Hepatitis B virus receptors and molecular drug targets

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Abstract

Chronic Hepatitis B virus (HBV) infection is a leading cause of liver disease worldwide. Virus-induced diseases include cirrhosis, liver failure and hepatocellular carcinoma. Current therapeutic strategies may at best control infection without reaching cure. Complementary antiviral strategies aimed at viral cure, are therefore urgently needed. HBV entry is the first step of the infection cycle, which leads to the formation of cccDNA and the establishment of chronic infection. Viral entry may thus represent an attractive target for antiviral therapy. This review summarizes the molecular virology and cell biology of HBV entry, including the discovery and development of new HBV entry inhibitors, and discusses their potential in future treatment of HBV infection.

Keywords: Antiviral targets; host-targeting agents; liver; therapy; treatment.

Introduction

With 250-350 million affected individuals, chronic hepatitis B virus (HBV) infection is a major public health problem. Chronic HBV infection can lead to advanced liver disease such as liver cirrhosis and liver failure (1-4). Furthermore, HBV infection is a leading cause of hepatocellular cellular carcinoma (HCC) worldwide (1-4). Despite a very efficient protective vaccine, HBV prevalence has only marginally declined. Current antiviral therapies involving pegylated type-I interferon (IFN) and nucleos(t)ide analogs (NUCs) may at best control viral infection. There is no anti-HBV treatment available for efficient viral clearance in HBV carriers. HBV is a small, enveloped DNA virus of the *Hepadnaviridae* family, which exclusively infects human hepatocytes (4). The viral coat consists of three envelope proteins: small S (S), middle M (S + preS2 region) and large L (S + preS2 region + preS1 region) (5). Unique features of the HBV life cycle include a genome replication mechanism that uses a reverse transcription step (targeted by NUCs) to convert pregenomic RNA to genomic DNA, and the formation of a covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes, the equivalent of proviral DNA in retrovirus-infected cells (6). The HBV cccDNA functions as a minichromosome in the nucleus of infected cells to serve as a template for transcription into viral mRNAs. Because it is responsible for viral persistence, HBV cccDNA is the target of choice for elimination of infection (6). HBV infection in humans may become more complex when patients are co-infected with the hepatitis delta virus (HDV). HDV is a subviral infectious agent, and an obligate satellite of HBV, which uses the HBV envelope proteins to assemble infectious particles and thereby propagate with the helper HBV. As for HBV, there is no curative treatment available (7), and chronic HBV/HDV co-infections lead to increased liver damage and HCC risk as compared to HBV mono-infections (8).

As current treatments may control, but not cure infection, new therapeutic strategies are needed, especially for treatment of chronic infections. Novel therapeutic approaches include host-targeting compounds that inhibit virus-host interactions as reviewed previously (9). As the first step of infection necessary for the formation of cccDNA and the establishment of chronic infection, viral entry represents an antiviral target in complement of

current therapies (9-11). As HDV and HBV share the same envelope and entry pathway, targeting viral entry is of particular interest for the treatment of HBV/HDV co-infection. Here, we review the recent developments in our understanding of the HBV/HDV entry process, including the consequent new antiviral strategies.

HBV entry: the quest for a viral receptor

Although the HBV DNA replication cycle has been described in detail, molecular interactions between virus and host factors are not fully understood. One reason for this limitation has been the lack of robust cell culture infection systems for the study of the full life cycle. Viral entry can be observed in primary cultures of human hepatocytes (PHH) and *Tupaia belangeri* (PTH) hepatocytes (12, 13). One human hepatic progenitor cell line (HepaRG) has also demonstrated susceptibility to HBV infection following DMSO-mediated differentiation, and it has been used extensively in recent years for HBV and HDV in vitro infection assays (14). However, HBV/HDV susceptible systems are limited by a number of constraints: susceptibility to infection is highly dependent on culture conditions and, in the case of PHH, subject to donor-to-donor variability (4, 10, 15). Besides viral entry, all other steps of the HBV or HDV replication cycles have been studied for many years in permissive hepatoma cell lines, such as Huh7 and HepG2 cells (15-18). These cell lines, however, are not susceptible to HBV and HDV infection, limiting their use to genome replication, transcription, translation, viral assembly and release, but not viral entry (19). Consequently, the HBV receptor(s) have remained unknown for many years (20, 21). Only in late 2012 was a first bona-fide HBV receptor identified at the basolateral membrane of differentiated human hepatocytes. Using PTH as target cells, and a labeled preS1 peptide as a bait to substitute for HBV envelope proteins, Yan et al. (22) identified the sodium taurocholate co-transporting polypeptide (NTCP), a bile-acid transporter mainly expressed at the basolateral membrane of hepatocytes (23) encoded by the *SLC10A1* gene, as an HBV-specific receptor (22, 24). In parallel, Ni et al. (24) had compared the transcriptomic pattern of differentiated (HBV susceptible) HepaRG cells to that of undifferentiated (not susceptible) HepaRG cells, and

found that NTCP mRNA was expressed upon differentiation. Interestingly, the overexpression of human NTCP in permissive hepatoma cells such as Huh7 and HepG2 cells that lack endogenous expression of NTCP, conferred susceptibility to both HDV and HBV infection, thereby providing a cell culture model for the study of the entire HBV life cycle, including viral entry (22, 24).

Molecular biology of HBV entry

The interaction between HBV or HDV virions and hepatocytes begins with attachment to cell surface heparan sulfate proteoglycans (HSPGs) (25) (Figure 1). HSPGs are glycoproteins that play major roles in development and physiology (26), but they are also used for cellular attachment of numerous pathogens, including viruses (27). In the case of HBV, HSPG binding function was demonstrated by the ability of soluble heparin and other sulfated polysaccharides to impair infectivity of HDV and HBV virions (28, 29). Moreover, heparinase treatment of susceptible cells could inhibit HDV and HBV infection (28, 30). Using HepaRG cells, and a heparin-binding assay, Sureau and Salisse (30) reported that HSPGs bind to the antigenic loop of the HBV envelope protein S domain. HBV-HSPG interaction is one explanation to the highly specific tissue tropism of the virus, as viral glycoproteins recognize specific patterns of hepatic proteoglycans (including the degree of sulfation of HSPG) (25, 29). Taking advantage of NTCP-based cell culture systems and using a siRNA library targeting all members of HSPG families, Verrier et al. (31) recently demonstrated that glypican 5 (GPC5) was an attachment factor for HDV and HBV at the cell surface of hepatocytes. The involvement of GPC5 in HBV binding was confirmed by the inhibitory effect of an anti-GPC5 monoclonal antibody and by the ability of soluble recombinant GPC5 protein to neutralize HBV in vitro infection (31). GPC5 is a member of the glypican family, consisting of extracellular HSPGs bound at the cell surface through a glycosyl-phosphatidylinositol anchor (32). GPC5 carries five insertion sites (compared to three for GPC3, for instance) for highly sulfated glycosaminoglycan chains (33) and is expressed in PHH (31), possibly explaining the specific affinity of GPC5 for HBV virions. Since HSPG, and particularly GPC5,

140 are involved in many cellular processes, including the control of cell division, growth
141 regulation, FGF2- or Hedgehog signaling, HBV-HPSG interactions may also play a role in the
142 pathogenesis of virus-induced liver disease (26, 33, 34). Further studies are needed for a
143 comprehensive overview of HSPG-HBV interactions.

144 Following initial attachment to HSPGs, HBV and HDV virions bind to NTCP (Figure
145 1). Being a liver-specific transporter, NTCP is likely to play the major role in HBV liver tropism
146 (35). In the liver, NTCP is responsible for the transport of conjugated bile salts, typically
147 taurocholate (36). The preS1 domain at the surface of HBV virions directly interacts with the
148 bile acid pocket site (aa 157–165) of NTCP (22, 24). The overlap between NTCP HBV
149 receptor function and NTCP bile acid transporter function was confirmed by the ability of
150 NTCP S267F mutation to disrupt not only bile acid transport but also HDV and HBV infection
151 in a cell culture model (37). Interestingly, two genetic studies showed that the S267F
152 *SLC10A1* allele was associated with resistance to chronic HBV infection (38) and with
153 decreased risk of cirrhosis and HCC in HBV carriers (39). However, the observation that
154 S267F homozygous patients can still be infected by HBV (38, 39) demonstrates that this
155 mutation does not totally suppress HBV entry *in vivo*, and suggests that other hepatic
156 factor(s) can function as surrogate HBV receptors in absence of functional NTCP. A recent
157 study identified another domain (aa 84–87) within NTCP that plays a crucial role in HBV
158 entry, probably at a postbinding step (40). Interestingly, the overexpression of human NTCP
159 in non-human cells is sufficient to confer susceptibility to HDV infection, suggesting that no
160 additional human-specific factor is required for viral attachment, fusion and trafficking of the
161 HDV genome to the cell nucleus (35, 40). However, hNTCP expression in mouse cells is not
162 sufficient to confer susceptibility to HBV infection. Using fusion between hNTCP-
163 overexpressing mouse cells and permissive non-susceptible HepG2 cells, Lempp et al. (41)
164 recently suggested that the absence of HBV infection was due to a lack a host factor
165 required for HBV replication. Following NTCP binding, HBV virions are thought to be
166 internalized by endocytosis. Both clathrin- (42) and calveolin-dependent (43) processes have

been proposed, but to date, the detailed mechanism of NTCP-mediated internalization remains unclear.

Currently, only a limited number of host proteins are known to be involved in HBV entry, and it is expected that the new NTCP-based infection models will allow the discovery of other entry factors. Given the relatively limited percentage of infected cells in this models, despite NTCP overexpression and high multiplicity of infection (22, 24, 31, 44), it is likely that additional factors indeed play an important role at viral entry.

HBV cell entry inhibitors

Entry inhibitors have emerged as attractive candidates for prevention and treatment of chronic viral infections (10, 35). Interestingly, in the context of hepatitis C virus (HCV), which also leads to chronic infection of the liver, an entry inhibitor used in monotherapy was shown to cure chronic infection in human liver chimeric mice (45). It is likely that hepatocyte turnover in the absence of *de novo* infection results in viral clearance (45). Furthermore, broadly neutralizing anti-envelope antibodies have been shown to control and eliminate persistent HCV infection in humanized mouse models (46). Indeed, by blocking reinfection and protecting uninfected hepatocytes from *de novo* infection, entry inhibitors may open perspectives for novel therapeutic approaches for HBV infection.

Neutralizing antibodies targeting viral envelope epitopes were the earliest entry inhibitors explored for HBV. Hepatitis B immunoglobulin (HBIG), a mixture of polyclonal antibodies targeting the HBV S protein, is currently used to prevent liver graft reinfection in HBV-positive patients (47). Monoclonal antibodies directed against the S protein (17.1.41 and 19.79.5) were shown to reduce HBV viral load and HBsAg levels in animal models (48) and in patients in a phase I clinical trial (49), but only for several days following treatment. Interestingly, a recent study demonstrated that the administration of a novel monoclonal anti-HBsAg antibody (E6F6) in a mouse model mimicking HBV infection not only suppressed the levels of HBsAg and HBV DNA for several weeks but also facilitated the restoration of anti-HBV T-cell response (50, 51). Other monoclonal antibodies targeting the preS1 domain

195 demonstrated marked anti-HBV activity *in vitro* (52) and in animal models (53) but have not
196 been pursued in clinical trials.

197 Targeting host cell entry factors is a novel strategy that has been proposed in the
198 context of HBV infection (10, 35). The attachment of HBV particles to the target hepatocyte
199 relies on low-affinity interactions with HSPGs (25, 29). Small molecules that non-specifically
200 interfere with HSPG binding therefore inhibit HBV infection. Highly sulfated compounds such
201 as heparin (25) and suramin (54) inhibit HBV attachment by competing with cellular HSPGs
202 for the binding sites in HBV surface proteins. GPC5 was recently identified as a HSPG
203 possibly involved in this process (31). Indeed, a monoclonal antibody targeting GPC5
204 inhibited HBV infection in cell culture (31). Furthermore, synthetic anti-lipopolysaccharide
205 peptides (SALPs) that bind to heparan sulfate moieties on the cell surface inhibit infection
206 with a variety of enveloped viruses (55).

207 Since its discovery as a receptor for HBV/HDV, NTCP has been extensively explored
208 as an antiviral target. The molecules targeting NTCP that exhibit antiviral functions against
209 HBV are listed in Table 1. Myrcludex B, a myristoylated preS1-derived peptide, binds to
210 NTCP and inhibits HBV infection by blocking binding of HBV to its receptor (10). PreS1-
211 derived peptides inhibit HBV/HDV infection in vivo (56), and Myrcludex B prevents HBV
212 spread in human liver chimeric mice (57). It is currently being evaluated in a phase IIa clinical
213 trial (58). The first data suggest that Myrcludex B is well tolerated. The inhibition of HBV entry
214 seems to be correlated with a decrease in both HBV DNA (> 1 log10) and HDV RNA, and
215 with an improvement of hepatic parameters (ALT) (58). Other molecules also target NTCP
216 and inhibit HBV infection. Cyclosporin A, a cyclic peptide, interferes with HBV entry as a
217 result of its interactions with NTCP (44, 59). Ezetimibe, a small molecule inhibitor of NTCP-
218 mediated bile acid uptake, also inhibits HBV/HDV entry by apparently similar mechanisms
219 (60). Among several FDA-approved drugs with NTCP-inhibiting activity, irbesartan, a drug
220 used for hypertension treatment, inhibited HBV infection in HepG2-NTCP cells as well as
221 HDV infection in Huh7-NTCP cells (61, 62). Vanitaracin A, a tricyclic polyketide identified
222 from a fungal secondary metabolite library, also interacts with NTCP to inhibit HBV/HDV

infection (63). Ritonavir, an antiretroviral drug active against HIV infection, and having a putative inhibitory effect on NTCP metabolic function (64) inhibits HDV infection in Huh7-NTCP cells (62). A small screen of phytochemicals identified the green tea polyphenol epigallocatechin gallate as another HBV inhibitor, which possibly interferes with HBV uptake by altering the localization and stability of NTCP (65).

Conclusion and perspectives

Chronic HBV infection is a major public health problem worldwide. Since current anti-HBV treatments do not effectively cure infection (i.e., eliminate cccDNA), novel treatment approaches are needed. Entry inhibitors may contribute to cccDNA elimination through inhibition of cccDNA synthesis and prevention of entry in non-infected hepatocytes. However, as viral entry inhibitors including host-targeting compounds do not directly target cccDNA, they should be considered as a complementary antiviral strategy in combination with other antiviral molecules, such as IFNs that can control cccDNA activity (9, 10, 35, 66-68). Recent progress in understanding the HBV entry process, particularly after the discovery of NTCP acting as a receptor, has lead to the identification of novel antiviral compounds for treatment of HBV and HDV infections. A key question will be whether entry inhibitors will help to cure HBV infection by preventing entry into cccDNA-free hepatocytes, while cccDNA-positive hepatocytes are eliminated by liver turnover. Further studies in state-of-the-art animal models and clinical trials are needed to address these questions, to ultimately establish the real place of entry inhibitors in the anti-HBV arsenal.

Compliance with Ethical Requirements

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Conflict of Interest: Eloi R Verrier, Che C Colpitts, Camille Sureau and Thomas F Baumert declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure legend

Figure 1. Molecular mechanisms of HBV entry and entry inhibitors. HBV viral particles first interact with hepatic HSPGs, including GPC5. This interaction is inhibited by a monoclonal anti-GPC5 antibody or heparinase treatment. HBV then binds directly to NTCP at the bile acid pocket site. Several NTCP inhibitors block this interaction and prevent HBV entry,such as cyclosporin A, ezetimibe, irbesartan, vanitaracin A, ritonavir or epigallocatechin gallate. A synthetic HBV-derived preS1 peptide (Myrcludex B) exhibits a strong antiviral activity by competing with HBV for NTCP binding. After binding to NTCP, the virus is internalized but the underlying mechanisms are still unknown.

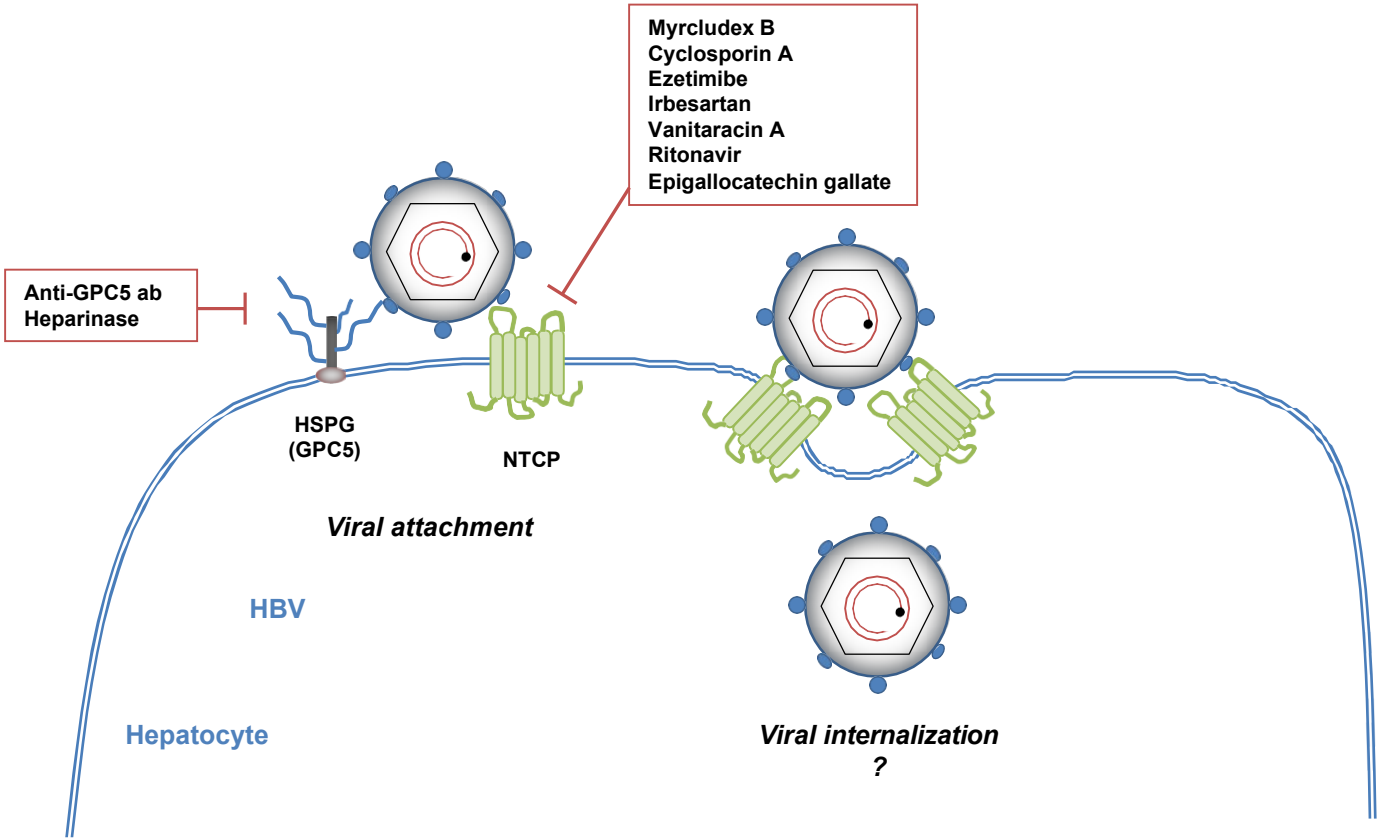
Table legend

Table 1. Examples of HBV entry inhibitors targeting NTCP in hepatocytes

Table

Name	Class	Development stage	References
Myrcludex B	Myristoylated lipopeptide	Clinical phase II	[57]
Cyclosporin A	Immunosuppressant	Cell culture	[44, 59]
Ezetimibe	b-lactam	Cell culture	[60]
Irbesartan	Angiotensin receptor blocker	Cell culture	[61, 62]
Vanitaracin A	Fungal tricyclic polyketide	Cell culture	[63]
Ritonavir	HIV protease inhibitor	Cell culture	[62]
Epigallocatechin gallate	Green tea polyphenol	Cell culture	[65]

Figure


Myrcludex B
Cyclosporin A
Ezetimibe
Irbesartan
Vanitaracin A
Ritonavir
Epigallocatechin gallate
Anti-GPC5 ab
Heparinase
HSPG
(GPC5)
NTCP
Viral attachment
HBV
Hepatocyte
Viral internalization
?