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**Hepatitis C virus cell entry: A target for novel antiviral strategies to  
address limitations of direct acting antivirals**

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## **Abstract**

**Hepatitis C virus (HCV) infection remains a major global health problem, with 130-170 million chronically infected individuals at risk to develop severe liver disease, including hepatocellular carcinoma. Although the development of direct-acting antivirals offers cure in large majority of patients, there are still a number of clinical challenges. These include DAA failure in a significant subset of patients, difficult-to-treat genotypes and limited access to therapy due to high costs. Moreover, recent data indicate that the risk for liver cancer persists in patients with advanced fibrosis. These challenges highlight the need for continued efforts towards novel therapeutic strategies for HCV. Over the past two decades, advances in HCV model systems have enabled a detailed understanding of HCV entry and its clinical impact. Many of the virus-host interactions involved in HCV entry have now been identified and explored as antiviral targets. Furthermore, viral entry is recognized as an important factor for graft reinfection and establishment of persistent infection. HCV entry inhibitors, therefore, offer promising opportunities to address the limitations of DAAs. Here, we summarize recent advances in the field of HCV entry and discuss perspectives towards the prevention and cure of HCV infection and virus-induced liver disease.**

**Global impact of HCV infection.** There are an estimated 130-170 million people worldwide who are chronically infected with hepatitis C virus (HCV) [1]. These individuals are at higher risk to develop severe liver disease, including cirrhosis and hepatocellular carcinoma (HCC) [1]. Although recent approval of direct-acting antivirals (DAAs) has improved the outlook for HCV patients, the risk for liver disease persists even after viral cure, once fibrosis has been established [2]. Furthermore, not all patients respond to therapy and the high costs of DAAs limit access to treatment even in high-resource countries [3-5]. A detailed, comprehensive knowledge of HCV entry will guide development of novel antiviral approaches [6, 7]. Here, we review recent insights into the HCV entry process and its clinical impact as an antiviral target. We also discuss perspectives to use our accumulating knowledge of HCV entry to develop strategies aimed at the prevention and cure of HCV infection and virus-induced liver disease.

**Molecular virology and cell biology of HCV entry.** HCV is a member of the *flaviviridae* family, classified in the *hepacivirus* genus. HCV, with a positive sense single-stranded RNA genome of 9.6 kilo-base pairs, is a cytoplasmic-replicating virus [8]. The HCV capsid is surrounded by a host-derived lipid envelope, in which the E1 and E2 glycoproteins are embedded, and is associated with serum lipoproteins such as apolipoprotein E (ApoE) [9].

The first step in HCV infection is low-affinity binding to heparan moieties in heparan sulfate proteoglycans on the surface of hepatocytes [10-12], an interaction at least partially mediated through virion-associated ApoE [13-15]. The cellular low-density lipoprotein receptor (LDL-R) also interacts with virion-associated apolipoproteins to facilitate further binding [16-19]. Furthermore, the scavenger receptor class B type I (SR-B1) binds to virion-associated lipoproteins [20] and the

HCV E2 protein [21]. The lipid transfer activities of SR-BI may expose regions of E2 involved in interactions with other cellular factors, such as cluster of differentiation 81 (CD81) [22, 23]. CD81, the first receptor identified for HCV, binds directly to E2 [24] and also mediates critical post-binding events [25, 26], including activation of signaling pathways. Indeed, CD81 engagement was shown to activate signaling through the epidermal growth factor receptor (EGFR) [27] and Rho and Ras GTPases [28, 29].

Tight junction (TJ) proteins claudin-1 (CLDN1) and occludin (OCLN) have also been identified as HCV entry factors [30, 31]. CD81 interacts with CLDN1 to form a co-receptor complex [32], which along with the HCV particle is ultimately internalized into clathrin-containing endosomes [26]. Given that CD81-CLDN1 co-receptor complex formation could be detected at basolateral membranes but not in TJ-associated pools of CLDN1 [32], it is likely that the nonjunctional pool of CLDN1 predominantly contributes to HCV entry. OCLN is another TJ protein required for a post-binding step of HCV entry [31, 33], although its specific role in entry has not yet been elucidated.

Other cellular factors have been implicated in HCV entry, yet their functions remain enigmatic. For example, the Niemann-Pick C1-like 1 cholesterol absorption receptor interacts with virion-associated cholesterol to mediate binding or internalization steps [34]. Transferrin receptor 1 and cell death-inducing DFFA-like effector b are thought to be involved in late entry steps [35, 36]. Recently, the provirus integration site for Moloney murine leukemia virus (Pim1) kinase was identified as an additional HCV entry factor, perhaps by contributing to CD81-CLDN1 receptor complex formation via PI3K-Akt signaling [37]. The serum response factor binding protein 1 (SRFBP1) also interacts with CD81 to coordinate

host cell penetration [38]. SRFBP1 was proposed to mediate actin-dependent translocation following HBV receptor binding [38], and may also contribute to CD81-CLDN1 co-receptor complex assembly.

Ultimately, these virus-host interactions lead to the internalization of HCV into Rab5-containing early endosomes, where low pH induces viral fusion [26, 39]. The HCV fusion protein is still unknown. Although initial predictions pointed to E2 as being the fusion protein, the recently solved crystal structure of the E2 core ectodomain suggests otherwise [40, 41]. Indeed, the E2 ectodomain is globular and did not undergo conformational changes when exposed to low pH [40, 41], suggesting that E2 acts through a novel mechanism or that E1 may be involved in inducing membrane fusion [42]. Further mechanistic studies, and likely a structure of the E1/E2 heterodimer, will be necessary to elucidate the fusion mechanism.

Another route of HCV entry relies on direct cell-to-cell spread [43]. In this context, CD81, CLDN1 and SR-BI likely play key roles [43, 44], as do the viral envelope glycoproteins [45]. However, CD81 may be dispensable for cell-to-cell spread, at least in hepatoma cell lines [45]. Signaling pathways activated by EGFR may also contribute to cell-to-cell spread [27, 29]. Furthermore, virion-associated host factors such as ApoE have been implicated in cell-to-cell transmission [46].

**HCV entry and liver disease.** Viral entry is thought to play a major role in the pathogenesis of HCV infection. In the context of liver transplantation – which is severely hampered by rapid reinfection of the graft – it has been shown that viral quasispecies are rapidly selected following transplantation [47] and the resulting selection of viral variants contributes to pathogenesis. Indeed, escape from antibody-mediated neutralization selects for viral variants with a highly efficient entry phenotype associated with altered receptor usage [48]. Mutations in E2 that modulate

interactions with CD81 were implicated in mediating viral evasion at a post-binding step [49]. Altered usage of SR-B1 has also been observed [50], and increased levels of CLDN1 and OCLN modulate recurrence of HCV infection following liver transplantation [51]. These findings highlight viral entry as an important determinant for graft reinfection and the establishment of persistent infection. They also point to entry as an attractive therapeutic target, including preventing reinfection of the liver graft.

**Entry as a therapeutic target to address current limitations of DAAs.** HCV entry offers many advantages as an alternative antiviral target. Entry inhibitors block the virus life cycle at a step before persistent infection can be established. Indeed, in the absence of *de novo* infection, hepatocyte turnover likely results in the elimination of infected hepatocytes and leads to clearance of infection [52]. Furthermore, host-targeting agents aimed at entry factors have a higher genetic barrier for resistance, as the targets are not encoded by highly mutable viral genomes. Entry inhibitors also act synergistically with DAAs [53, 54], which would allow their incorporation into combination regimens. Interestingly, adding an entry inhibitor to DAA therapy reduces breakthrough of DAA-resistant variants, and entry inhibitors have been shown to have strong antiviral activity against DAA-resistant variants [55, 56]. Furthermore, many of the entry inhibitors including natural compounds may be produced at low costs offering a perspective to improve access to therapy in particular in countries or patients with limited resources. The complex and multi-step HCV entry process offers many antiviral targets, and our accumulating knowledge of the virus-host interactions involved in HCV entry opens perspectives to develop antivirals targeting these steps.

**Entry inhibitors in preclinical and clinical development.** Several compounds have been shown to block HCV binding. These include negatively charged small molecules, such as heparin, heparin-like compounds and polyphenols, which non-specifically compete for binding to cell-surface HSPGs [10, 11, 57-63]. Other molecules target specific receptor binding. For example, the small molecule 281816 (a dibenzothiepin derivative) disrupts the interaction between the HCV E2 protein and CD81 [64]. Similarly, oleanane-type triterpenes and the terpenoid saikosaponin b2 bind to E2 and disrupt E2-CD81 interactions to inhibit HCV entry [65, 66]. Monoclonal antibodies against CD81 and SR-BI also interfere with HCV binding [67-69] and to protect human liver chimeric mice from HCV infection [67-69]. Antibodies targeting SR-BI also reduced viral spread in already infected mice [68, 69]. Conversely, neutralizing antibodies targeting highly conserved epitopes on the viral envelope are also able to inhibit viral binding to CD81 [70-72]. Polyclonal immunoglobulins against HCV have been shown to protect human liver chimeric mice from HCV infection [73,74] and are being evaluated in a clinical trial in the context of graft reinfection (NCT01804829). Antibodies targeting non-virally encoded virion-associated epitopes such as ApoE also interfere with virion binding [9], as do peptides derived from ApoE [75].

Following the binding step, CD81-CLDN1 co-receptor complex formation is a critical step in HCV entry and therefore a most promising antiviral target. Furthermore, the CD81-CLDN1 co-receptor complex has no known physiological role, thereby limiting off-target effects. Monoclonal antibodies targeting the extracellular loops of CLDN1 inhibit CD81-CLDN1 association at a post-binding step. These antibodies inhibit infection by all major genotypes of HCV as well as patient isolates [76-78]. Furthermore, anti-CLDN1 monoclonal antibodies prevent



HCV infection in human liver chimeric mice [52, 79]. Notably, one such antibody cured chronically infected mice in monotherapy [52], providing proof-of-concept for the use of entry inhibitors to cure chronic viral infection. Similarly, peptides targeting CLDN1 inhibit HCV entry at a post-binding step [80]. Small molecules targeting EGFR and EphA2, kinases involved in CD81-CLDN1 association, block HCV infection in cell culture and in human liver chimeric mice [27]. An EGFR inhibitor, erlotinib, is in clinical trials for chronic HCV infection (NCT02126137).

Other post-binding steps in the HCV entry process are also targets. SR-BI receptor antagonists, including the arylketoamide ITX5061, inhibit HCV infection following binding [81]. ITX5061 is in a phase 1b clinical trial (NCT01560468). Arbidol (a synthetic indole) and silibinin (a flavonolignan from milk thistle) inhibit HCV clathrin-dependent endosomal trafficking by interfering with dynamin-2-mediated membrane scission [82, 83]. Ezetimibe, a small molecule in clinical use as a cholesterol-lowering agent, inhibits HCV infection by interfering with NPC1L1 internalization [34]. A clinical trial has been initiated to study its effects on chronically infected patients (NCT02126137). Recently, an antihistamine approved for allergy treatment, chlorcyclizine hydrochloride, was shown to inhibit infection by all genotypes of HCV, likely by targeting a late entry step linked to fusion [84].

Fusion is a critical step in the entry of enveloped viruses, including HCV. Indeed, the fusion inhibitor enfuvirtide – a peptide preventing conformational rearrangements of the human immunodeficiency virus (HIV) fusion protein – is approved to treat HIV infections. Our limited understanding of the HCV fusion mechanism currently prevents similar rational design approaches for HCV. However, peptides derived from E2 were shown to inhibit HCV infection at a post-binding step [85]. Furthermore, flunarizine (a piperazine derivative approved for the treatment of

migraine headaches) inhibits HCV genotype 2 fusion by targeting E2 and a potential fusion peptide within E1 [42]. An antimalarial compound, ferroquine, inhibited HCV fusion, possibly through its interactions with E1 [86]. HCV infectivity inhibitor-1 (HCV-II1) is thought to lock the HCV envelope in a pre-fusion conformation, thus blocking HCV fusion [87]. An HCV-specific triazine inhibitor, EI-1, interacts with E2 to inhibit a post-binding pre-fusion entry step [88].

Given that lipids play a central role in membrane fusion, molecules that target lipids also modulate fusion of enveloped viruses [89]. Lipid-mimicking rigid amphipathic fusion inhibitors insert into the lipid core of virion envelopes, where they block curvature changes required for fusion of HCV [90, 91]. Membrane fluidity is another critical determinant of fusion. Indeed, modulators of membrane fluidity such as phenothiazine derivatives, benzhydrylpiperazines and curcumin inhibit HCV fusion [92-94]. Similarly, polyunsaturated endoplasmic reticulum-targeting liposomes deplete cellular cholesterol levels to inhibit HCV fusion [95]. Clinical cholesterol-lowering drugs such as statins and ezetimibe inhibit HCV infection [34, 96], perhaps at least partially by modulating membrane fluidity. Type II photosensitizers such as amphiphilic thiazolidine derivatives (e.g. LJ001) generate singlet oxygen species that oxidize phospholipids, leading to biophysical alterations in viral envelopes [97, 98]. These alterations were proposed to increase positive curvature and reduce membrane fluidity, both of which inhibit membrane fusion by increasing the energetics required.

Clearly, many compounds acting by distinct mechanisms show great promise in pre-clinical models; ultimately, clinical studies will determine the future role of HCV entry inhibitors. Several clinical trials are ongoing and will reveal the perspectives for entry inhibitors against HCV to prevent liver graft reinfection or to treat patients who fail DAA-based therapy.

**Perspectives.** Since the discovery of HCV approximately 25 years ago, major advances in HCV model systems have enabled a detailed understanding of HCV virology and virus-host interactions. These advances allowed the development of DAAs targeting virus replication steps, which have dramatically improved the standard of care for chronically infected patients. Recent advances in the understanding of HCV entry and its clinical impact have set the stage for further development of novel antiviral approaches, which could address the current limitations of DAAs including resistance/failure and access to therapy.

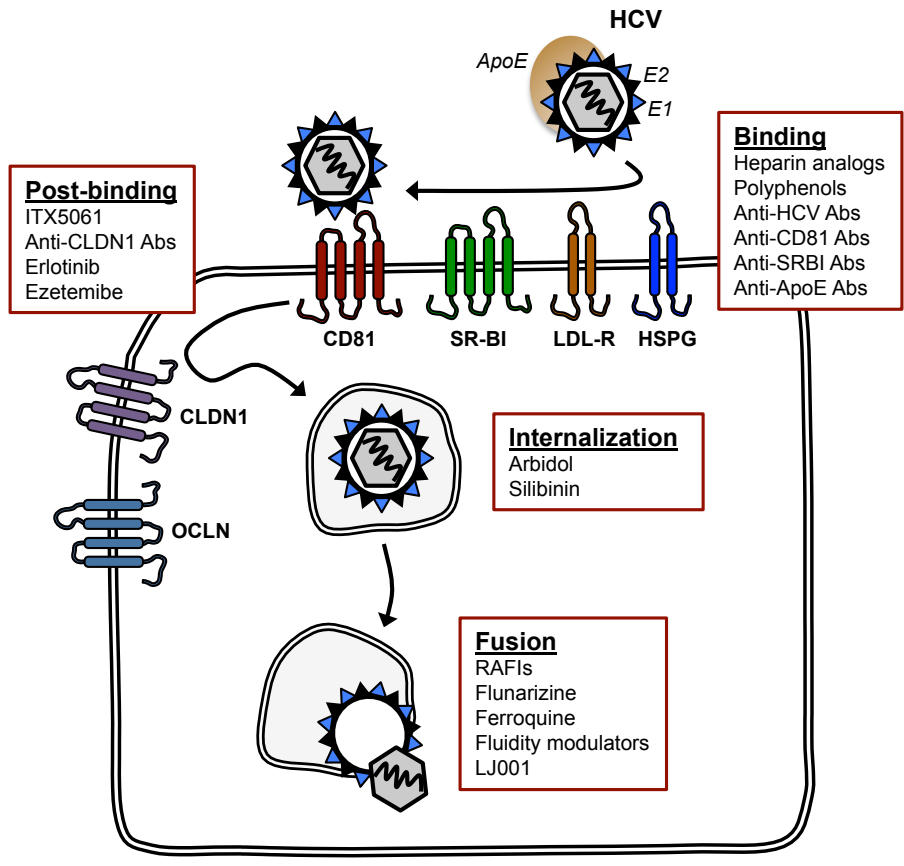
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**Conflict of interest:** the authors declare no conflict of interest. T.F.B. has served as an advisor on HCV antivirals for Biotest, Gilead and Vironexx. T.F.B is a co-inventor on a US patent on anti-Claudin-1 antibody for prevention and treatment of HCV infection filed by Inserm, University of Strasbourg and Genovac/Aldevron.

**Author contributions:** both authors contributed to the writing of the manuscript.

**Table 1.** HCV inhibitors targeting different steps of the HCV entry process. Inhibitors in **bold** are in clinical trials.

Entry step	Compound	Target/Mechanism	References
<b>Primary binding</b>	Heparin-like molecules	HCV-HSPG interaction	[6, 7, 8, 53]
	Epigallocatechin gallate	HCV-HSPG interaction	[54-56]
	Tannic acid	Docking of HCV at cell surface	[57]
	Gallic acid	Docking of HCV at cell surface	[58]
	Delphinidin	Docking of HCV at cell surface	[59]
<b>Specific binding</b>	281816	HCV E2-CD81 interaction	[60]
	Oleanane-type triterpenes	HCV E2-CD81 interaction	[61]
	Saikosaponin b2	HCV E2-CD81 interaction	[62]
	Anti-CD81 antibody	HCV E2-CD81 interaction	[63]
	Anti-SRBI antibody	HCV-SRBI interaction	[64, 65]
	Anti-ApoE antibody	HCV-associated ApoE-HSPG interaction	[5]
	Neutralizing antibodies	HCV E1/E2	[66-68]
<b>Polyclonal anti-HCV IgG</b>	HCV E1/E2	[69, 70]	
ApoE-derived peptide	HCV-associated ApoE-HSPG interaction	[71]	
<b>Post-binding</b>	Anti-CLDN1 antibodies	CD81-CLDN1 coreceptor complex	[48, 72-75]
	CLDN1-derived peptide	CD81-CLDN1 coreceptor complex	[76]
	<b>Erlotinib</b>	CD81-CLDN1 coreceptor complex; signaling	[23]
	<b>ITX5061</b>	SRBI lipid transfer activity	[77]
<b>Internalization</b>	Arbidol	HCV endosomal trafficking	[78]
	Silibinin	HCV endosomal trafficking	[79]
<b>Fusion</b>	Flunarizine	HCV genotype 2 fusion (E1 and/or E2)	[38]
	Ferroquine	HCV fusion (E1)	[81]
	RAFIs	HCV envelope curvature	[85, 86]
	Phenothiazines	HCV envelope fluidity	[87]
	Benzhydrylpiperazines	HCV envelope fluidity	[88]
	Curcumin	HCV envelope fluidity	[89]
	Polyunsaturated liposomes	Cellular membrane (cholesterol depletion)	[90]
	Statins	Cellular membrane (cholesterol depletion)	[91]
	<b>Ezetimibe</b>	Cellular membrane (cholesterol depletion)	[30]
	Photosensitizers (LJ001)	Lipid oxidation (viral envelope)	92, 93]



**Figure 1.** A simplified scheme of the HCV entry pathway, showing major viral and cellular determinants of viral entry. Inhibitors targeting the main entry steps are also shown.

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