infected patients.⁹³ HBIGs can also

prevent vertical transmission (from infected mothers to newborn infants) together with the HBV vaccine. However, prophylaxis is not effective for neonates of highly viremic mothers, indicating that protective neutralization depends on the virus dose. Therapy with nucleos(t)ide analogues during the last trimester of pregnancy reduces viral load, improving prophylaxis with the combination of HBIGs and the vaccine.^{94,95} However, these approaches are not effective against variants of virus with mutations in the AGL of the S domain, which escape HBIG recognition.^{96,97} These mutants can infect liver grafts and neonates despite prophylaxis.⁹⁸ Clinical trials of monoclonal antibodies against the S protein reported increased rates of virus suppression and HBsAg clearance. However, these trials were conducted at a time when the most potent nucleos(t)ide analogues were not yet available and were not continued.^{99,100}

Myrcludex B and other related HBV preS-derived lipopeptides were tested in uPA-SCID mice carrying PHH or PTH cells before infection.³⁴ Myrcludex B was also administered after infection and found to block virus spread in the liver.³³ These findings indicate that HBV spreads throughout the liver by an HBV receptor-dependent, Myrcludex B-sensitive pathway. Myrcludex B also blocked HDV infection in mice, making it the first selective drug against HDV.³²

Pharmacokinetic studies of Myrcludex B in different species showed its specific accumulation in the liver of even non-HBV-susceptible animals, such as mice, rats, and dogs.¹⁰¹ Peptides with mutations in the essential receptor binding site (9-NPLGF(F/L)P-15) do not accumulate in the liver, indicating the presence of a binding-competent HBV preS receptor in these animals. Surprisingly, Myrcludex B disperses to multiple organs in cynomolgus monkeys. Although they are more closely related to humans than other models, livers of cynomolgus monkeys do not have binding-competent HBV preS receptors.¹⁰¹ Researchers recently reported low-level HBV infection of macaques from Mauritius Island.¹⁰² These monkeys are closely related to cynomolgus monkeys, so it will be interesting to investigate receptor use in these animals. In vitro studies of primary hepatocytes from these different species confirmed the in vivo results.⁵⁹ Furthermore, the studies showed a selective binding of Myrcludex B to a basolaterally sorted receptor of only differentiated primary hepatocytes and HepaRG cells but not HepG2 and HuH7 cells. The receptor-Myrcludex B complex undergoes slow endocytosis, is linked to the actin cytoskeleton, and shows little lateral movement within the membrane. Myrcludex B binding requires myristoylation and the integrity of the NPLGF(F/L)P motif.⁵⁹ Remarkably, binding saturates at concentrations higher than those required to inhibit infection, indicating a dominant inhibitory effect. Myrcludex B did not produce any adverse effects in short-term and long-term toxicity studies of rats and dogs. Pharmacokinetic studies in 3 chimpanzees verified the hepatotropism of the peptide and the tolerability in a species closely related to humans (S. Urban, unpublished data, February 2012).

Researchers previously attempted to inhibit viral entry with suramin, which inhibits binding to heparan sulfate proteoglycans (HSPGs) on the membrane of hepatocytes and

blocks entry of HBV or HDV in vitro.^{20,47,48,103} Moreover, binding of synthetic antilipopolysaccharide peptides to heparin sulfate moieties inhibits infection with a variety of enveloped viruses, including human immunodeficiency virus, herpes simplex virus, hepatitis C virus, and HBV.¹⁰⁴

Host Determinants of Viral Entry

Researchers searched for the HBV receptor through biochemical analyses using the virus itself, SVPs, and recombinant surface protein fragments and peptides. PHH and hepatic cell lines were used as sources for receptor identification. More than 20 candidates that interact with L, M, or S protein have been described.¹⁰⁵ However, none of them fulfilled the criteria for a functional receptor, which mediates specific binding of virus and makes cells susceptible to HBV infection.

Studies of HepaRG cells, PHH, and PTH found that heparin and highly sulfated polysaccharides interfere with HBV^{20,48} and HDV infection.^{47,49} HBV and HDV infection are sensitive to heparinases or sodium chlorate, indicating that the viruses use HSPGs as attachment sites. Arg-122 and Lys-141 of the AGL are involved in HDV particle binding to HSPGs.⁴⁹ In addition, the preS1 domain has an important role and mediates selectivity for virions.²⁰ HSPGs, which are enriched in the space of Disse, are apparently required for close approximation of the virus before specific receptor engagement.

In identifying NTCP as the receptor for HBV and HDV, Yan et al²¹ took advantage of HBV preS-derived lipopeptides, cross-linking membrane proteins from PTH to the HBV preS receptor binding site¹⁰⁶ defined in infection inhibition assays.⁸⁷ They showed that expression of hNTCP in HEK293T cells allowed them to bind a Myrcludex B-derived peptide but not a mutant with an Asn-9-Lys exchange within the 9-NPLGF(F/L)P-15 motif. Knockdown of hNTCP with small hairpin RNAs significantly reduced infection of HepaRG cells by HBV and HDV, whereas constitutive expression of hNTCP in HepG2 and HuH7 cells made them susceptible to infection by these viruses. The investigators showed that NTCP from cynomolgus monkeys (cyNTCP) does not bind preS-derived lipopeptides, and its expression in HepG2 cells does not allow them to become infected. This is due to a sequence difference at aa 157–165 of cyNTCP. Schieck et al¹⁰¹ and Meier et al⁵⁹ showed that Myrcludex B cannot bind to hepatocytes from cynomolgus monkeys and is not targeted to the liver of this species.

Using a different approach, Ni et al²² took advantage of the observation that DMSO induces HepaRG cell differentiation and expression of the HBV receptor.⁵⁹ Comparing the expression profiles of undifferentiated and differentiated HepaRG cells and using stringent selection criteria (exclusive basolateral expression, slow endocytosis, and no lateral movement), they found hNTCP to be the most strongly induced membrane protein. They confirmed the findings of Yan et al,²¹ showing that hNTCP-expressing HepG2 and HuH7 cells support high-level infection by HBV and HDV when cultured in the presence of DMSO. A further increase of DMSO to 2.5% to 3.0% increased the proportion of

infected HepG2^{hNTCP} cells to more than 80%, a prerequisite for analyses based on bulk measurements.²² This effect was verified by another research group.¹⁰⁷

The identification of a sequence in cyNTCP that was required for HBV and HDV binding led to studies to determine whether other sequences in hNTCP versus mouse NTCP are required for infection. Mouse and human chimeric NTCPs were generated and analyzed for their ability to bind HBV and HDV and promote infection.^{22,108} As expected from in vitro and in vivo binding data,^{59,101} mouse NTCP bound HBV preS lipopeptides but did not make cells susceptible to infection with HDV or HBV. Analysis of mouse NTCP/hNTCP chimeras revealed that replacement of aa 84–87 of mouse NTCP with the human sequence allowed for HBV entry. Remarkably, expression of this chimera in CHO cells (non-hepatocytes) allowed for HDV infection.¹⁰⁸

These findings indicate that the species specificity of HBV is determined primarily by recognition of the NTCP receptor. At least 2 different sites are involved: one required for HBV preS binding (aa 157–165) and one involved in a postbinding event (aa 84–87). hNTCP allows for even non-hepatic, nonhuman cells to be infected with HDV, so it is unlikely that host- and liver-specific coreceptors are required for entry by this virus. However, HBV does not infect these cells, indicating that additional factors are required for infection of human cells with this virus.

NTCP

NTCP participates in the enterohepatic circulation of bile salts (Figure 3) and localizes to the sinusoidal/basolateral membrane of hepatocytes. Its expression is approximately 50% lower in female than male rats,¹⁰⁹ possibly due to transcriptional regulation by estrogens. No sex differences in expression have been observed in humans.¹¹⁰ The higher incidence of HBV in men is probably not a result of sex differences in expression of NTCP. Major substrates of NTCP are conjugated bile salts such as taurocholate, which is transported with a K_m of $\sim 6 \mu\text{mol/L}$.¹¹¹ Substances such as cyclosporin A, ouabain, vecuronium, pregnenolone sulfates, bumetanide, irbesartan, and ezetimibe^{112–114} inhibit NTCP-mediated transport of bile salts, whereas statins and the antimycotic drug micafungin^{115,116} are transported by NTCP. Bupivacaine, lidocaine, and quinidine increase the activity of NTCP without being transported.¹¹⁷ Etezimib¹¹⁸ and cyclosporin A^{119,120} interfere with HBV entry, possibly by blocking NTCP receptor function. Asp-115, Glu-257, and Cys-266 in NTCP are conserved and required for its transport activity¹²¹; they are proposed binding sites for sodium ions and bile salts, which are presumably transported in a 2:1 stoichiometry.¹²² A recent investigation indicates that aa residues, which are crucial for HBV and HDV entry, overlap with that for bile salt uptake by NTCP.¹²³

Regulation of NTCP Expression

Regulation of NTCP expression has mainly been studied in rodents, although mechanisms of transcriptional regulation in humans differ considerably. In human liver

sections, down-regulation of NTCP correlated with increased levels of TNF- α and interleukin 1 β after exposure to lipopolysaccharide.¹²⁴ In line with this observation, interleukin β ¹²⁵ or TNF- α and interleukin 6¹²⁶ reduced expression of NTCP in PHH. However, neither hepatocyte nuclear factor 1 α nor the retinoid X receptor–retinoic acid receptor heterodimer or small heterodimer partner 1 (SHP1, encoded by *PTPN6*) activated the promoter region of human *SLC10A1*¹²⁷ (which encodes NTCP), as expected from rat studies. Instead, a glucocorticoid response element was identified in the *SLC10A1* promoter,¹²⁸ which may contribute to the reactivation of HBV in glucocorticoid-treated patients.

Uptake of bile salts by NTCP increases their intracellular concentrations and leads to activation of the farnesoid X receptor. Farnesoid X receptor activates expression of SHP1, which reduces levels of *SLC10A1* mRNA and interferes with the glucocorticoid response element. These receptors have also been shown to be involved in HBV biosynthesis.¹²⁹ On the other hand, the peroxisome proliferator-activated receptor γ coactivator 1 α , an important integrator of external stimuli such as oxidative stress, low temperature, or exercise endurance, increases the effect of glucocorticoids on the *SLC10A1* promoter.¹²⁸ In healthy human liver, levels of *SLC10A1* mRNA vary approximately 40-fold.¹¹⁷ Livers of human fetuses at 14 to 20 weeks of gestation have <2% of the relative level of *SLC10A1* mRNA compared with adult liver.¹³⁰

Levels of NTCP in fetal liver might determine the risk of mother-to-infant transmission of HBV; vertical transmission accounts for almost 50% of new HBV infections. Livers of patients with inflammation-induced cholestasis, advanced primary biliary cirrhosis,¹³¹ progressive familial intrahepatic cholestasis,¹³² or biliary atresia¹³³ have reduced levels of NTCP,^{124,134} whereas livers of obese patients with nonalcoholic fatty liver disease or nonalcoholic steatohepatitis have up-regulated NTCP.¹³⁵ It is not clear how changes in NTCP expression under these conditions affect the course of HBV infection. Compared with healthy liver tissue, human hepatocellular carcinoma cells have significant reductions of *SLC10A1* mRNA and NTCP protein. NTCP was not detected on the membranes of hepatocellular carcinoma cells,¹³⁶ so it cannot be used as a direct therapeutic target molecule to address transformed hepatocytes. However, the exclusive expression in differentiated hepatocytes makes it a promising candidate for liver-specific drug targeting and liver imaging using HBV preS-derived peptides.^{101,137}

Intracellular Trafficking of NTCP

Understanding the intracellular trafficking pathways of NTCP will provide important insights into the cellular processes of HBV entry. NTCP is sorted in the basolateral membrane of hepatocytes. Its retrieval from intracellular pools regulates plasma membrane levels, a process that is inhibited by nocodazole and involves microtubules. In addition, insertion of NTCP into the plasma membrane is cytochalasin D sensitive, indicating the requirement of

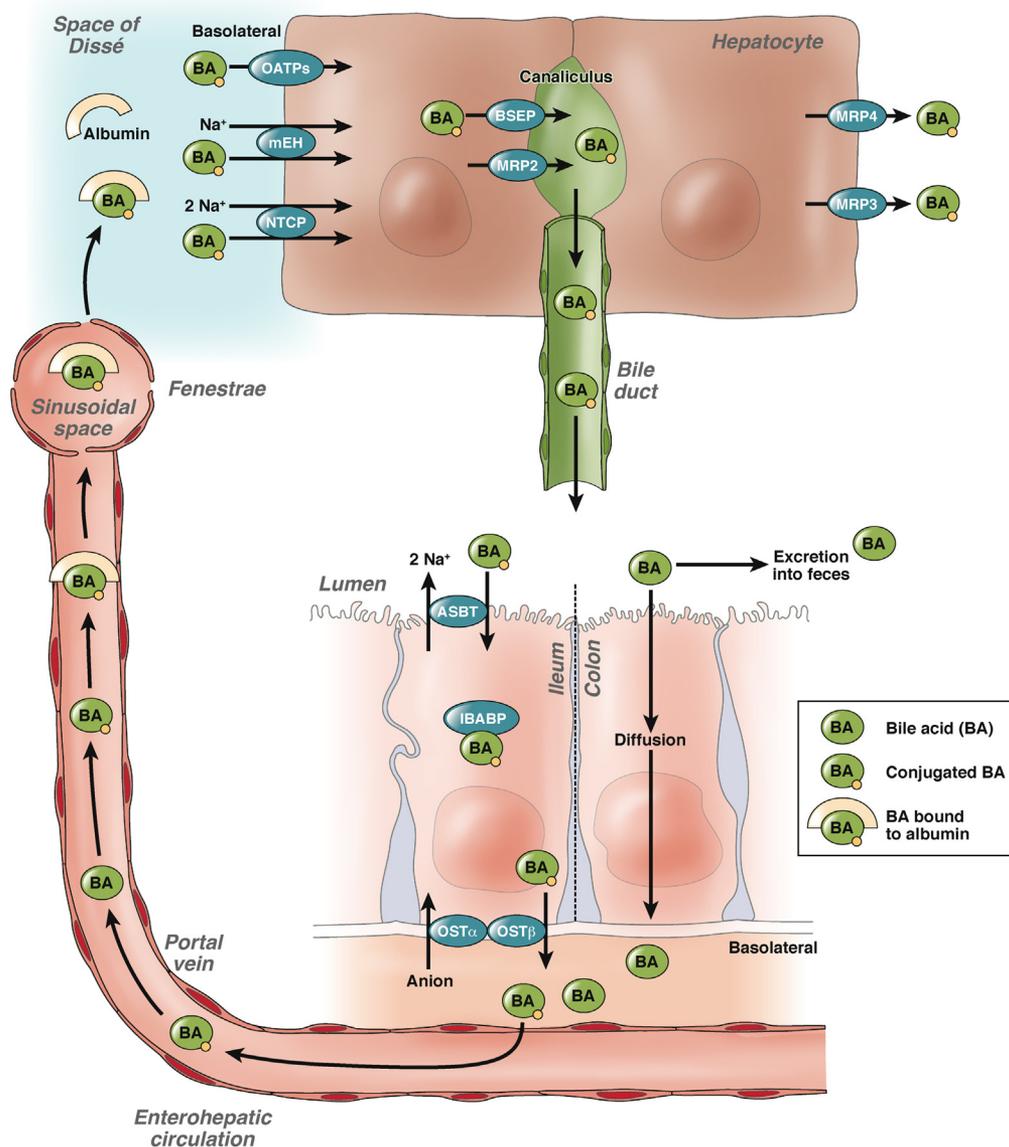


Figure 3. Enterohepatic circulation of bile acids. Primary bile acids are synthesized within hepatocytes and conjugated to glycine or taurine. They are secreted into bile canaliculi by the BSEP or the multi-drug resistance associated protein 2 (MRP2). Bile acids reach the small bowel via the bile duct. More than 90% of bile acids are reabsorbed within the terminal ileum by the apical sodium-dependent bile acid transporter (ASBT). At the basolateral membrane of ileocytes, bile acids are secreted into the circulation by the organic solute transporters (OST) α and β . On the way back to the liver via the portal vein, bile acids are partially bound to albumin. Bile acids reenter the hepatocytes mainly via NTCP and organic anion-transporting polypeptides (OATPs). As part of an overflow mechanism, bile acids may be resecreted from hepatocytes into the blood by the multi-drug resistance associated proteins 3 and 4 (MRP3 and 4). Bile acids that have not been reabsorbed by the terminal ileum enter the colon, where they are dehydroxylated and deconjugated by intestinal bacteria. Unconjugated bile acid may be reabsorbed via diffusion and enter the portal circulation.

microfilaments.^{138,139} On exposure to adenosine 3',5'-cyclic monophosphate, NTCP in the plasma membrane is dephosphorylated at Ser-226,¹⁴⁰ which requires phosphatidylinositol 3-kinase¹⁴¹ and protein kinase C (PKC)- ζ .¹⁴² NTCP is found in early and recycling endosomes and colocalizes with Rab4, Rab11, and transferrin receptor. These endosomes move along microtubules, driven by kinesin 1 and dynein.¹⁴³ NTCP was detected in early but not in late endosomes.¹⁴³ However, when PKC isoforms are activated with a phorbol ester, NTCP becomes degraded

within lysosomes,¹⁴⁴ so it must pass the late endosome compartment. Stimulation of Ca^{2+} -dependent PKC- α induces endocytosis of NTCP.¹⁴⁵ Likewise, taurochenodeoxycholate, but not taurocholate, induces NTCP retrieval in vivo in a PKC- and protein phosphatase 2B-dependent manner.¹⁴⁶ In rats, endocytosis depends on a dileucine motif at position 221 and 222, which is in close vicinity to Ser-226. Alanine substitution of Ser-226 or Thr-225 reduces PKC-dependent endocytosis and trafficking from the Golgi to the plasma membrane, indicating combined effects with

the dileucine motif.¹⁴⁴ In hNTCP, the corresponding region comprises 2 leucines followed by an isoleucine. In black subjects, a genetic variant (p.I223T) was detected with an allele frequency of 5.5%,¹⁴⁷ resulting in reduced surface expression and a defect in bile salt transport. Other NTCP single nucleotide polymorphisms such as p.S267F (c.800C>T), p.I279T (c.836T>C), and p.K314E (c.940A>G) have been identified.^{147,148} In American subjects of Chinese descent and Vietnamese subjects, the p.S267F variant was found at an allele frequency of approximately 7.5% and 9.2%, respectively.¹⁴⁸ This single nucleotide polymorphism has reduced ability for bile salt transport and is defective in HBV receptor function¹²³ (Yi Ni and Stephan Urban, unpublished data, February 2013). It will be interesting to analyze this and other single nucleotide polymorphisms and relate their prevalence with HBV infection.

NTCP Structure and Interaction With the HBV Surface Protein

Structural information on NTCP is based on topological analyses with antibodies, the localization of N- and C-terminal fusion tags, and model predictions based on comparison with other bile salt transporters. Using the

9-TM NTCP model predicted from the ASBT homologue from *Neisseria meningitidis* crystalized in an inward bound state (Figure 4), the crucial preS-binding region (aa 157–165, green) locates within helix 5 in the outer leaflet of the membrane at one side of the NTCP molecule. The second site, which is important for discrimination between mouse and NTCP, maps to the loop between TM-II and TM-III (red). The interaction between NTCP and HBV is complex and involves several contact sides within the receptor molecule. HBV preS lipopeptides of varying length differ substantially in both their median inhibitory concentrations and their binding affinity to NTCP, so it is likely that all N-terminal 75 aa of the preS1 domain participate in NTCP interactions to form a complex that tightly connects the viral and cellular membranes. Interestingly, it has been shown previously that NTCP forms oligomers.¹⁴⁹ Because enveloped viruses generally depend on the formation of trimeric fusion complexes, it will be interesting to see whether oligomerization of NTCP is required to promote HBV/HDV entry. Recently, the crystal structures of ASBT from *Yersinia frederiksenii* in the inward and outward oriented state were solved. These structures indicate that bile salt transport is coupled to a conformational change in the transporter

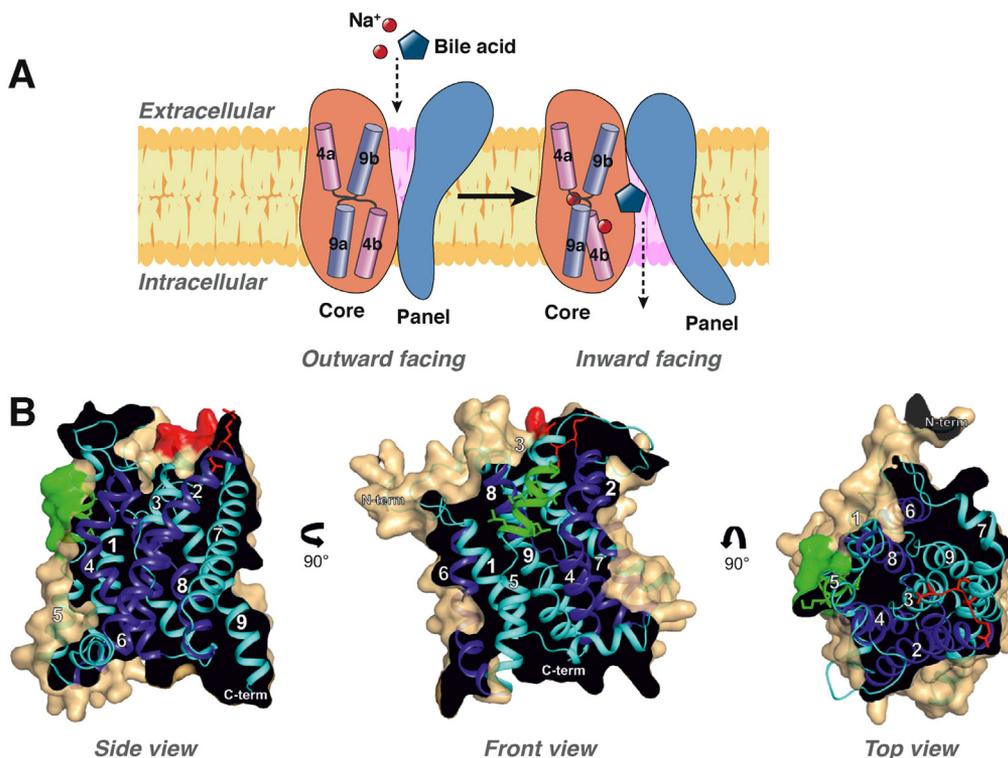


Figure 4. NTCP and NTCP-mediated bile salt transport and HBV preS binding sites. NTCP is a multi-pass TM protein. (A) Sodium-dependent transport of bile acids through the plasma membrane. In the outward-facing conformation, 2 sodium ions and 1 conjugate bile salt molecule bind an accessible pocket in the active sites of NTCP. The sodium ion promotes a conformational change that induces transmembrane transport and cytoplasmic release of the bile salt substrate. (B) 9TM-3D model of NTCP based on the crystal structure of ASBT from *Neisseria meningitidis*. Side view (left panel), front view (middle panel), and top view (right panel) of NTCP. The localization of the essential site for Myrcludex B binding (aa 157–165) is in green and locates within helix 5 at the side of the molecule. The region that differs between mouse and human NTCP (aa 84–87), allowing human but not mouse NTCP to serve as an HBV receptor, is in red and is in a loop between TM-II and TM-III at the outward (sinusoidal) side of the molecule.

promoted by the rotation of 2 intramembranous α -helices.¹⁵⁰ Because it is likely that NTCP-mediated bile salt transport proceeds by a similar mechanism, it will be interesting to study whether this conformational change, driven by the sodium gradient across the membranes, is required to promote infection.

Clinical Implications

Although there are many potential clinical applications of entry inhibitors and/or NTCP blockers, the following considerations are only concepts, which require preclinical and clinical studies. Inhibition of HBV and HDV entry with molecules that target NTCP could be a new prophylactic and therapeutic strategy, as summarized in Figure 5. These inhibitors could be an important alternative to HBIGs for prevention of viral recurrence during immunosuppressive therapies or after liver transplantation, in cases of accidental nosocomial infection, or to stop vertical transmission. They would be especially useful in highly endemic areas, where

effective and cheap prophylaxis is needed most. For patients with chronic viral infection, entry inhibitors could speed HBsAg clearance and provide the opportunity for treatment with a finite duration. Current treatments for chronic HDV infection provide low rates of HDV clearance, so new options are necessary.

Preventing Transmission

Entry inhibitors might replace HBIGs, which are expensive and not regularly supplied to some parts of the world, or could be administered with the HBV vaccine for people exposed to HBV-infected fluids. To prevent vertical transmission, entry inhibitors might be administered to neonates soon after birth together with the HBV vaccine; in mothers with high levels of HBV viremia, administration of nucleos(t)ide analogues might still be necessary during the third trimester of pregnancy. New strategies to prevent viral recurrence in transplanted organs might be tested with the administration of nucleos(t)ide analogues, before and transplantation, and entry inhibitors instead of HBIG right

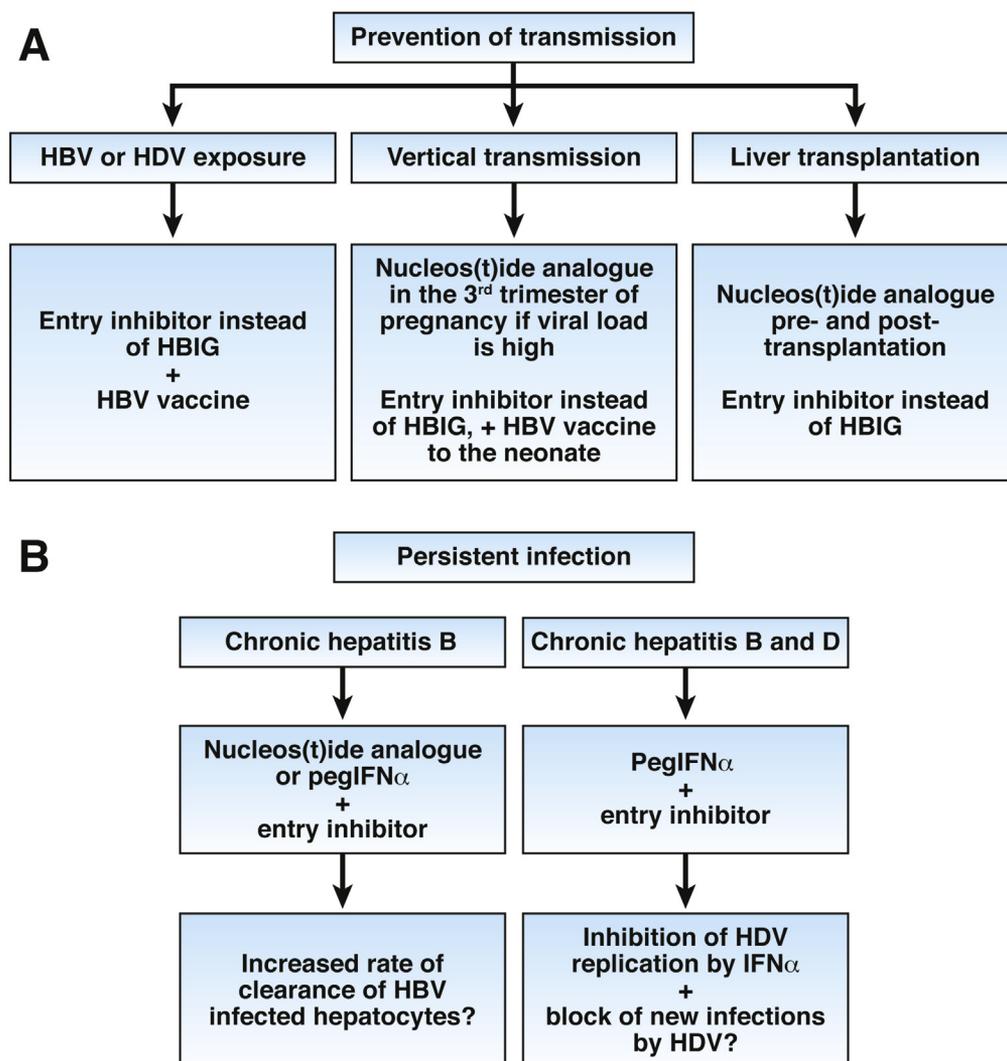


Figure 5. (A) Prophylactic and (B) therapeutic strategies for possible future combination strategies of entry inhibitors in combination with approved drugs.

after transplantation. In livers transplanted in patients with HBV and HDV coinfection, HDV was shown to establish latent, asymptomatic infections without the apparent assistance of active HBV replication. Entry inhibitors might therefore prevent recurrence of HBV infection in the graft.¹⁵¹

Treatment of Chronic Infection

As discussed earlier, current treatments for chronic HBV infection suppress viral replication but rarely result in viral clearance, loss of HBsAg, or even HBsAg seroconversion. New, more effective strategies are required, which might involve combinations of approved drugs or the development of novel agents that target thus far unaddressed steps of viral replication.

The mechanisms involved in viral clearance during natural infection include T cell-mediated killing of infected hepatocytes, noncytolytic clearance of cccDNA by unknown mechanisms, and hepatocyte turnover and replacement by noninfected cells. Blocking the spread of HBV to naïve cells could accelerate the process of virus elimination. Along with nucleos(t)ide analogue-based therapy, entry inhibitors might neutralize residual viruses that are still produced, sometimes below the detection limit of polymerase chain reaction-based assays.

In chronically infected patients, entry inhibition will terminate de novo infections of naïve hepatocytes and those that arise during hepatocyte turnover. The degree of virus elimination by entry inhibition will therefore depend on the grade of inflammation and accordingly the rate of cell killing or natural hepatocyte turnover. The half-life of infected hepatocytes in the liver of chronic HBV carriers is not known but is presumably shorter than that of healthy hepatocytes.

Although the latest generation of nucleos(t)ide analogues suppress viral replication, it is likely that hepatocytes can still become infected. Because these drugs cannot prevent the formation of new cccDNA during de novo virus entry into cells,¹⁵² the combination of nucleos(t)ide analogues and entry inhibitors might speed up elimination of infected cells. Because removal of nucleos(t)ide analogues in chronically infected patients results in virological and biochemical relapse but provokes a marked percentage of viral clearance,¹⁵³ the addition of an entry inhibitor that prevents reformation of cccDNA could increase this therapeutic effect. The immune modulator IFN- α might also be combined with an entry inhibitor. IFN- α , which decreases HDV replication, might be combined with an entry inhibitor to block the spread of HDV; this interesting approach requires testing in clinical trials.

Inactivation of NTCP

Prolonged inactivation of the receptor function of NTCP with antibodies, cyclosporin A, ezetimibe, or Myrcludex B presumably affects bile transport by the molecule. This might increase serum levels of conjugated bile salts, which are primarily transported by NTCP. In the long-term, these

changes could affect expression levels of compensating transporters such as organic anion-transporting polypeptides and other pathways that change with intrahepatic levels of bile salts and are controlled by farnesoid X receptor or other nuclear receptors.

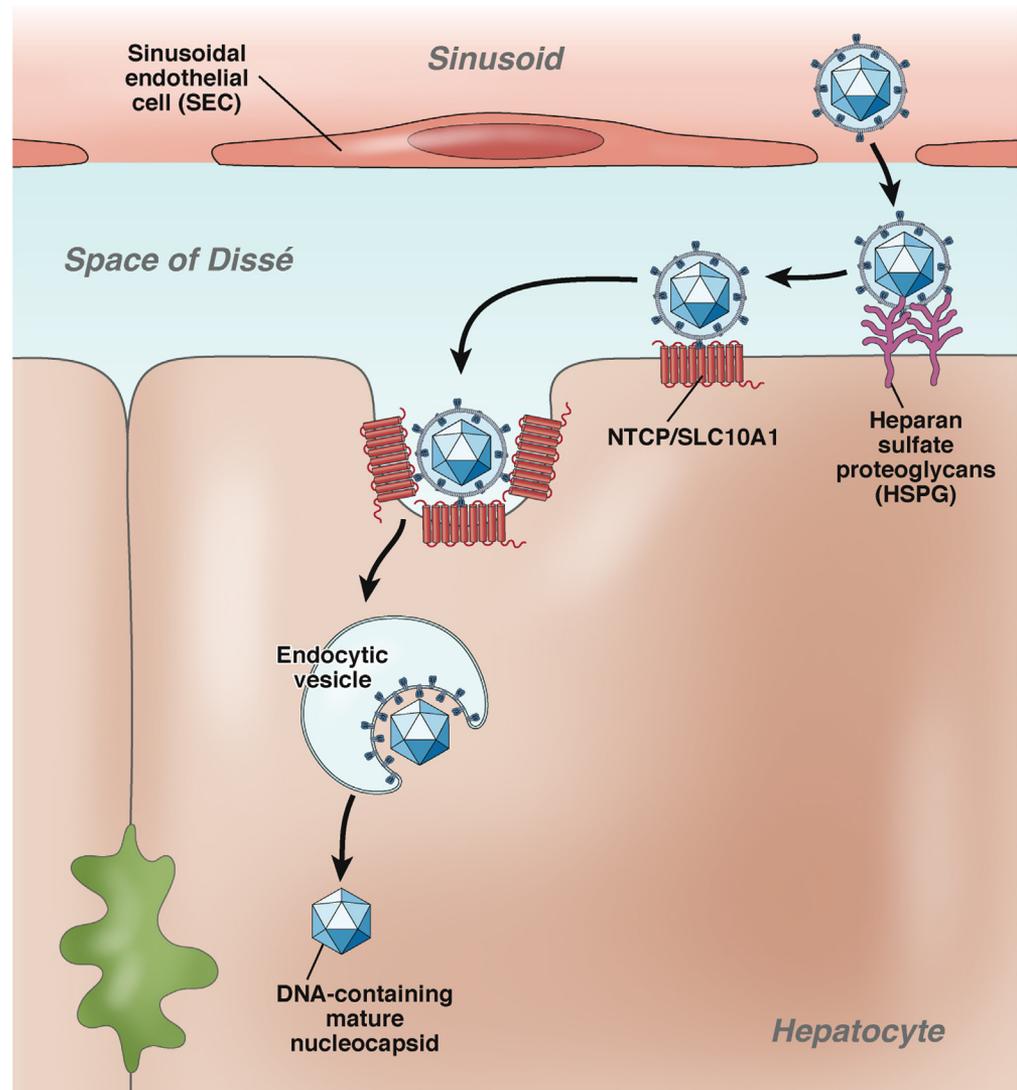
Thus, the clinical consequences of NTCP inhibition are unclear. However, subjects who are homozygous for the single nucleotide polymorphism c.800C>T, which almost completely disrupts NTCP-mediated transport of bile salts, have been described.¹⁴⁸ Myrcludex B inactivates NTCP function at concentrations far below those required to block bile salt transport. Thus, a therapeutic window exists that allows only partial inactivation of NTCP bile salt transport but complete block of HBV or HDV entry.¹¹⁹ NTCP also transports substrates other than bile salts, so blockade of NTCP could affect the pharmacokinetics and effectiveness of certain drugs. These aspects need to be considered when Myrcludex B or other NTCP inhibitors are used in the clinic. Interestingly, inhibitors of NTCP-mediated bile salt transport might be used to treat certain forms of cholestasis. Preventing reimport of bile salts into hepatocytes that do not secrete them into canaliculi might be used to treat acute biliary cholestasis.

Future Directions

The identification of NTCP as the bona fide HBV and HDV receptor was a major breakthrough. Basic researchers now have an improved system for studying HBV entry (Figure 6) and can establish cell lines that are easily infectable and support efficient replication of HBV and HDV. These systems will be instrumental for studying the early steps of HBV and HDV replication and identifying other cell components required for productive infection. These could include factors involved in intracellular trafficking of virus particles, nuclear import of HBV nucleocapsids, and formation of cccDNA. The identification of this receptor can also be used to establish immune-competent transgenic mice that can be infected with either virus. Although mouse cells support HBV assembly and secretion after artificial delivery of the HBV genome, it remains to be determined which other factors besides NTCP (eg, factors required for nucleocapsid delivery to the nucleus or DNA repair) codetermine species specificity.

Robust infection systems are urgently needed to establish assays to characterize clinical isolates and increase our knowledge about viral determinants of host range and pathogenesis. Moreover, it is now possible to develop screening assays to identify cellular factors that promote or restrict HBV and HDV replication cycles. These assays could use RNA interference or transgenic overexpression approaches, like those conducted in HIV-1¹⁵⁴ or hepatitis C virus research.^{155,156} These types of high-throughput screening formats are also suitable to search for agents that inhibit HBV replication in a more general way, such as cell factors that are exploited by the viruses for their own replication. This approach was used to identify cyclophilin A as a target for anti-hepatitis C virus therapy¹⁵⁷ and the

Figure 6. Model of HBV entry into hepatocytes. Enveloped HBV and HDV particles circulating in the sinusoidal blood enter the space of Disse, probably by passage through the fenestrae of sinusoidal endothelial cells. Through reversible attachment to HSPGs, L protein–enriched virions are concentrated at the surface of hepatocytes, allowing them to contact the integral membrane protein receptor NTCP. In a slow transition process, the N-terminus of the HBV L protein is subsequently released from its intra-membranous state and forms an irreversible receptor complex with NTCP. After endocytosis by an unknown mechanism, the viral and cellular membranes fuse. Formation of a fusion-active NTCP virus complex can be inhibited by Myrcludex B.



human immunodeficiency virus coreceptor CCR5, which is targeted by Maraviroc.¹⁵⁸ The ability of Myrcludex B to target the NTCP provides hope that new antiviral strategies will become available in the near future.

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Author names in bold designate shared co-first authorship.

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Reprint requests

Address requests for reprints to: Prof Dr Stephan Urban, Department of Infectious Diseases, Molecular Virology, University Hospital Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany. e-mail: stephan.urban@med.uni-heidelberg.de; fax: (49) 6221-561946.

Conflicts of interest

Stephan Urban is co-applicant and co-inventor on patents protecting HBVpreSderived lipopeptides (Myrcludex B) for the use of HBV/HDV entry inhibitors. The remaining authors disclose no conflicts.