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Review

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Targeting Viral Entry for Treatment of Hepatitis B and C Virus Infections

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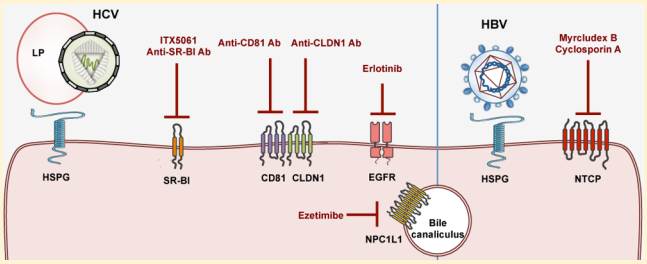
ABSTRACT: Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections remain major health problems worldwide, with 400−500 million chronically infected people worldwide. Chronic infection results in liver cirrhosis and hepatocellular carcinoma, the second leading cause of cancer death. Current treatments for HBV limit viral replication without efficiently curing infection. HCV treatment has markedly progressed with the licensing of direct-acting antivirals (DAAs) for HCV cure, yet limited access for the majority of patients is a major challenge. Preventative and curative treatment strategies, aimed at novel targets, are needed for both viruses. Viral entry represents one such target, although detailed knowledge of the entry mechanisms is a prerequisite. For HBV, the recent discovery of the NTCP cell entry factor enabled the establishment of an HBV cell culture model and showed that cyclosporin A and Myrcludex B are NTCP-targeting entry inhibitors. Advances in the understanding of HCV entry revealed it to be a complex process involving many factors, offering several antiviral targets. These include viral envelope proteins E1 and E2, virion-associated lipoprotein ApoE, and cellular factors CD81, SRBI, EGFR, claudin-1, occludin, and the cholesterol transporter NPC1L1. Small molecules targeting SR-BI, EGFR, and NPC1L1 have entered clinical trials, whereas other viral- and host-targeted small molecules, peptides, and antibodies show promise in preclinical models. This review summarizes the current understanding of HBV and HCV entry and describes novel antiviral targets and compounds in different stages of clinical development. Overall, proof-of-concept studies indicate that entry inhibitors are a promising class of antivirals to prevent and treat HBV and HCV infections.



KEYWORDS: *virus−host interactions, hepatitis viruses, viral entry, entry inhibitors, novel antiviral targets*

HEPATITIS B AND C INFECTIONS REPRESENT A MAJOR GLOBAL HEALTH BURDEN

Globally, hepatitis B virus (HBV) and hepatitis C virus (HCV) chronically infect 350 million and 160 million people, respectively.[1,2] Viral hepatitis due to chronic HBV and HCV infection leads to progressive liver disease, including cirrhosis and hepatocellular carcinoma (HCC)—the second leading and fastest rising cause of cancer death worldwide.[1,3,4] Furthermore, between 5 and 10% of HBV-infected individuals are co-infected with the hepatitis delta virus (HDV), which increases the risk of HCC.[5] Virus-induced liver cirrhosis and HCC are major burdens on global health and are the leading indications for liver transplantation.[6]

Great strides in the understanding of HCV virology enabled the development of direct-acting antiviral agents (DAAs), which have improved the standard of care for chronic HCV infection. These therapies enable the elimination of HCV in most (but not all) patient groups.[7] However, their high cost, adverse side effects, and lack of efficacy in certain patient groups preclude their widespread use.[7] End-stage liver cirrhosis due to chronic HCV infection remains the leading cause of liver transplantation.[8] Graft reinfection is universal, due in part to the absence of preventive antiviral strategies, which are emerging but are still poorly defined.[8]

Despite the existence of a protective vaccine, the prevalence of HBV infection has remained virtually unchanged, as a result of limited access to vaccination, the high efficiency of vertical transmission, and the lack of curative treatment. Current treatment approaches are based on nucleos(t)ide analogues, including tenofovir and entecavir (for a total of seven FDA-approved agents). These therapies suppress HBV replication but have no meaningful ability to cure chronic infection.[3,9] Thus, the development of efficient antiviral strategies to cure HBV infection is a critical unmet medical need.

VIRAL ENTRY INTO HEPATOCYTES OFFERS NEW TARGETS FOR CURATIVE ANTIVIRAL THERAPY

As the first step in viral infection, viral entry has many advantages as an antiviral target. With entry inhibitors, it is possible to block viral infection before the virus produces its

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Table 1. Examples of HBV and HCV Entry Inhibitors[a]

name	chemical structure	target	development stage	refs
		HCV		
ITX5061	arylketoamide	SRBI	clinical phase Ib	66
erlotinib	quinazoline	EGFR	clinical phase I/II	34
ezitimibe	β-lactam	NPC1L1	clinical phase I	43
EGCG	catechin	viral glycoprotein(s)	cell culture	54, 55
281816	dibenzothiepin	viral E2 glycoprotein	cell culture	56
HCV-II1	tetrahydroquinoline	viral envelope	cell culture	57
EI-1	triazine	viral E2 glycoprotein	cell culture	58
ferroquine	aminoquinoline	viral E1 glycoprotein	cell culture	59
aUY11	nucleoside analog	virion envelope lipids	cell culture	60
phenothiazines	phenothiazine	membrane fluidity	cell culture	61
benzhydryl-piperazines	benzhydrylpiperazine	membrane fluidity	cell culture	62
curcumin	diarylheptanoid	membrane fluidity	cell culture	63
arbidol	bromindole	endosomal trafficking	cell culture	64
silibinin	flavonolignan	endosomal trafficking	cell culture	65
anti-CD81	monoclonal antibody	CD81	in vivo (mouse)	14, 69
anti-SRBI	monoclonal antibody	SRBI	in vivo (mouse)	70, 71
anti-CLDN1	monoclonal antibody	CLDN1	in vivo (mouse)	12, 73
		HBV		
Myrcludex B	myristoylated peptide	NTCP	clinical phase II	18
cyclosporin A	nonribosomal peptide	NTCP	cell culture	91, 92
EGCG	catechin	NTCP	cell culture	93
ezetimibe	β-lactam	NTCP	cell culture	94, 95

[a]Chemical structures, targets, and development stage are indicated.

genomic material or alters infected cells. Entry inhibitors inhibit the viral life cycle at a step before persistent reservoirs are established or resistant variants are generated by error-prone replication of the viral genomes. Furthermore, host entry factor usage is highly conserved among viral variants,[10] resulting in a high genetic barrier to resistance. Because entry is required for dissemination and maintenance of infection, targeting viral entry could also allow treatment of chronic HCV and HBV infections, including control and cure of infection, as shown in humanized mouse models.[11−14] In the absence of de novo infection, there is thought to be a time-dependent elimination of virus-infected hepatocytes, with hepatocyte turnover and replacement by noninfected hepatocytes. Turnover may be promoted by apoptosis of infected hepatocytes.[15,16] Furthermore, chronicity of infection depends on the de novo infection of new cells due to the immune-induced clearance of infected hepatocytes.[17] Indeed, an HCV entry inhibitor resulted in the elimination of infected hepatocytes, leading to viral clearance in a mouse model.[12] An HBV inhibitor resulted in the control of infection with a marked decrease of viral spread.[18] Entry inhibitors protect naïve hepatocytes from infection, especially important in the context of liver transplantation and, for HBV, viral transmission from mothers to children.[17] Entry inhibitors targeting HBV also inhibit HDV entry,[19,20] as HDV is a satellite virus that uses the envelope proteins of HBV. Treatment options for HDV infection, which is associated with more rapid progression of liver disease, are currently lacking.[5] Recent advancements in the understanding of virus−host interactions during HBV, HCV, and HDV entry provide multiple opportunities to inhibit infection.

HCV ENTRY

HCV is a positive-sense single-stranded RNA virus in the Flaviviridae family. A host-derived membrane containing E1 and E2 glycoproteins surrounds the nucelocapsid core, and viral particles are associated with serum lipoproteins. Like many viral infections, HCV infection is initiated by low-affinity interactions between viral particles and heparan sulfate-containing proteoglycans (HSPGs) on the cell surface.[21,22] The cellular low-density lipoprotein receptor (LDL-R) is thought to further facilitate binding[23−25] and most likely interacts with virion-associated lipoproteins (LP).[26] The human scavenger receptor class B type I (SRBI) also interacts with virion-associated lipoproteins[27] and HCV E2 protein,[28] thereby contributing to virion binding. Furthermore, the lipid transfer activities of SRBI are thought to prime the viral particle for interactions with other cellular factors.[27,29]

Cluster of differentiation 81 (CD81) is a member of the tetraspanin family, a group of cell-surface proteins that mediate signal transduction events.[30] CD81 was the first cellular receptor identified for HCV and is thought to interact with the virion E2 protein.[31] However, time course studies with CD81-specific antibodies indicated that CD81 also mediates postbinding events.[32,33] CD81 engagement triggers signaling through the epidermal growth factor receptor (EGFR)[34] and Rho and Ras GTPases.[35,36] Activation of these signaling pathways likely induces actin remodeling, thereby facilitating the lateral movement of CD81-bound HCV particles along the cell surface to tight junctions and promoting coreceptor complex assembly.[36]

HCV particles are internalized into hepatocytes at tight junctions, via clathrin-mediated endocytosis.[33] Claudin 1 (CLDN1), a tight junction protein highly expressed in the liver,[37] is required for HCV infection.[38] CLDN1 interacts with CD81 to form a coreceptor complex,[39] which ultimately leads to clathrin-mediated endocytosis of the CLDN1−CD81−HCV complex.[33] Occludin (OCLN), another tight junction protein,[40] is likely involved in a postbinding step of HCV entry,[41,42] although its specific functions in HCV entry are unknown. Entry of HCV requires other cellular factors, although their

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specific roles are not yet known. The Niemann−Pick C1-like 1 cholesterol adsorption receptor is thought to contribute to virion binding or internalization through its interactions with virion-associated cholesterol.[43] Transferrin receptor 1 and cell death-inducing DFFA-like effector b are also required for a late entry step.[44,45] However, the detailed mechanistic contributions of these factors are still unknown.

Clathrin-mediated endocytosis ultimately delivers the HCV−receptor complex to Rab5-containing early endosomes. The low endosomal pH induces fusion between the viral envelope and endosomal membranes,[33] mediated by the HCV glycoproteins.[46] Recent crystal structures for E2 revealed a globular architecture that differs from that of other viral fusion proteins.[47,48] Further studies are necessary to elucidate the HCV fusion mechanism.

HCV is also able to spread to neighboring cells via direct cell-to-cell transfer.[49] Cell-to-cell transmission likely relies on CD81, CLDN1, and SRBI,[49] although CD81-independent spread has also been observed.[50] This process allows viral escape from neutralizing antibodies, or compounds targeting free virions, and thus represents a challenge to the development of anti-entry therapeutics that target cell-free virions, such as anti-envelope antibodies. In contrast, inhibitors of host cell entry factors, including CD81, SRBI, CLDN, OCLN, and EGFR, efficiently inhibit both cell-free and cell-to-cell entry of HCV.[34,51] Nevertheless, broadly neutralizing anti-E2 antibodies have been shown to control and partially clear HCV infection in human liver chimeric mice,[13] suggesting that cell-to-cell transmission can be inhibited by defined neutralizing anti-envelope antibodies or that cell-to-cell transmission may be dispensable for control of viral infection by entry inhibitors.

INHIBITORS OF HCV ENTRY

The virus−host interactions involved in HCV entry offer many therapeutic targets. Indeed, inhibitors acting against each step of the HCV entry process have been identified, including synthetic small molecules, natural products, peptides, and antibodies (Table 1).

Several small molecules in preclinical development inhibit HCV entry steps by interactions with virion glycoproteins. Negatively charged small molecules, including heparin and heparin-like compounds, nonspecifically compete for binding of the viral envelope proteins to glycosaminoglycans on the cell surface.[21,52,53] The green tea polyphenol epigallocatechin gallate (EGCG) inhibits HCV attachment by similar mechanisms.[54,55] Other small molecules target specific virus−receptor interactions. One such molecule, a dibenzothiepin derivative termed 281816, binds the viral E2 protein and disrupts its interaction with CD81, thereby blocking both cell-free entry and cell-to-cell transmission.[56] HCV infectivity inhibitor-1 (HCV-II1), a substituted tetrahydroquinoline, interacts with viral particles and blocks HCV endosomal fusion, potentially by locking the viral envelope in a prefusion conformation.[57] Triazine derivatives, including one molecule named EI-1, also interact with E2 to inhibit HCV infection at a postattachment step.[58] Ferroquine, a ferrocenic analogue of chloroquine that is in phase II clinical trials for malaria, inhibits a postbinding and postinternalization step of HCV entry, likely by targeting E1.[59]

Small molecules that interact with virion envelope lipids or host factors can also block entry steps. As this group of inhibitors targets factors that are not encoded by highly mutable viral genomes, they offer a very high genetic barrier to resistance. Synthetic rigid amphipathic nucleoside derivatives insert into the lipid core of virion envelopes to prevent curvature changes required for fusion,[60] whereas some phenothiazine derivatives, benzhydrylpiperazines, and a diarylheptanoid curcuminoid (curcumin) modulate membrane fluidity to inhibit HCV fusion.[61−63] Another approach is to target the host cell factors involved in HCV entry. Arbidol, a substituted indole used in the treatment of influenza virus infections, also inhibits HCV clathrin-dependent endosomal trafficking by inhibiting dynamin-2-mediated membrane scission.[64] Silibinin, a flavonolignan isolated from milk thistle, similarly interferes with endosomal trafficking.[65]

Some small-molecule entry inhibitors have entered into clinical trials for HCV. Small-molecule SRBI receptor antagonists inhibit HCV infection at a postbinding step.[66] Among them, ITX5061—an arylketoamide that increases HDL levels by targeting the SRBI pathway[67]—is in phase 1b clinical trials for HCV infection (clinical trial NCT01560468). EGFR and EphA2, receptor tyrosine kinases that regulate CD81−CLDN1 coreceptor complex formation, are also promising targets, as their kinase activities can be inhibited by clinically approved protein kinase inhibitors. The 4-anilinoquinazoline derivative erlotinib, which blocks the kinase activity of EGFR, inhibits HCV infection in vitro and in human liver chimeric mice.[34] Erlotinib is currently in clinical trials for chronically infected HCV patients (clinical trial NCT01835938). Ezetimibe, a 2-azetidinone that is already approved as a cholesterol-lowering medication,[68] inhibits NPC1L1 internalization and consequently blocks HCV infection in cell culture and in human liver chimeric mice.[43] A phase I clinical trial to study the effects of ezetimibe in patients with chronic HCV infection was recently initiated (clinical trial NCT02126137).

Monoclonal antibodies targeting host cell factors have shown great promise as HCV entry inhibitors. Prophylactic treatment with antibodies against CD81 protected human liver chimeric mice from HCV infection, although it had no effect if administered after viral challenge.[69] Antibodies targeting SRBI also protected mice from challenge with HCV and, unlike anti-CD81 antibodies, also reduced viral spread.[70,71] Humanized mice infected with HCV strains resistant to SRBI-targeting molecules in vitro were still protected by anti-SRBI antibodies in vivo.[72] Finally, monoclonal antibodies directed against the CLDN1 extracellular loops efficiently inhibited infection by all major genotypes of HCV as well as highly variable HCV quasispecies isolated from individual patients.[73] Strikingly, treatment with this antibody not only prevented but also cured chronic HCV infection in human liver chimeric mice.[12] Similarly, viral clearance was observed in some human liver chimeric mice treated with anti-CD81 antibody.[14] Anti-CLDN antibody-induced modulation of signaling pathways in virus-infected cells may contribute to viral clearance. However, the specific mechanisms involved remain unclear, and the efficacy of these antibodies in humans needs to be determined.

Viral-targeted antibodies also block HCV entry. Neutralizing antibody responses play an important role in viral clearance for spontaneous resolvers of HCV infection,[74] although the highly error prone HCV polymerase generates a pool of viral quasispecies which may contain variants that allow escape of host immune responses. However, polyclonal immunoglobulins isolated from a chronically infected patient protected up to 50% of human liver chimeric mice from HCV infection.[75,76] Polyclonal immunoglobulins have been explored in clinical trials.[77] Human monoclonal antibodies have also shown efficient cross-neutralizing activity against HCV.[78−80] Thus,

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antibodies targeting highly conserved epitopes on the viral envelope may provide effective antiviral strategies. Alternatively, antibodies targeting nonvirally encoded epitopes would overcome the limitation of high viral diversity. As a proof-of-concept, monoclonal antibodies targeting virion-associated host-derived apolipoprotein E inhibit HCV entry.[81]

Peptides have also been explored as inhibitors of HCV entry. Peptidic antivirals have a proven track record in the human immunodeficiency virus (HIV) field with enfuvirtide, a clinically approved peptide that inhibits HIV fusion by preventing conformational rearrangements of the viral fusion protein.[82] A better understanding of the HCV fusion mechanism is a prerequisite for analogous strategies against HCV. However, peptides derived from the HCV E2 protein inhibited HCV infectivity at a postbinding step,[83] providing proof-of-concept for this approach. Host-directed peptides offer an alternative peptide-based strategy. Indeed, peptides derived from ApoE and CLDN1 were shown to inhibit entry of HCV at binding and postbinding steps, respectively.[84,85] Peptides can also be designed to disrupt virus−host signaling interactions at the level of entry.[86]

AN IMPROVED UNDERSTANDING OF HBV/HDV ENTRY ENABLES THE IDENTIFICATION OF ANTIVIRAL TARGETS AND COMPOUNDS

HBV is a small, enveloped DNA virus that belongs to the Hepadnaviridae family.[4] The viral membrane, which is the same for HBV and HDV, contains three forms of the viral envelope protein: large (L), middle (M), and small (S).[4] As for HCV, HBV and HDV entry begins with attachment to HSPGs, as demonstrated by treatment of target cells or virus with heparinase or soluble heparin, respectively.[87,88] Following initial attachment, HBV binds to its first identified cellular receptor, the sodium taurocholate cotransporting polypeptide (NTCP/SLC10A1),[19,20] which is a hepatocyte-specific sodium-dependent bile salt transporter. NTCP overexpression in hepatoma cell lines that lack endogenous NTCP confers susceptibility to HBV/HDV infection.[19,20,89] NTCP is expressed on the basolateral membrane of hepatocytes, likely contributing to HBV tropism for the liver. Following binding, HBV virions are internalized, probably by endocytosis, but the detailed entry mechanisms are still poorly understood.[90] In particular, the existence of coreceptors or other entry factors, which may provide additional antiviral targets, remains to be determined.

INHIBITORS OF HBV/HDV ENTRY

The discovery of NTCP as an HBV/HDV receptor opened new avenues for the development of therapeutic strategies aimed at viral entry (Table 1). For example, cyclosporin A (CsA), a cyclic nonribosomal peptide widely used in organ transplantation as an immunosuppressant, specifically targets NTCP and inhibits HBV and HDV entry in vitro.[91,92] More importantly, Myrcludex B, an HBV pre-S1 NTCP-targeting peptide, markedly inhibits HBV and HDV infection in vivo[18] and is currently being evaluated in a phase IIa clinical trial.[17] When administered after infection, Myrcludex B prevented the spread of HBV in the liver of human liver chimeric mice.[18] Further preclinical and clinical studies are necessary to assess the ability of entry inhibitors to cure chronic HBV infection, as was recently shown for HCV. Interestingly, some previously identified HCV entry inhibitors also interfere with HBV entry. Green tea extract (containing EGCG)[93] and ezetimibe[94,95] have been shown to inhibit HBV entry. These studies suggest the possibility of using one molecule to treat both HBV and HCV infections, which would be particularly useful in the case of co-infected patients.[96]

CONCLUSIONS AND CLINICAL IMPACT

Significant progress in recent years to understand HCV entry enabled the identification of many novel strategies aimed at blocking HCV entry. Indeed, many HCV entry inhibitors are in preclinical and clinical stages of development. In contrast to DAAs, entry inhibitors have been shown to prevent[69,70,75,78] and cure[12] HCV infection in monotherapy in preclinical models. The combination of entry inhibitors with DAAs results in synergy in cell culture and in a humanized mouse model,[97] and entry inhibitors are effective against DAA-resistant mutants that rely on cell-to-cell transmission for viral spread.[98] Therefore, entry inhibitors may complement DAAs for prevention of liver graft infection and in patients nonresponsive or resistant to DAAs. Host-targeting entry inhibitors in particular pose a higher genetic barrier to resistance, although viral escape has been described for anti-SRBI[99] and anti-CLDN[100,101] antibodies in vitro. Nonetheless, the in vivo relevance remains unclear. Clinical studies, some of which have already been initiated, are the next step to define the role of entry inhibitors in HCV-infected patients (e.g., erlotinib, clinical trial NCT01835938). For HBV, an NTCP inhibitor has demonstrated proof-of-concept as an antiviral in humans[17] and the recent development of NTCP-based infection model systems opens a new perspective to develop further HBV and HDV entry inhibitors for viral cure.

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Notes

The authors declare the following competing financial interest(s): T.F.B. has served as an advisor on HCV antivirals for Biotest, Gilead, and Vironexx and is a co-inventor on a U.S. patent on anti-Claudin-1 antibody for the prevention and treatment of HCV infection filed by Inserm, University of Strasbourg, and Genovac/Aldevron.

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