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Chapter 13

Strategies to Preclude Hepatitis C Virus Entry

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Abstract

Without a preventive vaccine, hepatitis C virus (HCV) remains an important pathogen worldwide with millions of carriers at risk of end-stage liver diseases. Despite the introduction of novel direct-acting antivirals (DAAs), resistance problems, challenges with the difficult-to-treat populations and high costs limit the widespread application of these drugs. Antivirals with alternative mechanism(s) of action, such as by restricting viral entry or cell-to-cell spread, could help expand the scope of antiviral strategies for the management of hepatitis C. Transfusion-associated HCV infection remains another issue in endemic and resource-limited areas around the world. This chapter describes some of the latest developments in antiviral strategies to preclude HCV entry, such as through monoclonal antibodies and small molecules, as well as measures to enhance the safety of therapeutic plasma products in blood transfusion.

Keywords: hepatitis C virus, viral entry, antivirals, entry inhibitors, monoclonal antibodies, small molecules, therapeutic plasma products

1. Introduction

Hepatitis C virus (HCV) is a major pathogen that predisposes about 170–300 million people worldwide to risks of end-stage liver diseases (ESLD), including cirrhosis and hepatocellular carcinoma (HCC). The hepatotropic virus remains one of the top indications for liver transplantation in treating ESLD [1]. While a preventive vaccine remains unavailable, the recent introduction of direct-acting antivirals (DAAs) has revolutionized the treatment for hepatitis C, phasing out the decade-old interferon (IFN)-based regimens. The majority of DAAs, however, focus on targeting viral replication such as via inhibition of the HCV NS3/4A protease, the NS5A cofactor, and the NS5B polymerase [2]. Although the DAAs have significantly improved the rate of sustained virological response (SVR) in the most prevalent genotype 1 patients, several challenges persist in real-world setting including high cost, drug-drug

interactions, emergence of drug resistance, hard-to-treat populations (e.g., human immunodeficiency virus [HIV] coinfection, ESLD, and transplant patients), and management of DAA failures [3–5]. With the advent of hepatitis C treatment in larger populations and borrowing from the experience with HIV cocktail therapy, it is becoming clear that developing therapeutic strategies with different modes of action would be necessary to address the various limitations of current DAAs. In addition, HCV transmission due to transfusion of contaminated blood products remains an issue in endemic areas around the world. This is particularly the case in resource-limited countries that face inadequate supply of safe blood products or have poorly controlled blood screening practices, leading to significant risk of transfusion-associated HCV infection [6]. Measures to enhance the safety of therapeutic plasma products such as through the implementation of viral inactivation treatments are therefore a necessity to reduce such risk.

The multistep process of HCV entry makes it an attractive target since it is the foremost fundamental prerequisite in establishing an infection. Following successful entry, the viral life cycle initiates to produce more virions, and with this development the underlying disease begins its progression. Blocking HCV infection by targeting its entry therefore has important implications for both prophylactic and therapeutic purposes since it abolishes the viral life cycle. As a prophylactic treatment, it can be used to prevent infection or reinfection. This is particularly useful in liver transplant setting of hepatitis C wherein the liver allograft is inevitably reinfected [7, 8]. As a therapeutic treatment, precluding HCV entry via de novo infection or cell-to-cell transmission helps to restrict viral spread in an infected person which could slow the progression of the disease. In addition, incorporation of strategies to block HCV entry into existing DAA treatments is expected to maximize the treatment response rate, even producing a synergistic effect [9], as with the experience of using multiple inhibitors in HIV cocktail therapy to concomitantly target various stages of the viral life cycle. Since more steps are being targeted in such a multipronged approach, the inclusion of entry inhibitors to existing DAAs could impose a higher genetic barrier to drug-resistance development. Such tactic not only aids in disrupting persistent HCV infection but could also help to ultimately achieve viral clearance. These aspects therefore make the development of HCV entry blocking strategies highly advantageous in both expanding the scope of antiviral treatments against hepatitis C and providing new insights into antiviral management. This chapter describes some of the latest development of strategies in precluding HCV entry for the management of hepatitis C.

2. Overview of HCV entry

Owing to the development of infectious HCV culture systems (e.g., cell-culture-derived HCV, HCVcc) and viral pseudoparticles bearing HCV glycoproteins (e.g., HCV pseudoparticles, HCVpp), a scenario of how HCV entry occurs has slowly emerged over the last decade of research. It is widely recognized that the HCV particle undergoes a series of intimate and well-orchestrated interactions with various receptors/coreceptors on the hepatocyte host cell surface as well as in the tight junctions, which ultimately lead to the attachment, internalization, and

fusion of the virion with the cellular membrane. A number of these receptor interactions are thought to be attributed to the highly lipidated nature of the HCV virion. Specifically, HCV exists as a lipo-viro particle (LVP) with a lipid composition that includes the apolipoproteins and resembles that of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) [10–15]. The association with lipids on the viral particle is thought to contribute to the shielding of HCV glycoproteins from neutralization by the host antibody-mediated response. In addition, the presence of the apolipoproteins on the virion has a large influence on the production of infectious HCV and also its tissue tropism [13, 16–22].

Following circulation in the blood, the HCV viral particles reach the liver and begin the interactions with molecules at the surface of the hepatocytes (**Figure 1**). The initial contacts are with nonspecific receptor(s) including the glycosaminoglycan (GAG) heparan sulfate moieties [23–25] that can be found on the transmembrane core proteins syndecans [26, 27]. These early interactions facilitate the attachment of the HCV virion and its accumulation on the hepatocytes for subsequent binding to more specific receptors. Although the LDL receptor (LDLR) has also been suggested as a potential initial attachment factor [28–30], recent evidence suggests that it may play a more essential role in viral replication [31, 32].

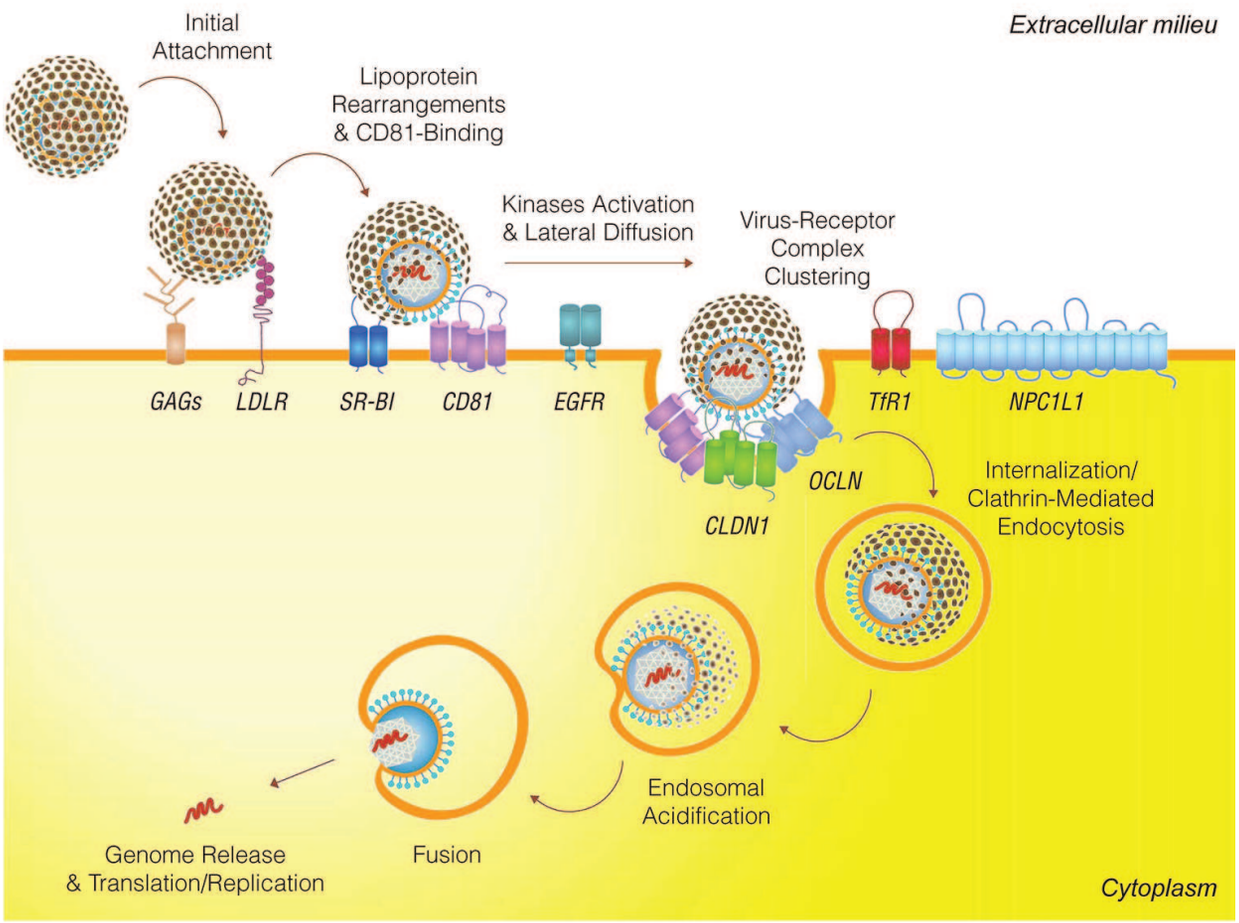

Figure 1. Overview of HCV entry.

Nevertheless, these initial interactions have been shown to be mediated via apolipoprotein E (apoE) on the virion [29, 33–36]. The capturing process of the HCV particle is finalized by its interaction with the scavenger receptor class B type I (SR-BI) [37, 38], which is able to associate with the virion's lipoproteins [37, 39] as well as the HCV E2 glycoprotein [40, 41]. Binding of HCV with SR-BI induces lipoprotein rearrangements that help prime the virion for subsequent binding to other host cell factors and promote entry. This process is proposed to occur via SR-BI's lipid transfer activity between the viral particle and the plasma membrane [37, 42] and/or by direct interaction with the hypervariable region 1 (HVR1) domain on E2 [37, 43], which ultimately leads to conformational change and the exposure of functional glycoprotein epitopes for additional receptor binding. Closely following this event is the engagement of the HCV particle with the tetraspanin receptor CD81 [44, 45], which is an important entry factor for the virus [41, 46, 47]. HCV binding to CD81 is proposed to induce a dynamic lateral diffusion of virus-receptor complexes toward the tight junction area for further interactions with additional entry factors and viral internalization [22, 48]. Specifically, CD81 forms a coreceptor complex with the tight junction protein claudin-1 (CLDN1) [49, 50] and is engaged in late events of HCV entry [51]. This re-localization and virus-receptor complex association with CLDN1 involves multiple signaling pathways (e.g., Rho GTPases, PI3K/AKT, and ERK/MAPK) [52, 53], includes the activation of host cell kinases such as the epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) [54, 55], and is influenced by the absence of the CD81-associated partner EWI-2wint on the hepatocytes [56, 57]. The EWI-2wint molecule is normally bound to CD81 on most cell type surfaces and inhibits its diffusion which is required to promote HCV entry; however, it is not expressed in the hepatocytes, and hence its absence has been suggested to contribute to the restricted tropism of the virus [56]. Following interaction with the CD81/CLDN1 complex, the HCV particle is presumed to then interact with the tight junction protein occludin (OCLN) prior to viral internalization [58]. Additional proteins that take part in influencing virion entry into the hepatocyte include the transferrin receptor 1 (TfR1) [59] and the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) [60], although their specific role and interplay with other entry factors in the HCV entry process remain to be defined. The HCV particle finally enters the cell via clathrin-mediated endocytosis [61]. The HCV-receptor complexes then migrate to endosomal compartments [62, 63] where acidification occurs to induce membrane fusion, which allows the release of viral RNA into the host cytosol.

The above sequential and multistep entry process consequently yields the successful release of the HCV genome into the host cytoplasm for direct translation and the ensuing launch of viral replication. The roles played by several of these entry factors including SR-BI, CD81, CLDN1, and OCLN not only mediate HCV entry but also presumably help to define tissue and species tropism of the virus [64–67]. The understanding of how HCV achieves viral entry has led to the possibility of antiviral targeting. From docking to virus internalization, essentially all steps are targetable to prevent HCV infection of the host cell. In addition, given the association of HCV with lipoproteins and the viral particle's interaction with lipoprotein and lipid receptors (LDLR, SR-BI, and NPC1L1), the lipidic nature of HCV virion also offers various methods of pharmacological intervention. Finally, many of the entry factors including CD81, SR-BI, CLDN1, OCLN, and NPC1L1 also play a role in mediating HCV cell-to-cell transmission

between intercellular junctions [68–71], and therefore targeting these molecules could help restrict both cell-free entry and cell-to-cell spread of HCV.

3. Current development in inhibition of HCV entry

3.1. Use of monoclonal antibodies to target host cell receptors or viral antigens

Recent insight into the molecular interactions of HCV at the cellular membrane has significantly enhanced the understanding of the HCV entry paradigm and revealed potential targets for drug intervention, including the use of monoclonal antibodies (mAbs) to mask HCV entry receptors/coreceptors or viral antigens. As described below and summarized in **Table 1**, the use of mAbs targeting CD81, SR-BI, CLDN1, or the HCV E2 has been shown to have prophylactic/therapeutic effects against HCV infection in both cell culture and animal models.

3.1.1. Anti-CD81 monoclonal antibodies

CD81 is the first putative receptor identified for HCV entry [72, 73] and plays an important role in the virus infection. The molecule is a member of the tetraspanin superfamily with four transmembrane domains and two extracellular loops and is expressed in most human tissues [74]. Commercial CD81 mAb JS-81 has been applied in human liver-chimeric mouse model and shown prophylactic protection but no postexposure effect inhibiting HCV infection [75]; nonetheless, this experimental test inspired subsequent studies of anti-CD81 mAbs as antiviral agents. Of the newly generated antibodies, mAb QV-6A8-F2-C4 produced by genetic immunization could efficiently inhibit HCVcc infection and pan-genotypic HCVpp entry in a similar range as mAb JS-81 [76]. The antibody also appeared to block neutralizing antibody-resistant HCV cell-to-cell transmission and viral dissemination in a dose-dependent manner, with a less cytotoxic or antiproliferative property than JS-81 in vitro. In a recent study, another mAb K04 generated with hybridoma technique not only showed inhibitory effect against HCVpp and HCVcc infection in hepatoma cells and primary human hepatocytes (PHH), but also surprisingly blocked HCV infections in both prophylactic setting and postinfection stage in human liver-chimeric mice [77]. This is probably due to the improved intrinsic binding affinity of mAb K04 to CD81 large extracellular loop (LEL) and a different binding epitope as compared to mAb JS81. However, treatment-associated reductions in body weight and human serum albumin levels were observed in this study. Further research will be needed to determine the minimal dose of antibodies needed to provide protection and to evaluate the toxicology of anti-CD81 mAbs for long-term development.

3.1.2. Anti-SR-BI monoclonal antibodies

SR-BI is a member of the CD36 family primarily expressed in liver and non-placental steroidogenic tissues which facilitates selective cholesterol uptake [78]. The molecule has been proposed to be a horseshoe-like glycoprotein with a large extracellular loop anchored to the plasma membrane at both N- and C-termini with short extensions into the cytoplasm [79]. It was first identified as the alternative E2 receptor on HepG2 cells which efficiently recognize

Candidates	Effect(s)	Stage of Development	Reference
mAbs Against Host Entry Factors			
Anti-CD81 mAbs	Inhibit CD81-E2 interaction	Mouse model	[75–77]
Anti-SR-BI mAbs	Inhibit SR-BI-E2 interaction	Mouse model	[80–82]
Anti-CLDN1 mAbs	Inhibit E2-CD81-CLDN1 association	Mouse model	[84–87]
Passive Immunotherapy Against HCV			
Anti-E2 mAbs	Neutralize circulating virion	Phase II	[89–96]
Polyclonal IgG	Neutralize circulating virion	Phase III	[190, 192, 193]
Small Molecule Inhibitors			
Heparin, heparin-derived compounds	Heparan sulfate competitors	Cell culture	[24, 25]
Heparinases	Heparan sulfate enzyme	Cell culture	[25]
EGCG	Compete with heparan sulfate; alter viral shape; inhibit cell-to-cell spread	Cell culture	[99–101]
Delphinidin	Alter viral shape	Cell culture	[101]
SSb2	Inhibit attachment & viral fusion	Cell culture	[102]
GA	Inactivate virion	Cell culture	[103]
Hydrolysable tannins CHLA & PUG	Inactivate virion; inhibit attachment & cell-to-cell spread	Cell culture	[104]
LOD	Inactivate virion; inhibit attachment	Cell culture	[105]
DHMD	Inactivate virion; inhibit attachment	Cell culture	[106]
Curcumin	Decrease viral envelope fluidity; inhibit cell-to-cell spread	Cell culture	[107]
CV-N	E1/E2 glycan-binding protein	Cell culture	[109]
Griffithsin	E1/E2 glycan-binding protein	Mouse model	[110]
MBL	E1/E2 glycan-binding protein	Cell culture	[111]
Recombinant L-ficolin	E1/E2 glycan-binding protein	Cell culture	[112]
BA-LNCs	E2 glycan-binding protein	Cell culture	[114]
Oleanolic acid	E2 glycan-binding protein	Cell culture	[115]
CD81-derived peptides	Interact with E2	Cell culture	[116, 117]
CLDN1-derived peptide (CL58)	Interact with E1 & E2	Cell culture	[81]
E2-derived peptide	Interfere with E1/E2 hetero-dimerization	Cell culture	[119]
Terfenadine	CD81 competitor	Cell culture	[120]
ITX 5061	SR-BI inhibitor	Phase Ib	[121–124]
Aspirin	Down regulates CLDN1	Cell culture	[125]
Erlotinib	EGFR inhibitor; inhibit cell-to-cell spread	Mouse model	[54]
Dasatinib	EphA2 inhibitor; inhibit cell-to-cell spread	Cell culture	[54]

Candidates	Effect(s)	Stage of Development	Reference
Tipifarnib, sorafenib	Ras, Raf inhibitor	Cell culture	[126]
Ferristatin	TfR1 inhibitor	Cell culture	[59]
Ezetimibe	NPC1L1 inhibitor	Mouse model	[60]
PF-429242	Down regulate NPC1L1 & LDLR	Cell culture	[127]
Phenothiazines	Modulate host cell membrane	Cell culture	[128]
Chlorpromazine	Clathrin-coated pit formation inhibitor	Cell culture	[61]
Arbidol	Trap virion in clathrin-coated vesicles	Cell culture	[129]
Bafilomycin A1, concanamycin A	Disturb acidic endosomal compartments	Cell culture	[130]
Chloroquine, ammonium chloride	Disturb acidic endosomal compartments	Cell culture	[131]
RAFI dUY11	Inhibit viral fusion	Cell culture	[132]
Ferroquine	Inhibit cell-to-cell spread	Cell culture	[133]
Triazine-based compounds	Inhibit post- binding step & cell-to-cell spread	Cell culture	[134, 135]
Silibinin	Inhibit viral fusion & cell-to-cell spread	Clinical Legalon® SIL	[136–140]
Tamoxifen	Inhibit attachment & post-binding step	Cell culture	[142]
HCV II-1 (GS-563253)	Inhibit attachment & post-binding step	Cell culture	[143]
EI-1 (BJ486K)	Inhibit post-binding step	Cell culture	[144]

Table 1. Antiviral strategies to preclude HCV entry.

soluble E2 proteins but do not express CD81 on their surface [40]. As described above, both CD81 and SR-BI are considered necessary for HCV entry, since the overexpression of CD81 on HepG2 cells restores HCVpp entry in these originally poorly permissive cells [41]. Monoclonal antibodies targeting SR-BI that inhibited HCV infections include mAb C167, mAb16-71, mAb8, and mAb151. For HCV inhibitory activities in vitro, mAb C167 effectively prevented infection in hepatoma cells with HCVcc and ex vivo virus recovered from HCVcc-infected chimpanzees [80]; mAb16-71 exhibited preventive effect against HCVcc infection in both hepatoma cells and PHH [81]; mAb8 and mAb151 also prevented HCVcc infection in reporter Huh-7 cells [82]. Additionally, mAb16-71, mAb8, and mAb151 all showed their ability in blocking HCV cell-to-cell spread in vitro and in vivo. Human liver-chimeric mouse models challenged with serum-derived HCV isolates of different genotypes revealed the anti-HCV property in vivo of the three antibodies in both prophylactic and postexposure settings. Specifically, mAb16-71 showed complete blockage of infection and intrahepatic spread of HCV isolates with a prophylactic treatment, but had no effect on chronically infected chimeric mice; mAb151, on the other hand, appeared to be effective against an HCV variant escaped from adaptive immune response in a liver transplant patient and displayed better antiviral activity in inhibiting viral spread and amplification in the postexposure setup.

3.1.3. Anti-CLDN1 monoclonal antibodies

The CLDN1 tight junction protein has four transmembrane domains and is highly expressed in the liver [83]. Its role in HCV entry is proposed to occur in the post-binding steps [64]. Anti-CLDN1 antibodies directed against the CLDN1 extracellular loops were found effective in neutralizing HCV infection in hepatoma cells through disrupting CD81-CLDN1 association and therefore inhibiting E2 binding to the cell surface [84]. A CLDN1 mAb OM-7D3-B3 targeting CLDN1 extracellular loop was found to be effective in inhibiting HCV isolates in vitro [85]. Further experiments in human liver-chimeric mouse models confirmed its potency in preventing HCV infection and eliminating persistent infection in vivo [86]. Pretreatment of another anti-CLDN1 mAb 3A2 targeting CLDN1 extracellular loop also showed protective effect in a chimeric mouse model [87]. Safety profiles of these antibodies were also assessed regarding the levels of human albumin, aspartate transaminase, alanine transaminase and total bilirubin, and potential side effects on the other organs and tight junction integrity. Further studies were suggested to assess potential immune-mediated adverse effects to ensure its relevance for clinical use [86, 87].

3.1.4. Anti-HCV E2 monoclonal antibodies

Another approach to developing entry-inhibiting mAbs is to target the glycoproteins on the HCV virion surface. Albeit HCV glycoproteins exhibit high variability and are protected by glycosylation and lipids on the viral particle, neutralizing mAbs have been designed to target more conserved and accessible regions, specifically on the E2 glycoprotein [88]. Effects of E2 mAbs have been shown in vitro and in vivo [89–94]. Clinical trials have been carried out to assess the protective function of human anti-E2 mAbs HCV-Ab^{XTL}68 and MBL-HCV1 in liver transplant settings of HCV-positive patients. With a higher dose and daily infusion of HCV-Ab^{XTL}68, HCV RNA in patient serum showed transient reduction in the first week post-transplantation but not yet below the detectable limits [95]. MBL-HCV1, on the other hand, successfully suppressed the viral load from 7 to 28 days after transplantation in genotype 1a-infected patients with multiple infusions. Although the primary endpoint at day 42 was not met, the viral rebound was significantly delayed, and the magnitude of the viral load reduction was greater than the previous HCV-Ab^{XTL}68 therapy [96]. The result indicates that mAbs may be a promising class of entry inhibitors that adsorbs circulating virions to protect the new liver from reinfection after transplantation. A study of combination therapy with DAAs to prevent allograft HCV infection is currently underway [96].

Current obstacles to the development of mAbs as therapeutic antiviral agents include the high cost of production, storage, and administration, which can only be done by injection so far [88]. Nevertheless, the associated immune responses such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) may help to clear the viruses and infected cells [88, 97]. Antibodies that directly block host cell entry factors are more likely to be effective for the diverse circulating viral strains; however, due to the distribution and multiple functions of such molecules, the blockage may cause potential adverse side effects [97]. As for antibodies targeting viral antigens, designing suitable candidates may be a challenging issue due to the heterogeneity of the HCV glycoproteins [98], but such

antibodies may provide a safer option for the synergistic therapy with other antivirals of different modes of action to suppress the development of resistance, particularly at the early post-transplantation stage [96]. Additional neutralizing antibodies against other entry factors have also been reported to antagonize HCV infection in vitro, such as anti-TfR1 [59] and anti-NPC1L1 [60] antibodies, suggesting they could also be potentially developed for treatments against hepatitis C.

3.2. Small-molecule inhibitors of HCV entry

In addition to the mAbs, great efforts have been put into identifying small molecules with potent antiviral effects against HCV entry. The source of such entry inhibitors includes clinically approved medications, synthetic molecules, and natural product-based compounds. These small molecules could be further evaluated for development as drug candidates or drug leads. Below is a panel of small molecules that have been investigated with their activities inhibiting HCV entry (**Table 1**).

3.2.1. Small molecules inhibiting viral attachment

The attachment step represents the primary interaction of an HCV virion with its host cell surface. Since the GAG heparan sulfate moieties dominate the capturing of HCV virions, the heparan sulfate homologue heparin and its derivatives as well as the enzyme heparinases which degrade the molecule were all shown to inhibit the viral binding to hepatoma cells [24, 25]. (-)-Epigallocatechin-3-gallate (EGCG), a green tea catechin, was speculated to exert its inhibitory effect on viral attachment [99] by competing with heparan sulfate for HCV binding [100] or altering the viral shape [101]. Delphinidin, an anthocyanidin extracted from plant pigment, was also demonstrated to inactivate HCVpp by altering its shape and was particularly potent when added concurrently with the viral inoculation [101]. The natural terpenoid saikosaponin b2 (SSb2), isolated from the root of *Bupleurum kaoi*, was observed to specifically block HCV particle binding and early viral entry without affecting other stages of the viral life cycle [102]. SSb2 could inactivate cell-free HCV particles and was suggested to target the glycoprotein E2 in mediating its antiviral effect against HCV infection. Several other natural compounds including the gallic acid (GA) extracted from *Limonium sinense* [103], the hydrolyzable tannins chebulagic acid (CHLA) and punicalagin (PUG) [104], and the hepatoprotective plant *Phyllanthus urinaria*-derived monolactone loliolide (LOD) [105] and butenolide (4R,6S)-2-dihydromenisdaurilide (DHMD) [106] were also found to efficiently inactivate cell-free HCV viral particles and impede viral attachment. Another natural compound curcumin extracted from turmeric was shown to decrease the fluidity of viral envelope and therefore prevent the binding and fusion [107], possibly by inserting into the membrane in a manner similar to cholesterol [108].

3.2.2. Small molecules blocking viral glycoproteins

A variety of broad-spectrum antiviral agents have exhibited their ability to interact with the glycans on viral glycoproteins. In the case of HCV, glycan-binding proteins interfere with the association between the E1/E2 heterodimer and the host cell receptor CD81. Lectins such as cyanovirin-N (CV-N) and griffithsin, isolated from cyanobacterium *Nostoc ellipsosporum* and

the red alga *Griffithsia* sp., respectively, were reported to have such effect. CV-N was shown to interact with N-linked glycans of HCV glycoproteins and disrupt E1/E2 binding to CD81 [109]. The inhibitory effect of griffithsin on HCV entry was also quenched when N-linked high-mannose oligosaccharides were present, indicating a pattern similar to CV-N of affecting the glycoproteins-CD81 interaction [110]; pretreatment of griffithsin was shown to delay the viral infection in chimeric mouse model. Humoral lectins of the innate immune systems including the mannan-binding lectin (MBL) and L-ficolin were also considered to have analogous effect neutralizing HCV particles. MBL [111] and recombinant oligomeric L-ficolin [112] were found to interact with the glycans on the E1/E2 heterodimer in a calcium-dependent manner, thereby inhibiting the viral entry. Notably, the MBL-associated complement system was activated upon its binding to HCV E1/E2, suggesting the use of humoral lectins as viral entry inhibitors may also help facilitate viral clearance. However, the detailed mechanism and specific target of the humoral lectins remain to be defined. The boronic acid (BA)-modified nanoparticles were also found to suppress HCV entry in a way that acted similar to lectins [113], with the incorporation of lipid nanocapsule (BA-LNC) techniques enhancing their stability and solubility [114]. Chemically modified oleanolic acid, a triterpene compound originally extracted from *Dipsacus asperoides*, was found able to interrupt the E2-CD81 interaction by binding to E2 [115].

Besides the glycan-binding proteins, molecules imitating HCV host entry factors or viral glycoproteins were also developed in the attempt to block the viral entry. An imidazole-based scaffold presenting CD81 helix D amino acid side chains [116] and stapled peptides based on CD81 LEL [117] were designed to antagonize the E2-CD81 interaction by mimicking the putative E2-binding region of CD81. A CLDN1-derived peptide, CL58, was also found to inhibit HCV entry in the post-attachment stage by interacting with HCV E1 and E2 [118]. As for viral glycoprotein-based molecules, an E2-derived peptide was found able to block E1/E2-mediated fusion by targeting E1 and therefore interfere with the hetero-dimerization of the glycoproteins [119].

3.2.3. Small molecules targeting host entry factors and CD81-triggered signaling pathway

In addition to the therapeutic antibodies mentioned in the previous section, several small molecules have been suggested to exert their inhibitory activity of HCV entry by targeting cellular receptors/coreceptors. Terfenadine, an antihistamine, was found able to prevent HCV infection by competing with the CD81 antibody JS81 binding to the LEL of CD81 protein on the hepatoma cell surface [120]. ITX 5061, a clinical stage compound originally characterized as a p38 MAPK inhibitor, was identified with its capability of antagonizing SR-BI [121] and further validated for its potency of inhibiting HCV entry at post-binding step [122]. The anti-HCV effect of ITX 5061 was found additive to synergistic in combination with several standard-of-care therapeutics, and the resistant mutant was defined on the viral glycoprotein E2 [123]. A latest phase 1b clinical trial [124] revealed that the ITX 5061-treated patients, especially the genotype 1-infected patients, had a significant reduction in HCV RNA through the first week after liver transplantation and viral evolution were restricted; however, the viral RNA levels became comparable in both ITX 5061-treated and untreated patients, suggesting the need to incorporate other antiviral agents using different modes of actions to eliminate HCV infection. Aspirin, alternatively, inhibited HCV entry by downregulating CLDN1 [125].

Since the receptor tyrosine kinases are also involved in the HCV entry process, two clinically approved protein kinase inhibitors were evaluated for their ability to abrogate the viral entry. Both erlotinib, an EGFR inhibitor, and dasatinib, an EphA2 inhibitor, could successfully block HCV entry in a dose-dependent manner as well as the cell-to-cell transmission. Specifically, erlotinib was shown to inhibit the membrane fusion of hepatoma cells overexpressing HCV glycoproteins. In vivo treatment of erlotinib resulted in a significant suppression of the viral load in PHH-chimeric mouse model with HCV infection [54]. Furthermore, inhibitors of EGFR downstream kinases Ras (tipifarnib) and Raf (sorafenib) were also assessed and found effective in blocking HCV entry [126].

Inhibitors of other entry factors were also shown to be effective in hampering the viral entry. Pretreatment of ferristatin, a TfR1 inhibitor that binds to the molecule and causes its internalization and degradation, was shown to decrease HCVcc infection in vitro [59]. The NPC1L1 internalization inhibitor ezetimibe, which is also an FDA-approved cholesterol-lowering medication, diminished HCVcc foci formation before and during the viral challenge. Daily oral administration of ezetimibe starting two weeks before infection also delayed the viral growth of a genotype 1 clinical isolate in PHH-chimeric mouse model [60]. PF-429242, an SKI-1/S1P inhibitor, potentially impeded HCV entry by downregulating NPC1L1 and LDLR expression [127]. On the other hand, phenothiazines, a group of synthesized nitrogen- and sulfur-containing tricyclic compounds, inhibited HCV fusion into the cell by modulating the host cell membrane. Insertion of phenothiazines into the cholesterol-rich membrane increased its fluidity, thus possibly decreasing the local inhomogeneity of the cell required for the viral fusion [128].

3.2.4. Inhibition of clathrin-mediated endocytosis and viral fusion

Since HCV fusion has been discovered to be facilitated by clathrin-mediated endocytosis and requires an acidic environment, several reagents were assessed for their effectiveness in preventing HCV entry through blocking such pathways. Chlorpromazine, an inhibitor of clathrin-coated pit formation, was shown to inhibit both HCVpp and HCVcc infection in vitro in the validation of clathrin-mediated endocytosis pathway of HCV fusion to the host cell membrane [61]. Arbidol, a broad-spectrum antiviral agent that blocks viral entry and has been licensed in some regions for influenza, was described to trap the HCV virion in clathrin-coated vesicles, thereby hindering the release of viral genome and the following infection [129]. It was also suggested that arbidol could generally cause the intracellular accumulation of clathrin-coated structures and restrain the formation of clathrin-coated pits on the cell surface [129], possibly due to its tropism for lipid bilayers.

Small molecules disturbing the acidic endosomal compartments were also identified as HCV entry inhibitors in the discovery of the low pH-triggered entry. These include bafilomycin A1 and concanamycin A, which are inhibitors of vacuolar H+-ATPases [130]. Weak bases such as chloroquine and ammonium chloride were also found to inhibit the low pH-dependent conformational change required for the viral fusion, based on their ability to penetrate lysosomes and increase the pH [131]. Finally, dUY11, one of the rigid amphipathic fusion inhibitors (RAFIs), was suggested to inhibit HCV entry by interacting with the hydrophobic structures in virions and preventing the formation of negative curvature required for viral fusion [132]. Curcumin [107] is also able to affect the fusion step as previously mentioned.

3.2.5. Small molecules inhibiting cell-to-cell transmission

Besides inhibiting the HCV entry in de novo infection, blocking cell-to-cell spread of the viral particles is also important as this mode of transmission facilitates efficient spread of the virus in the liver escaping from neutralizing antibodies [68, 69]. Ferroquine was speculated to interact with HCV glycoprotein E1 and abrogate cell-to-cell spread of the virus [133]. Triazine-based compounds indicated to be closely related to the amino acids on the glycoprotein could also selectively inhibit genotype 1 HCV entry at the post-attachment step along with cell-to-cell transmission [134, 135]. Several molecules also block cell-to-cell spread in addition to their activities in hindering HCV viral entry. For instance, besides impeding viral attachment, CHLA and PUG exhibit pronounced antiviral effects at the postinfection stage, especially in restricting HCV foci expansion [104]. Others include EGCG [99], curcumin [107], erlotinib, and dasatinib [54]. Silibinin, the major component of *Silybum marianum* that has been designated as an orphan drug for the prevention of recurrent hepatitis C in liver transplant patients [136], was also suggested to possess a prominent effect blocking transmission of the viral particles between intercellular junctions [137, 138], although other studies have proposed that it may slow down clathrin-mediated endocytosis [139] as well as inhibit viral membrane fusion [140]. This could be useful since DAA-resistant HCV variants have been suggested to escape via cell-to-cell transmission route [141]. Therefore, the choice of inhibitors exhibiting mechanistic effect against both HCV cell-to-cell spread and cell-free entry, or a combination of such two types of inhibitors, should facilitate viral clearance.

3.2.6. Additional candidate entry inhibitors

Some other molecules were found able to prevent the infection at different steps of HCV entry. The estrogen receptor modulator tamoxifen [142] and HCV infectivity inhibitor 1 (HCV II-1 or GS-563253) [143] were shown to inhibit the HCV infection at both attachment and post-binding steps. HCV II-1 was also found capable of impeding infectious virion propagation [143]. HCV entry inhibitor 1 (EI-1 or BJ486K), a flavonoid ladanein, was shown to interrupt the viral entry at post-attachment stage [144]. The exact mechanisms of these molecules require further investigations. Other compounds such as serum amyloid A [145, 146], p7 ion channel-derived peptide H2-3 [147], amphipathic DNA polymers [148], lactoferrins [149], tellimagrandin I and its derivatives [150], indole derivatives [151], and imidazo[1,2α][1,8]naphthyridine derivatives [152] were found able to inhibit HCV entry with mechanisms that remain to be clarified.

3.3. Control of HCV infection risks in human blood-derived therapeutic products

Many viruses can contaminate human blood. HCV, along with HIV and HBV are a major cause of infectious complications of blood product transfusion therapy. HCV contamination in patients by transfusion of blood components such as red blood cells, platelets or clinical plasma, as well as industrial fractionated plasma products, has been well documented. At the time of the "tainted blood scandal," numerous recipients of blood components and hemophiliacs receiving plasma-derived factor VIII concentrates were contaminated through transfusion of nonvirally inactivated products prepared from blood products that were not HCV-tested.

HCV transmission through blood transfusion is a major medical issue, as infection can lead to high risk of liver cirrhosis and eventually cancer complications.

3.3.1. HCV safety nets for blood components

There are now over 100 million whole blood donations collected each year in the world. Collected blood is most often separated by "blood establishments" into red blood cell concentrates, platelet concentrates, and plasma that are transfused at nearby hospitals. Plasma, which can be obtained from whole blood collection or drawn by specialized apheresis procedures, can also be used as raw material for the production of "industrial" plasma protein products. These protein drugs include immunoglobulins G (IgGs), various coagulation factors, albumin, and many others. Industrial plasma products are manufactured from pools of plasma of several thousand liters, making them statistically more susceptible to contamination by HCV and other viruses as one highly infectious donation would contaminate the whole plasma pool and potentially the derived products.

Today in developed economies benefiting from strict regulatory oversight, several measures are in place to decrease the possibility for patients to acquire HCV by transfusion. Blood transfusion HCV safety nets for blood components rely on complementary measures encompassing (a) epidemiological control of the population, (b) individual screening of candidate blood donors to defer those identified as presenting potential risk factors, and (c) individual blood donation testing to identify and eliminate donations reactive to anti-HCV antibodies and/or HCV RNA nucleic acid test (NAT) [153]. In technology-advanced countries applying such procedures, this has allowed to decrease the risk of acquiring HCV by transfusion of single blood components down to approximately 1 per 1.8 million. The remaining risk reflects the inevitable presence of "window-phase" donations for which all markers to detect donor infection by HCV, either indirect or direct, are found nonreactive [154, 155]. Understandably, HCV transmission risks are substantially higher in less developed economies (a) lacking a safe blood donor base, (b) relying on paid or "replacement" donors to increase the blood supply, (c) with a deficient blood collection system, and (d) with a lack of reliable viral testing procedures [6]. The ultimate barrier to avoiding HCV transmission risks from blood products collected during the window-phase period relies on the implementation of dedicated viral reduction treatments. Those have been developed for industrial plasma protein products, plasma for transfusion, and platelet concentrates. Until now, however, no treatment is available commercially for whole blood and red blood cell concentrates.

3.3.2. HCV reduction treatment of industrial plasma protein products

Development and implementation of dedicated viral/HCV reduction treatments of industrial plasma protein products took place in the 1980s and early 1990s [156]. In the early 1980s, albumin, a relatively heat-stable protein, was the only plasma product subjected to specific HCV inactivation by heat treatment at 60 °C for 10 h in the liquid state (a process called pasteurization), in the presence of fatty acid stabilizers. From the mid-1980s to the early 1990s, heat treatment of freeze-dried coagulation factors at 60–68 °C for 24–96 h or 80 °C for 72 h were developed to inactivate HIV and HCV concomitantly [156]. Although pasteurization

has successfully been adapted to several plasma products (such as antithrombin and alpha 1-antitrypsin), a milestone in the safety of industrial plasma products was the development of the solvent/detergent (S/D) incubation procedure at 20–37 °C [157] designed to dissolve the lipid envelope of viruses, including HCV, without affecting plasma protein functions. This technique is still largely used for a wide range of industrial plasma products owing to well-proven efficacy and a safety profile established by years of industrial and clinical practices [158]. Other HCV viral inactivation treatments include low pH incubation and caprylic acid precipitation/incubation of immunoglobulin products [159]. An additional milestone to enhance plasma protein product safety is nanofiltration, a procedure of filtration of protein solutions on 15–35 nm nanopore membrane devices designed to entrap and remove viruses [160]. This dedicated virus removal methodology is well established, including for HCV, and is currently applied to most plasma products [156]. Thanks to the implementation of such reduction treatments, most often combined in a complementary manner at different stages of the manufacturing process, no case of HCV transmission by industrial plasma products has been reported since 1993 [154].

3.3.3. HCV reduction treatment applied to plasma and platelet concentrates for transfusion

3.3.3.1. Plasma

Several viral inactivation treatments of clinical plasma are licensed in various countries [161]. The S/D technology was adapted to 100–500 l of pooled industrial plasma in the early 1990s [162] and demonstrated, prior to HCV identification, to efficiently inactivate non-A-non-B hepatitis virus [163]. The removal of the S/D agents is typically achieved by oil extraction and column hydrophobic interaction chromatography [162]. A miniaturized version of the S/D process using a different detergent (Triton X-45 instead of Triton X-100) has been developed allowing its implementation in single-use equipment, thereby facilitating its application in developing countries, such as Egypt, currently lacking industrial capacity [164]. The efficacy of such method to inactivate HCV has been specifically demonstrated using an in vitro culture assay [165].

A procedure consisting in adding methylene blue and illuminating acellular plasma was made available in the early 1990s [166]. The method leads to inactivation of free HCV particles through photochemical alteration of nucleic acids and incapacity of replication [154, 167].

Two other photoinactivation procedures of plasma have been licensed more recently. One combines the addition of psoralen S-59 (amotosalen) with ultraviolet light A illumination [168]. The other is based on the addition of riboflavin followed by UV irradiation [169]. These small molecules can penetrate membranes and intercalate with helical regions of HCV nucleic acids. Subsequent UV illumination irreversibly alters nucleic acids, making HCV particles unable to replicate [154, 170].

3.3.3.2. Platelets

Development of HCV inactivation methods in cellular blood products in general, and platelet concentrates in particular, has been more challenging due to the difficulty to inactivate

intracellular viruses without affecting cell function for transfusion. The two photoinactivation methods applied to plasma could nevertheless be adapted to the inactivation of HCV and other viruses in platelet concentrates [170–172].

3.3.3.3. Cryoprecipitate

Cryoprecipitate, obtained by a freeze-thaw process of plasma, is rich in factor VIII, von Willebrand factor, and fibrinogen. This plasma fraction is still largely used in many developing countries for substitution therapy in hemophilia A, von Willebrand factor disease, or fibrinogen deficiency, respectively. The frequency of treatment of patients with congenital deficiency exposes them to a high risk of infection in countries such as Egypt with a close to 10% HCV incidence [173, 174]. Similar mini-pool methods of HCV inactivation used for clinical plasma are applied to cryoprecipitates [164].

3.3.3.4. Red blood cell concentrates and whole blood

No methodology is licensed yet for HCV inactivation in red blood cell concentrates or whole blood. However, the riboflavin/UV pathogen reduction technology is being adapted to the treatment of whole blood [175] and has been shown recently to contribute to lower the risk of malaria transmission in a clinical study in Ghana [176]. It is still uncertain whether a pathogen reduction technology can be developed to substantially inactivate HCV in whole blood or red blood cell concentrates without detrimentally affecting their transfusion quality and functionality or immunogenic potential.

3.4. Therapeutic apheresis and passive immunotherapy

Additional methods of precluding HCV infection are to remove circulating virus through therapeutic apheresis or attempting to neutralize HCV infectivity by administering plasma-derived anti-HCV immunoglobulins. These strategies are aimed at reducing the infectious viral load and have been explored in clinical trials.

3.4.1. Therapeutic apheresis for the removal of HCV virions

Therapeutic apheresis is the process of transiently circulating the blood outside the body and removing the components causing particular diseases by membrane separation and adsorption separation technologies. In the case of HCV, immunoadsorption apheresis was first applied to treat the chronic hepatitis C-related cryoglobulinemia that causes autoimmune symptoms [177]. The technique of heparin-induced extracorporeal LDL precipitation (HELP) apheresis, which could eliminate apolipoprotein B-containing lipoproteins, was then discovered to reduce HCV viral load [178]; however, the decline was found not correlated with LDL reduction in plasma and appeared to be transient due to the high turnover rate of HCV [179]. Studies using combination therapy of antiviral agents and double-filtration plasmapheresis (DFPP) that selectively removes substances with high molecular weight including HCV particles and therefore, happened to display better effects of suppressing the viral kinetics and therefore have been substantially explored during the past decade. Patients who underwent the prophylactic combination treatment of low-dose IFN, ribavirin, and

DFPP had no evidence of HCV recurrence or fibrosing cholestatic hepatitis exacerbation for more than 1 year after liver transplantation [180]. Combination of DFPP and IFN also achieved impressive SVR in difficult-to-treat patients (i.e., relapsed, nonresponder, or HIV-coinfected patients) [181–184] and may also be safe for the elderly population [185]. However, the approach of apheresis for decreasing HCV viral load requires specialty equipment and possesses potential risk of adverse events (e.g., blood pressure lowering, puncture site hematoma, or infection) [181, 185].

3.4.2. Passive immunotherapy using plasma-derived polyclonal HCV immunoglobulins

Passive immunotherapy, also known as antibody therapy, is a very well-established treatment based on the administration of polyclonal hyperimmune immunoglobulins extracted from plasma or mAbs prepared by genetic engineering technologies. One application of passive immunity is to prevent or treat infections due to viruses or to reduce the pathologies associated with bacterial or venom toxins. Human immunoglobulins for passive immunotherapy are fractionated from the plasma of immunized donors having high-titer antibodies against a particular organism or antigen. For the fractionation process, plasma donations from hundreds or thousands of donors are pooled and subjected to various purification and viral inactivation steps, as described in this chapter, to isolate an essentially pure Ig preparation [159, 186]. Current human plasma-derived hyperimmune globulin products are used for the prophylaxis and treatment of viral diseases due to hepatitis B virus (HBV), rabies virus, cytomegalovirus, hepatitis A virus, or respiratory syncytial virus [187]. Human plasma-derived polyclonal hepatitis B immunoglobulin for intravenous use has been made available commercially for over 20 years in some countries. These licensed preparations are efficacious to predictably prevent HBV recurrence after liver transplantation and vertical HBV transmission from mother to child and are used as prophylactic treatment to prevent infection following contact with HBV-contaminated body fluids [188].

The possibility to use polyclonal HCV immunoglobulin to treat or prevent HCV infection has been proposed for many years [189], but no commercial preparation is available yet as it is not proven whether such immunoglobulin can prevent HCV infection or control viremia in infected patients. The rationale in polyclonal HCV immunoglobulins made from large pool of plasma units is to have a preparation that contains neutralizing antibodies to various strains of HCV [189]. However, the presence of neutralizing antibodies has been unclear initially as their presence in plasma was just considered to reflect the occurrence of an infection. Data have suggested that HCV-neutralizing antibodies exist in anti-HCV-positive plasma, but the anti-HCV antibody titer does not correlate with neutralizing capacity [190]. In vitro and animal experiments in a mice model have nevertheless suggested the presence of neutralizing antibodies in polyclonal IgG from a patient with a long-standing HCV infection [191]. A clinical study was initiated in the USA to evaluate the capacity of polyclonal plasma-derived HCV immunoglobulins to "prevent post-transplantation HCV infection of the liver graft and related progression of HCV-related liver disease." This clinical trial was "designed to evaluate a polyclonal human hepatitis C immune globulin given during and post liver transplantation for preventing or reducing the impact of recurrent HCV infection" [192]. However the trial was terminated in 2012 after treatment of seven patients (five receiving the immunoglobulins and

two standard-of-care treatment alone) and no data reported. A new trial has begun in 2013 and was recently completed [193]. It is unclear whether plasma-derived polyclonal HCV immunoglobulin will be developed. If this occurs, clear donor screening and donation testing criteria should be defined to determine the specifications of the plasma donations suitable for fractionation, as well as the fractionation methodology itself to exclude any infectious risks from the fractionation of plasma donations. It should be noted that several mAbs for clinical use in HCV-infected patients have been proposed and one has undergone a clinical trial [190]. The future will indicate whether any HCV immunoglobulin, either polyclonal or monoclonal, has a role to play in the control of HCV infection.

4. Prospects of targeting HCV entry in clinical setting

Treatment options against hepatitis C have significantly improved owing to recent advances in the development of anti-HCV therapeutics. Nevertheless, there is still much room for improvement due to potential drug resistance and possibility of viral rebound, which usually require long periods of monitoring and analysis to uncover. More importantly, there is currently no immunization or prophylactic treatment against hepatitis C. Introducing novel antivirals with a different mode of action, such as targeting viral entry using mAbs or small molecules, not only helps expand the spectrum of anti-HCV drugs but also in developing novel treatment modalities. Many of the mAbs targeting HCV receptors/coreceptors as well as small-molecule inhibitors of HCV entry impede both viral attachment and cell-to-cell transmission; this is useful in providing protection against de novo infection and at the same time in helping restrict viral spread. The inclusion of viral entry inhibitors to current DAAs has already been shown to produce synergistic treatment effect [9]. Furthermore, taking a multistep targeting approach would help elevate the genetic barrier against selection of resistant variants, thus facilitating viral clearance. Finally, the advantage of developing entry inhibitors is its potential prophylactic application against hepatitis C, which is particularly useful in protecting liver allografts from recurrent HCV infection. Other protective measures of hepatitis C transmission in clinical scenarios include implementation of viral inactivation methods for the removal of HCV infectivity in therapeutic plasma products [165]. In addition, therapeutic apheresis [180] and protective anti-HCV immunoglobulins [192, 193] have also been suggested for prevention of HCV reinfection in liver transplant patients. In the absence of an approved hepatitis C vaccine, these approaches could be explored as preventive and prophylactic measures against HCV infection. With the above-described strategies to preclude HCV entry, it is foreseeable, in a not-too-distant future, that these tactics under development will help provide a better management of chronic and recurrent hepatitis C, particularly in liver transplant setting.

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