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4 **Hepatitis C virus entry into hepatocytes:**

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6 **molecular mechanisms and targets for antiviral therapies**

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49 **Abbreviations:**

50
51 CLDN1: claudin-1; HCV: hepatitis C virus; HCVcc: cell culture-derived HCV; HCVpp: HCV

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53 pseudoparticle; HCV-LP: HCV-like particle; JFH1: Japanese fulminant hepatitis 1; OCLN:

54
55 occludin; SR-BI: scavenger receptor class B type I

Abstract

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma. Preventive modalities are absent and the current antiviral treatment is limited by resistance, toxicity and high costs. Viral entry is required for initiation, spread, and maintenance of infection, and thus is a promising target for antiviral therapy. HCV entry is a highly orchestrated process involving viral and host cell factors. These include the viral envelope glycoproteins E1 and E2, CD81, scavenger receptor BI and tight junction proteins claudin-1 and occludin. Recent studies in preclinical models and HCV-infected patients have demonstrated that the virus has developed multiple strategies to escape host immune responses during viral entry. These include evasion from neutralizing antibodies and viral spread by cell-cell transmission. These challenges have to be taken into account for the design of efficient antiviral strategies. Thus, a detailed understanding of the mechanisms of viral entry and escape is a prerequisite to define viral and cellular targets and develop novel preventive and therapeutic antivirals. This review summarizes the current knowledge about the molecular mechanisms of HCV entry into hepatocytes, highlights novel targets and reviews the current preclinical and clinical development of compounds targeting entry. Proof-of-concept studies suggest that HCV entry inhibitors are a novel and promising class of antivirals widening the preventive and therapeutic arsenal against HCV infection.

1 Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. The current therapy
2 against HCV infection, consisting of an association of pegylated interferon alpha (PEG-IFN)
3 and ribavirin, is limited by resistance, adverse effects and high costs. Although the clinical
4 development of novel antivirals targeting HCV protein processing has been shown to
5 improve sustained virological response, toxicity of the individual compounds and
6 development of viral resistance remain major challenges [1-3]. To date, a vaccine is not
7 available. The absence of preventive strategies is a major limitation for patients undergoing
8 liver transplantation (LT) for HCV-related end-stage liver disease. Re-infection of the graft is
9 universal and characterized by accelerated progression of liver disease [4]. Moreover,
10 treatment of recurrent HCV infection after LT is challenging due to enhanced adverse effects
11 and limited efficacy of IFN-based therapies in LT recipients [4, 5]. Recurrent HCV liver
12 disease in the graft with poor outcome has become an increasing problem facing
13 hepatologists and transplant surgeons. Thus, novel antiviral preventive and therapeutic
14 strategies are urgently needed.

15 Viral entry is the first step of virus-host cell interactions leading to productive infection
16 and thus represents an interesting target for antiviral therapy. HCV entry is believed to be a
17 highly orchestrated process involving several viral and host cell factors, thereby offering
18 multiple novel targets for antiviral therapy. However, multiple strategies evolved by the virus
19 in order to escape the host immune system, such as escape from neutralizing antibodies and
20 direct cell-cell transmission, have to be taken into account for the design of efficient novel
21 antiviral strategies. Understanding the mechanisms of viral entry and escape are thus a
22 prerequisite to define viral and cellular targets that will give broad protection against HCV
23 infection.

24 HCV is an enveloped single-strand RNA virus that mainly targets hepatocytes. Due to
25 the difficulty to grow HCV *in vitro* and the species specificity of this virus, surrogate model
26 systems have been developed to study HCV entry into hepatocytes: recombinant envelope
27 glycoproteins [6], HCV-like particles (HCV-LP) [7], HCV pseudoparticles (HCVpp) [8, 9] and
28 recombinant infectious HCV (HCVcc) [10-12] have been used to study interactions of the

1 viral envelope with human hepatoma cells or primary human hepatocytes. Moreover, the use
2 of transgenic immunodeficient mice with hepatocyte-lethal phenotype (Alb-uPA/SCID [13]
3 and Fah/Rag2/IL2 γ mice [14]) that can be successfully transplanted with primary human
4 hepatocytes allowed to establish a small animal model to study certain aspects of HCV
5 infection *in vivo* [15, 16].

6 Using the above described model systems, tremendous progress has been made
7 over the past years in deciphering the mechanisms of HCV-host interactions leading to viral
8 entry. The understanding of these mechanisms has allowed researchers to identify novel
9 targets for antivirals and several compounds are reaching early clinical development. The
10 aim of this review is to summarize the current knowledge about the complex mechanisms of
11 HCV entry into host cells, highlight antiviral targets and review the current development of
12 HCV entry inhibitors that represent a novel important class of antivirals. Developing efficient
13 HCV entry inhibitors may hold promise to improve sustained virological response in chronic
14 HCV-infected patients and prevent HCV re-infection during LT.

16 **Hepatitis C virus evades host immune responses to enter the hepatocyte**

17 Viral entry is the first step of HCV infection that requires interaction of the HCV envelope
18 glycoproteins E1 and E2 and the host cell membrane. E1 and E2 are type I transmembrane
19 proteins with a N-terminal ectodomain and a short C-terminal transmembrane domain (TMD).
20 Functional virion-associated E1E2 envelope glycoproteins mediating viral entry form large
21 covalent complexes are stabilized by disulfide bridges [17]. The TMD plays a major role in
22 the biogenesis of the E1E2 complexes and membrane fusion process [18]. The N-terminal
23 ectodomains of E1 and E2 are heavily glycosylated. The glycans play a major role in E1E2
24 folding as well as HCV entry [19] and are of crucial importance for the evasion from the host
25 immune responses by masking immunogenic envelope epitopes [20]. Moreover, HCV exists
26 in heterogenous forms in human serum and may be associated with VLDL, LDL and HDL
27 [21-24] also shielding the virus from neutralizing antibodies targeting the HCV envelope
28 glycoproteins.

1 Both E1 and E2 contain putative fusion domains [25, 26]. While the role of E1 in HCV
2 entry is not completely understood, several E2 domains play pivotal roles in viral entry, i.e.
3 putative domain binding to two HCV entry factors, CD81 and scavenger receptor class B
4 type I (SR-BI), and escape from host immune responses. Hypervariable regions (HVR) have
5 been identified in E2. The first 27 amino acids of E2 called hypervariable region 1 (HVR1),
6 are most divergent among HCV isolates. HVR1 plays an important role in viral fitness, likely
7 due to an involvement in SR-BI-mediated entry [27], assembly and release of virus particles
8 [28] as well as HCV membrane fusion process [28]. HVR1 is a target for neutralizing
9 antibodies. However, due to its high variability, antibodies targeting HVR1 exhibit poor cross-
10 neutralization potency across different HCV isolates [29]. Broadly neutralizing antibodies are
11 directed against conserved conformational epitopes within E2 [30, 31] and mostly inhibit E2-
12 CD81 interaction [32]. The region located immediately downstream of HVR1 contains a
13 potent and highly conserved epitope. This epitope defined by the mouse monoclonal
14 antibody (mAb) AP33 and a rat mAb 3/11, is involved in E2-CD81 [33] and E2-heparan
15 sulfate interaction [34]. Importantly, mutated variants that escape from AP33 neutralization
16 show very low infectivity [35]. Recently, new conformational and conserved epitopes were
17 identified in the N-terminal part of E2. Antibodies targeting these epitopes neutralize
18 genetically diverse HCV isolates and protect against heterologous HCV quasispecies
19 challenge in the human liver-chimeric Alb-uPA/SCID mouse model [31]. Since these epitopes
20 are thought to be involved in HCV entry, viral mutation could induce escape from broadly
21 neutralizing antibodies but at a substantial cost in viral fitness [35]. The conserved nature of
22 these epitopes makes them of interest for vaccine and immunotherapeutic development.

23 *In vivo*, humoral responses are thought to play an important role in controlling HCV
24 infection. Indeed, spontaneous responders tend to have early induction of neutralizing antibody
25 responses, whereas chronically evolving subjects have delayed initiation of neutralizing
26 antibody responses [36-38]. Furthermore, the generation of cross-reactive humoral
27 responses is associated with protection against HCV reinfection [39]. These data suggest
28 that protective immunity following HCV infection is possible and highlights the plausibility of

1 preventive antiviral strategies including a vaccine [39]. However, the accelerate evolution [40]
2 and diversity of HCV as well as the variety of strategies the virus evolved to escape antibody-
3 mediated neutralization (reviewed in [41]) are a major challenge. Indeed, due to its very high
4 replication rate and the highly error prone viral polymerase, HCV circulates as a pool of
5 genetically distinct but closely related variants known as viral quasispecies. The capacity of
6 HCV to mutate continuously allows a high plasticity, an ability of the virus to adapt to variable
7 environmental conditions and escape the host's immune responses leading to HCV
8 persistence [42, 43]. Noteworthy, a recent longitudinal analysis of six HCV-infected patients
9 undergoing LT suggests that efficient entry and escape from host neutralizing antibodies
10 represent important mechanisms for selection of HCV during LT [43]. As strains selected
11 during LT could be neutralized by broadly neutralizing antibodies, the major challenge for
12 developing efficient antiviral strategies targeting the HCV envelope glycoproteins will be to
13 identify epitopes largely conserved among genotypes and selected isolates.

14 15 **Hepatitis C virus uses multiple host factors to enter its target cell**

16 HCV attachment and entry into host cells is a complex and multistep process. Using various
17 model systems, several cell surface molecules have been identified interacting with HCV.
18 These include CD81 [44], the low-density lipoprotein (LDL) receptor [21], highly sulfated
19 heparan sulfate (HS) [45], SR-BI [46], DC-SIGN (dendritic cell-specific intercellular adhesion
20 molecule 3 grabbing non integrin)/L-SIGN (DC-SIGNr, liver and lymph node specific) [47,
21 48], claudin-1 (CLDN1) [49] and occludin (OCLN) [50-52].

22 *In vivo*, HCV enters the liver through the sinusoidal blood. Capture of circulating HCV
23 particles by liver sinusoidal cells may thus facilitate viral infection of neighbouring
24 hepatocytes which are not in direct contact with circulating blood. This process may be
25 mediated by DC-SIGN, expressed in Kupffer cells that localize close to liver sinusoidal
26 endothelial cells (LSEC) and hepatocytes [53], and L-SIGN that is highly expressed in LSEC.
27 DC-SIGN and L-SIGN have been shown to bind envelope glycoprotein E2 with high affinity
28 [47, 54]. On hepatocytes, HS glycosaminoglycans represent first attachment sites [34, 45,

1 55] that may help to concentrate the virus on the target cell surface and allow further
2 interactions with other host factors triggering viral entry.

3 CD81 is a ubiquitously expressed 25 kDa tetraspanin, containing a small extracellular
4 and a large extracellular loop (LEL). CD81 has been the first molecule described to interact
5 with a soluble truncated form of HCV E2 and to be a critical host cell factor for viral entry [11,
6 56, 57]. The LEL seems to play an important role in this process as soluble recombinants
7 forms of CD81 LEL have been shown to inhibit HCVpp and HCVcc infection [58]. Several
8 amino acid residues critical for E2-CD81 binding have been identified throughout the CD81
9 LEL and HCV E2 [33, 44, 59-61]. In recent years, studies using HCVpp and HCVcc have
10 provided additional valuable information about E2-CD81 interactions and highlighted the
11 importance of E2 residues at positions 415, 420, 527, 529, 530, 535 [62, 63] for virus
12 particle-CD81 interaction.

13 Human SR-BI or CLA-1 (CD36 and LIMPII Analogous-1) is a 82 kDa glycoprotein with
14 a large extracellular loop highly expressed in the liver and steroidogenic tissues [64]. SR-BI
15 binds a variety of lipoproteins (HDL, LDL, oxLDL) and is involved in bidirectional cholesterol
16 transport at the cell membrane. The SR-BI extracellular loop has been demonstrated to
17 interact with E2 HVR1 [46]. Recent evidence suggests that amino acids 70 to 87 and the
18 single residue E210 of SR-BI are required for E2 recognition [65]. SR-BI may play a dual role
19 during the HCV entry process, during both binding and postbinding steps [65, 66].
20 Physiological SR-BI ligands have been shown to modulate HCV infection: HDL is able to
21 enhance HCVpp and HCVcc infection [67, 68] whereas oxidized LDL inhibits HCVpp and
22 HCVcc infection [69]. Interestingly, high concentrations of HDL and LDL inhibited HCV
23 replication in human hepatocytes infected with serum-derived HCV [70]. Moreover, using
24 serum-derived HCV, it has been suggested that the virus-associated lipoproteins rather than
25 the E2 protein interact with SR-BI on transfected CHO cells [71]. A recent mapping study
26 reported that HCV and HDL binding to SR-BI as well as the lipid transfer properties of SR-BI
27 are required for SR-BI function as HCV entry factor [72]. This study also suggests that the C-
28 terminal cytoplasmic tail of SR-BI modulates the basal HCV entry process, but seems not to

1 influence HDL-mediated infection-enhancement whereas the extracellular domain is required
2 for E2 binding and lipid transfer function [72]. Taken together, these results suggest the
3 existence of a complex interplay between lipoproteins, SR-BI and HCV envelope
4 glycoproteins for HCV entry that needs to be taken into account for the development of
5 antivirals targeting SR-BI.

6 CLDN1, a 23 kDa four transmembrane protein, has been identified as a critical HCV
7 hepatocyte entry factor by expression cloning [49]. Interestingly, CLDN6 and CLDN9 are also
8 able to mediate HCV entry in hepatoma cells [73, 74]. CLDNs are critical components of tight
9 junctions (TJ) regulating paracellular permeability and polarity. CLDN1 is expressed in all
10 epithelial tissues but predominantly in the liver [75]. Noteworthy, CLDN1 may localize to TJ of
11 hepatocytes but also to basolateral surfaces of these cells [76]. Recent studies suggest that
12 non-junctional CLDN1 may be involved in HCV entry [49, 77] probably during a post-binding
13 step [49, 78]. So far, no direct HCV-CLDN1 interaction has been demonstrated [49, 78].
14 Mapping studies suggest that the first extracellular loop (ECL1), and more particularly
15 residues in the highly conserved claudin motif W(30)-GLW(51)-C(54)-C(64), are critical for
16 HCV entry [49, 77]. CLDN1 associates to CD81 in a variety of cell types and the formation of
17 CLDN1-CD81 complexes is essential for HCV infection [79, 80]. Mutations at residues 32
18 and 48 in CLDN1 ECL1 ablate the association with CD81 and the viral receptor activity [80].

19 OCLN has been identified as another host cell factor critical for HCV entry, probably
20 at a late post-binding event [50, 51, 81]. OCLN is a 65 kDa four transmembrane protein
21 expressed in TJ of polarized cells. To date, there is no evidence of a direct interaction with
22 HCV. It is worth noting that OCLN has been reported to be one of the two HCV host entry
23 factors responsible for the species specificity of HCV: expression of human OCLN and
24 human CD81 may confer HCV permissivity to mouse cell lines [50]. The species-specific
25 determinants of this protein have been mapped to the second extracellular loop [50].
26 Interestingly, OCLN expression on hepatocytes as well as HCV entry are increased upon
27 glucocorticoid treatment [82] while OCLN expression is downregulated upon HCV infection to

1 prevent superinfection [51]. Further studies are needed to decipher the interplay between
2 HCV, OCLN and the other known host factors.

3 As HCV circulates in the blood in association with LDL and very low-density
4 lipoproteins (VLDL), the LDL receptor has also been proposed as an attachment and/or entry
5 factor for HCV [21, 83]. As HCVpp are not associated with lipoproteins, studies investigating
6 the role of LDLR in HCVpp entry did not show a major role for LDLR [8]. Moreover, no direct
7 interaction between envelope glycoprotein E2 and LDL or LDLR was demonstrated [83].
8 However, the LDLR has been shown to mediate internalization of serum-derived HCV into
9 CD81-deficient HepG2 cells by binding virus-LDL particles [21]. This will have to be taken
10 into account for the development of antiviral therapies targeting HCV host factors.

11 **HCV entry is a multistep process**

12 *In vivo*, HCV most likely first interacts with the basolateral surfaces of hepatocytes. HS
13 glycosaminoglycans represent first attachment sites [34, 45, 55] before the virus interacts
14 with several entry factors, SR-BI [27, 46, 66, 68, 84], CD81 [44, 55], CLDN1 [49, 78] and
15 OCLN [50-52] (Figure 1). It is worth noting that all these entry factors are required for
16 productive HCV infection. This suggests that HCV entry may be mediated through the
17 formation of a tightly orchestrated HCV-entry factor complex at the plasma membrane [66,
18 78]. First evidence for such co-entry factor complexes has been provided by fluorescence
19 resonance energy transfer (FRET) studies demonstrating the role of CLDN1-CD81
20 complexes in HCV infection [79, 80]. The fact that only members of the CLDN family
21 supporting HCV entry, i. e. CLDN1, CLDN6 and CLDN9, were able to form complexes with
22 CD81 suggests that CLDN-CD81 complex formation is essential for HCV entry [78, 80]. To
23 date, our knowledge about the molecular mechanisms of potential other co-factor
24 association(s) is still rudimental. First studies showed that the majority of CLDN1 proteins at
25 the plasma membrane interact with OCLN but did not show any relationship between
26 CLDN1-OCLN association and HCV infection [80]. In addition, it has been demonstrated that
27 cell contacts modulate SR-BI and CLDN1 expression levels and favours HCV internalization

1 through facilitation of entry factor complexes [85]. Further studies are thus necessary to
2 assess which set of host factors are present with HCV in these complexes.

3 To date, the sequence of events leading from HCV-interaction with host factors on the
4 plasma membrane to internalization, viral fusion and replication still remains elusive. Studies
5 using HCVpp and HCVcc have demonstrated that HCV entry into both hepatoma cells and
6 primary human hepatocytes depends on clathrin-mediated endocytosis [86-90], the most
7 commonly route of endocytosis for viruses that require internalization. Moreover, actin and
8 clathrin-actin associations have also been shown to be involved in efficient HCV endocytosis
9 [90]. The question whether all or part of the plasma membrane expressed HCV host factors
10 internalize together with HCV still remains unanswered. A recent study suggests that during
11 internalization, HCV associates with CD81 and CLDN1 [90]. Moreover, PKA has been
12 suggested to play a role during this process as inhibition of PKA lead to reorganization of
13 CLDN1 from the plasma membrane to intracellular vesicular location(s) and disrupted CD81-
14 CLDN1 co-receptor association [91]. Interestingly, in line with the fact that polarization
15 restricts HCV entry [92] and that HCV co-entry factors are co-expressed on basolateral sites
16 of hepatocytes but not at TJ [76], imaging studies suggest that HCV internalization does not
17 preferentially take place at sites of cell-cell contacts [90].

18 Clathrin-mediated endocytosis transports incoming viruses together with their
19 receptors into early and late endosomes [93]. HCVpp have been suggested to be delivered
20 to early but not late endosomes [87]. This is in line with recent imaging data showing
21 colocalization between HCV and Rab5a, an early endosome marker [90]. The acidic pH in
22 endosomes provides an essential cue that triggers penetration and uncoating. Penetration of
23 enveloped virus occurs by membrane fusion catalyzed by fusion peptides embedded in the
24 viral envelope glycoproteins [94]. To date, the mechanisms of HCV fusion have not been
25 completely elucidated but it has been suggested that similar fusion mechanisms as
26 described for other flaviviridae may apply to HCV [95-98]. This hypothesis is supported by
27 the observation that HCVpp entry [8, 9, 99] and HCVcc infection [86, 100] are pH-dependent,
28 suggesting that a pH-dependent membrane fusion process may be required for delivery of

1 the HCV genome into the host cell cytosol. It is worth noting that although HCV entry
2 requires an acidification step, extracellular HCV is resistant to low pH treatment [87, 100]. As
3 HCV fusion kinetics are delayed as compared to other viruses, it has been suggested that
4 after internalization, HCVpp entry necessitates additional, low-pH-dependent interactions,
5 modifications, or trafficking [87]. However, neither HCVpp nor HCVcc require cleavage by
6 endosomal proteases for fusion [87, 100]. Several *in vitro* fusion assays have been set up in
7 the last years [25, 26, 49, 99]. Liposome/HCVpp fusion assays suggest that HCVpp-induced
8 fusion was low pH and temperature dependent and facilitated by cholesterol [99].
9 Interestingly, patient-derived anti-HCV antibodies were able to inhibit liposome/HCVpp fusion
10 [101] highlighting the importance of HCV envelope glycoproteins in this process. These data
11 have been recently confirmed in a novel liposome/HCVcc fusion assay showing that HCVcc
12 fusion was dependent on pH, lipid composition of both viral and target membranes and HCV
13 E2 [102]. However, in this kind of assay no host cell factor is necessary to allow fusion to
14 occur. To study the role of both viral and host factors in HCV fusion, cell-cell fusion assays
15 have been used where HCV envelope glycoproteins are expressed on one cell type and host
16 entry factors on another cell type [25]. Cell-cell fusion assays are also pH-dependent and
17 most interestingly, these assays highlighted the importance of CD81 and CLDN1 in this
18 process [25, 49]. To date, it still remains unclear whether these host factors directly
19 participate in the HCV fusion process or whether they play a role in an earlier entry steps
20 required to enable efficient subsequent fusion. Taken together, these data suggest that HCV
21 internalization and fusion offer multiple targets for the development of HCV entry inhibitors.

22 23 **An alternative route of entry and spread by cell-cell transmission**

24 The above described entry mechanisms have been unravelled using cell-free HCV, i. e. the
25 virus infects surrounding cells after the formation of viral particles that are released from
26 infected cells and enter naïve cells by a host factor-dependent mechanism. In addition,
27 viruses may also use direct cell-cell transfer to infect neighbouring cells [93] thereby
28 escaping potential interaction with neutralizing antibodies in the extracellular milieu.

1 Direct cell-cell transfer or neutralizing antibody-resistant transmission has been
2 described for HCV [103]. CLDN1, CD81 and probably SR-BI are involved in this process [85,
3 103]. Interestingly, CD81-independent routes of cell-cell transport have also been described
4 [103, 104]. Direct cell- cell transfer has an important impact for the development of antivirals
5 as this process allows viral spreading by escaping extracellular neutralizing antibodies as
6 well as defined antibodies interfering with host cell entry factors. It will be challenging to
7 develop novel anti-HCV therapeutics interfering with this process.

9 **Viral entry offers promising targets for antiviral therapy**

10 In contrast to the current standard of care therapy for HCV infection, new therapeutic
11 approaches aim at the development of more specific compounds targeting the virus and/or
12 host cell factors. This represents the concept of specifically targeted antiviral therapy for HCV
13 (STAT-C). This concept consists in developing more efficient and better tolerated
14 combination therapies that need shorter treatment periods. To date, several small molecule
15 compounds targeting the HCV non-structural proteins including protease, polymerase and
16 NS5A have been developed and are at various stages of clinical development [1-3, 105].
17 First clinical trial data are promising but toxicity of the individual compounds and emergence
18 of resistance against these drugs limit their use in monotherapy. This suggests that
19 additional drugs, ideally targeting different steps of the viral life cycle, are needed for efficient
20 anti-HCV therapy.

21 HCV entry into target cells is a promising target for preventive and therapeutic
22 antiviral strategies since it is essential for initiation, spread and maintenance of infection.
23 Interfering with HCV entry holds promise for drug design and offers several targets: (i)
24 blocking virus-target cell interaction during attachment and binding, (ii) interfering with post-
25 binding events, and (iii) interfering with viral fusion (Figure 1). Various modalities may be
26 developed as HCV entry inhibitors: these include neutralizing antibodies targeting the viral
27 envelope and inhibitory/blocking antibodies targeting host cell surface factors as well as
28 small molecule compounds or siRNAs against host cell factors or viral proteins [106, 107].

1 Several non-HCV specific molecules interfering with HCV envelope glycoproteins and
2 abrogating viral attachment have been described. As HCV envelope proteins are highly
3 glycosylated, molecules interfering with glycoproteins may possess antiviral activity against
4 HCV. As shown for HIV, targeting the glycans may represent a new therapeutic concept for
5 controlling HCV infection [108]. Carbohydrate-binding agents that interact with the viral-
6 envelope glycans may compromise the efficient entry of the virus into its susceptible target
7 cells and induce a progressive creation of deletions in the envelope glycan shield, thereby
8 triggering the immune system to act against previously hidden immunogenic epitopes of the
9 viral envelope [108]. The lectin cyanovirin-N (CV-N) interacts with high-mannose
10 oligosaccharides on viral envelope glycoproteins and has been demonstrated to have
11 antiviral activity against several enveloped viruses [109-111]. It has been shown that
12 oligomannose glycans within the HCV envelope glycoproteins interact with CV-N resulting in
13 HCV antiviral activity by blocking HCV entry into target cell [112]. As most of the HCV
14 glycosylation sites are highly conserved, drugs that target glycans on HCV glycoproteins may
15 not lead so rapidly to viral escape/resistance as it is the case for HIV [113]. Other
16 carbohydrate-binding agents that have been shown to prevent HIV infectivity [108] might also
17 be efficient against other viruses that require a glycosylated envelope for entry into target
18 cells. Interfering with interaction of viral envelope proteins and glycosaminoglycans on the
19 cell surface is another way to abrogate viral attachment. HS glycosaminoglycans mediate
20 HCV and dengue virus binding to host cells and heparin, a structural analogue of HS, has
21 been demonstrated to inhibit dengue virus infection as well as HCV E2, HCVpp, HCV-LP
22 and HCVcc binding to hepatoma cells [34, 45, 55, 114]. HS-like molecules and semisynthetic
23 derivatives are already explored as an antiviral approach against dengue virus infection
24 [115]. Such molecules may also have antiviral activity against HCV.

25 Neutralization of the viral particle may be achieved by targeting the HCV envelope or
26 host derived factors associated with the mature viral particle. The molecular mechanisms of
27 viral assembly and the exact composition of released HCV particles still remain elusive but
28 recent studies suggest that HCV and VLDL assembly are closely linked [116, 117].

1 Noteworthy, apolipoprotein E (apoE) is required for HCV assembly [118, 119] and is also part
2 of infectious HCV particles [118]. Interestingly, anti-apoE antibodies are able to inhibit HCV
3 entry [21, 118] suggesting that HCV may be neutralized using compounds directed against
4 the lipoprotein moiety of the viral particle (Table1).

5 Viral attachment and entry is a major target of adaptive host cell defenses and anti-
6 HCV antibodies represent unique tools to interfere with the HCV entry process. Virus-specific
7 neutralizing antibodies are defined by their antiviral activity enabling them to block viral entry
8 and control viral spread. Neutralizing antibodies may interfere with different steps of the viral
9 entry process such as attachment, post-binding steps and fusion [120]. Two studies of large-
10 scale accidental HCV infections demonstrated that rapid induction of neutralizing antibodies
11 in the early phase of infection correlates with viral clearance or control of infection [36, 38].
12 These studies suggest that neutralizing antibodies represent an interesting approach for the
13 development of novel preventive and therapeutic antiviral strategies (Table 1). In line with
14 this concept, it has been shown that immunoglobulins prepared from unscreened donors or
15 from selected patients with chronic HCV infection have prevented HCV infection in recipients
16 when administered before exposure to the virus [6, 121]. Moreover, administration of
17 polyclonal immunoglobulins from a chronically infected patient conveyed sterilizing immunity
18 toward a homologous strain in human liver-chimeric Alb-uPA/SCID mouse model [122].
19 Human mAbs provide an attractive alternative to polyclonal immune globulin for
20 immunotherapy, since mAbs can be more readily standardized. The recent production of
21 human mAbs efficiently cross-neutralizing HCV may represent an important step for the
22 development of immunopreventive strategies against HCV infection [31, 123-125] as such
23 antibodies have been demonstrated to protect against HCV quasispecies challenges *in vivo*
24 in the human liver-chimeric Alb-uPA/SCID mouse model [31] (Table1). However, due to the
25 high variability of HCV, it will be a major challenge to develop efficient cross-neutralizing
26 antibodies able to target conserved epitopes across all genotypes to avoid escape.
27 Examples for neutralizing antibodies in preclinical or clinical development are provided in
28 Table 1.

1 Targeting the host entry factors, which are indispensable for the propagation of the
2 virus, represents an additional approach for the development of antivirals because they may
3 impose a higher genetic barrier for resistance. HCV interaction with host entry factors offers
4 multiple targets for the development of specific entry inhibitors (Table 1).

5 CD81 is one of these potential targets. Imidazole based compounds mimicking an
6 alpha helix in the LEL of CD81 compete for HCV E2-CD81 binding. These drugs bind E2 in a
7 reversible manner and block E2-CD81 interaction while having no effect on CD81 expression
8 nor on CD81 interaction with physiological partner molecules [126]. Interestingly, anti-CD81
9 antibodies inhibiting HCV infection *in vitro* have also been demonstrated to prevent HCV
10 infection in the human liver-chimeric Alb-uPA/SCID mouse model [127]. This study suggests
11 that targeting CD81 may be an efficient strategy to prevent HCV infection *in vivo* and
12 demonstrates the proof-of-concept that anti-receptor antibodies prevent HCV infection in a
13 clinically relevant animal model,.

14 SR-BI binds a wide variety of molecules and is thus another interesting target for anti-
15 HCV drugs. SR-BI binds and internalizes serum amyloid A (SAA), an acute phase protein
16 produced in the liver [128, 129]. SAA inhibits HCV entry by interacting with the virus thereby
17 reducing its infectivity [130]. Anti-SR-BI antibodies blocking interaction with HCV are another
18 interesting strategy to prevent HCV entry. Anti-SR-BI antibodies have been demonstrated to
19 inhibit HCVcc infection *in vitro* [66, 67, 131]. Finally, small molecule inhibitors of SR-BI have
20 recently been developed. ITX5061 is a compound that inhibits entry of HCVpp from all major
21 genotypes and HCVcc infection without affecting viral replication [132]. Kinetic studies
22 suggest that this small molecule inhibitor targets HCV entry during an early post-binding
23 stage [132]. The safety of this compound has been evaluated in patients for another clinical
24 indication [132] allowing future clinical trials in HCV infected patients.

25 CLDN1 is a promising antiviral target since it is essential for HCV entry and to date
26 there is no evidence for CLDN1-independent HCV entry. Furthermore, CLDN1 has been
27 suggested to play an important role in cell-cell transmission. In contrast to other HCV entry
28 factors such as CD81 or SR-BI, CLDN1 is predominantly expressed in the liver. Recently,

1 anti-CLDN1 antibodies inhibiting HCV infection *in vitro* have been developed [78, 133]. Anti-
2 CLDN1 antibodies inhibit HCV infectivity by reducing HCV E2 association with the cell
3 surface and disrupting CLDN1-CD81 interactions [78]. Interestingly, monoclonal anti-CLDN1
4 antibodies efficiently block cell entry of highly infectious escape variants of HCV that are
5 resistant to host neutralizing antibodies [133]. These data suggest that anti-CLDN1
6 antibodies might be used to prevent HCV infection, such as after liver transplantation, and
7 might also restrain virus spread in chronically infected patients. [133].

8 Finally, OCLN may also be considered as a potential target for interfering with HCV
9 entry. To date, no anti-OCLN antibodies inhibiting HCV infection have been described.
10 Further characterization of the role of this host cell factor in the HCV entry process may lead
11 to designing compounds interfering with OCLN and inhibiting HCV entry.

12 In addition to cell surface expressed host factors, HCV internalization and fusion are
13 complex processes that also offer several targets for antivirals. Long phosphorothioate
14 oligonucleotides (PS-ON), are a promising new class of antiviral compounds. These
15 amphipathic DNA polymers display a sequence-independent antiviral activity against HIV by
16 blocking virus-cell fusion [134]. A recent study demonstrated that PS-ON inhibited HCV
17 internalization without affecting viral binding and replication [135]. Noteworthy, PS-ON block
18 *de novo* HCV infection in the human liver-chimeric Alb-uPA/SCID mouse model [135]
19 highlighting the promise of PS-ON as future clinical HCV entry inhibitors (Table 1). A peptide-
20 based HIV fusion inhibitor (Enfuvirtide) has already been approved for treatment of HIV
21 infected patients [136]. As HCV fusion requires acidification of the endosome, molecules able
22 to prevent acidification of endosomes, such as chloroquine, prevent HCV fusion *in vitro* [86,
23 100]. In the last years, other compounds interfering with HCV fusion have been described
24 (Table 1). Arbidol is a broad-spectrum antiviral that has already been evaluated in humans
25 indicating good safety and tolerability (for review see [137]). Arbidol inhibits HCV fusion in the
26 HCVpp-liposome assay and prevents HCVpp and HCVcc infection *in vitro* [137]. In addition,
27 this molecule also targets other steps of the viral life cycle such as replication [138].
28 Silymarin is another compound inhibiting HCVpp-liposome fusion as well as other steps of

1 the HCV life cycle, such as replication, protein expression and infectious virus production
2 without affecting viral assembly [139, 140]. Interestingly, silymarin inhibited HCV infection *in*
3 *vitro* irrespective of the entry route, i. e. cell-free and cell-cell transmission [140], highlighting
4 the potential of such drugs for *in vivo* use.

6 **Conclusions and perspectives**

7 In recent years, substantial progress unravelling the molecular mechanisms of HCV entry
8 has been made and revealed a multitude of novel targets for antivirals. Several compounds
9 interfering with HCV entry have been demonstrated to efficiently inhibit HCV infection using
10 *in vitro* assays or state of the art animal models and may thus be valuable for future anti-HCV
11 therapy or prevention of HCV infection during LT. As for other chronic viral infections such as
12 HIV, the future therapeutic and preventive approach for HCV infection will probably be based
13 on the combination of several drugs [2, 3]. HCV entry inhibitors represent a promising class
14 of novel antivirals since they are complementary to current approaches and target an
15 essential step of the viral life cycle. Indeed, the first compounds have reached the early stage
16 of clinical development. Moreover, recent data suggest that combination of antivirals
17 targeting the virus and host factors such as CLDN1 act in an additive manner in suppressing
18 HCV infection [133]. Thus, combining compounds targeting viral and host cell factors and
19 complementary steps of the viral life cycle such as entry and replication is a promising
20 approach for prevention of infection in LT and cure of chronic HCV infection.

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Conflict of Interest

The authors declare no conflict of interest. Inserm, the University of Strasbourg and Genovac have filed a patent application on monoclonal anti-claudin 1 antibodies for the inhibition of hepatitis C virus infection.

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Figure legend

Figure 1. Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets

for antiviral therapies. A model of HCV life cycle with potential targets for virus neutralizing antibodies and other entry inhibitors is shown. HCV is believed to first interact with HS and LDLR on the basolateral membrane surface of hepatocytes to allow concentration of the virion. Subsequently, interaction with other host factors such as SR-BI, CD81, CLDN1 and OCLN ultimately leads to viral internalization via clathrin-mediated endocytosis. For CLDN1 both junctional and non junctional forms have been described (for review see [141]). Fusion between viral and endosomal membranes is followed by release of the viral genome into the cytosol where translation and replication take place. HCV particles are then assembled and released from the host cell. An alternative route of viral entry is direct cell-cell transmission which is resistant to neutralizing antibodies. Entry inhibitors can potentially interfere with the viral life cycle at different steps, i. e. viral binding, post-binding and fusion. Ab: antibody; apo: apolipoprotein; BC: bile canaliculi; CLDN-1: claudin 1; HCV: hepatitis C virus; HS: heparan sulfate; JAM: junction-associated adhesion molecule; LDLR: low-density lipoprotein receptor; nAb: neutralizing antibody; OCLN: occludin; PS-ON: phosphorothioate oligonucleotides; SR-BI: scavenger receptor class B type I; ZO: zona occludens.

Table Legend

Table 1. Examples of compounds targeting viral entry. Targets within the HCV entry process are depicted, followed by examples of compounds targeting the respective entry step. Stage of development and references are indicated. CLDN: claudin; mAb: monoclonal antibody; pAb: polyclonal antibodies; SR-BI: scavenger receptor class B type I; PS-ON: phosphorothioate oligonucleotides.

1 **Key points 1**

- 2 • HCV entry into hepatocytes is a highly coordinated and multistep process requiring viral
3 and host cell factors.
- 4 • The viral envelope glycoproteins E1 and E2 are essential for HCV entry.
- 5 • Lipoproteins have been shown to associate with the viral particle and interfere with viral
6 entry.
- 7 • Host factors mediating viral attachment and binding to hepatocytes include highly
8 sulfated heparan sulfate and the low-density lipoprotein receptor.
- 9 • CD81, scavenger receptor BI and the tight junction proteins claudin-1 and occludin act on
10 a postbinding step and are essential for HCV entry.
- 11 • Host factors such as CD81 and CLDN1 form co-receptor complexes.
- 12 • HCV entry into hepatocytes depends on clathrin-mediated endocytosis.
- 13 • HCV appears to be delivered to early but not late endosomes where the acidic pH
14 provides an essential cue that triggers penetration and uncoating.
- 15 • An alternative route of viral entry is direct cell-cell transmission which is resistant to
16 neutralizing antibodies.

1 **Key points 2**

- 2 • New therapeutic approaches for HCV infection aim at the development of more specific
3 compounds targeting the virus and/or host cell factors.
- 4 • HCV entry into target cells is a promising target for preventive and therapeutic antiviral
5 strategies since it is essential for initiation, spread and maintenance of infection.
- 6 • The clinical impact of HCV entry for pathogenesis of HCV infection has been confirmed in
7 clinical cohorts of acute and chronic HCV infection.
- 8 • Interfering with HCV entry offers several targets including attachment/binding, post-
9 binding events and viral fusion.
- 10 • Entry inhibitors comprise neutralizing antibodies targeting the viral envelope,
11 inhibitory/blocking antibodies targeting host cell surface factors as well as small
12 molecules, peptides and siRNAs.
- 13 • HCV entry inhibitors are a promising class of novel antivirals since they are
14 complementary to current approaches focussing on viral protein processing and
15 replication.
- 16 • Combining compounds targeting viral and host cell factors and complementary steps of
17 the viral life cycle will increase the genetic barrier to resistance.

Table

Target	Examples of compounds	Stage of development	References
HCV E1E2	Neutralizing antibodies		
	- Polyclonal HCV IgG (Civacir)	Phase II	[142]
	- HCV-Ab ^{XTL} 68	Phase II	[143-145]
	- Human mAb AR3	Mouse model	[31]
	- CBH and HC antibodies	Cell culture	[146]
	- IGH antibodies	Cell culture	[34, 101, 125]
	- AP33	Cell culture	[33, 147]
	- 3/11	Cell culture	[59, 148]
	- Fab e137	Cell culture	[123]
	- mAbs 1:7 and A8	Cell culture	[124]
	- HCV1 and HCV95-2	Cell culture	[149]
	Heparin and HS analogues	Cell culture	[34, 45, 55]
	Lectins	Cell culture	[112]
HCV particle	Anti-apoE mAb	Cell culture	[118]
SR-BI	Anti-SR-BI pAb and mAb	Cell culture	[66, 67, 131]
	ITX5061	Cell culture	[132]
	Serum amyloid A	Cell culture	[130]
CD81	Anti-CD81 mAb	Cell culture	[59, 80, 148]
		Mouse model	[122]
	Imidazole based compounds	Cell culture	[126]
CLDN1	Anti-CLDN1 pAb and mAb	Cell culture	[78, 133]
Internalization/ fusion	PS-ON	Mouse model	[135]
	Arbidol	Cell culture	[137, 138]
	Chloroquine	Cell culture	[86, 100]
	Silymarin	Cell culture	[139, 140]

Figure

