The HCV Life Cycle: In vitro Tissue Culture Systems and Therapeutic Targets

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Abstract
Hepatitis C virus (HCV) is a highly variable plus-strand RNA virus of the family Flaviviridae. Viral strains are grouped into six epidemiologically relevant genotypes that differ from each other by more than 30% at the nucleotide level. The variability of HCV allows immune evasion and facilitates persistence. It is also a substantial challenge for the development of specific antiviral therapies effective across all HCV genotypes and for prevention of drug resistance. Novel HCV cell culture models were instrumental for identification and profiling of therapeutic strategies. Concurrently, these models revealed numerous host factors critical for HCV propagation, some of which have emerged as targets for antiviral therapy. It is generally assumed that the use of host factors is conserved among HCV isolates and genotypes. Additionally, the barrier to viral resistance is thought to be high when interfering with host factors. Therefore, current drug development includes both targeting of viral factors but also of host factors essential for virus replication. In fact, some of these host-targeting agents, for instance inhibitors of cyclophilin A, have advanced to late stage clinical trials. Here, we highlight currently available cell culture systems for HCV, review the most prominent host-targeting strategies against hepatitis C and critically discuss opportunities and risks associated with host-targeting antiviral strategies.

Introduction
Chronic infection with hepatitis C virus (HCV) affects an estimated 160 million individuals worldwide [1]. In the course of 10–25 years, one fifth of these patients are at risk of developing severe liver disease including cirrhosis and hepatocellular carcinoma [2]. As a consequence, chronic hepatitis C is among the most frequent indications for liver transplantation in many countries [3]. The viral agent causing hepatitis C was originally identified in 1989 [4] and it is now grouped within the genus Hepacivirus of the family Flaviviridae. It encodes an RNA genome of plus-strand orientation that is ca. 9.6 kb in length and encodes ten distinct viral proteins, which are translated as a single polyprotein. This viral polyprotein is expressed by way of an internal ribosome entry site encoded at the 5\textsuperscript{\prime} end of the HCV genome which folds into intricate secondary structures, thus directly recruiting cellular ribosomes to the viral RNA to initiate translation. Sev-
eral co- and post-translational cleavage steps mediated by both host and viral proteases liberate ten distinct viral proteins, namely core, envelope proteins 1 and 2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins (fig. 1).

Among these viral factors, two proteins have been key targets for development of directly acting antivirals (DAAs) for treatment of hepatitis C, namely the viral serine protease complex consisting of NS3 and 4A which is responsible for all polyprotein cleavage steps downstream of NS3 and the RNA-dependent RNA polymerase (RdRp) NS5B, the catalytic center of HCV genome replication. In fact, in 2011, NS3/4A protease inhibitors were the first licensed DAAs against HCV and recently powerful NS5B polymerase inhibitors have been approved. Selection of these two viral proteins as targets for development of antivirals was favorable for several reasons. First, recombinant purified and enzymatically active proteins were available relatively early, tests to evaluate enzymatic function were rapidly developed and the respective crystal structures were obtained. Moreover, the successful development of drugs targeting the protease and reverse transcriptase of HIV raised hopes that the HCV counterparts would be suitable drug targets as well. In addition, inhibitors of the regulatory NS5A protein will become available for patients in the near future. Finally, aiming for viral targets that are absent from non-infected cells and thus have no physiological role for the host provides unprecedented therapy options with minimal or completely absent side effects.

Nevertheless, concentrating solely on virus-encoded proteins has limitations: the most important are the variability of these viral targets across different HCV strains and the potential threat of viral drug resistance. Regarding the former, HCV is a particular challenge since the six epidemiologically relevant genotypes display a variability of HCV is a high replication rate combined with an error-prone replication machinery. As consequence of its pronounced genomic variation, HCV efficiently evades immune control, becomes resistant to antiviral drugs, and many DAAs exhibit pronounced genotype and strain-dependent antiviral activity.

The recent development of HCV cell culture systems (see below) not only provided the urgently needed armamentarium to identify and validate the most promising DAAs, it was also instrumental for characterization of numerous cellular factors that aid in HCV propagation. As obligate intracellular parasites, viruses are strongly dependent on host cell functions for their own propagation. This of course is no different for HCV, which utilizes multiple host-encoded factors for cell entry, genome replication and virus assembly. In principle, all these so-called HCV dependency factors are potential drug targets for HCV therapeutics provided that blocking their function arrests HCV propagation. Numerous studies have firmly evidenced that this can be achieved with a number of host-targeting antivirals (HTAs) – at least in cell culture. Translation of these observations into safe and efficacious treatments is, however, many steps away in most cases. Nevertheless, HIV-1 inhibitors, which target host-encoded chemokine receptors thus precluding viral cell entry, provide proof of concept that safe and well-tolerated HTAs can be developed.

While interference with key physiological functions of such host-encoded targets is an obvious limitation of HTA strategies, two major advantages argue for their clinical development: firstly, the resistance barrier to such therapies is assumed to be high since host factors are genetically stable. Exceptions to this will be critically discussed below. Secondly, usage of host factors by HCV is thought to be genotype-independent and thus HTAs should have pan-genotypic activity. Importantly, this as-

![Fig. 1. HCV genome organization. The HCV-positive strand viral RNA genome is depicted at the top. 5′- and 3′-terminal genome portions are not translated regions (NTRs), which fold into extensive secondary structures needed for genome replication and translation. The viral polyprotein is depicted in blue and encodes ten distinct viral proteins which are liberated by proteolytic cleavage mediated by host and viral proteases. A schematic drawing of the HCV virus particle and the membrane-bound replicase complex is given below. Note that HCV particles incorporate both viral factors and host-derived lipoproteins. Colors refer to the online version only.](Image 334x660 to 362x697)

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HCV Cell Culture Systems

The targeted design of HTAs against HCV was spurred by stepwise advances in cell culture systems for the study of individual life cycle steps. In the past 14 years, the generation of various in vitro systems for HCV facilitated the discovery of numerous host factors involved in HCV infection.

In 1989, 14 years after the first description of non-A, non-B hepatitis, HCV was discovered as the etiological agent of the disease. Despite the rapid delineation of the genome and polyprotein organization of HCV, the development of tissue culture infectious models proved to be difficult. Subgenomic replicons, i.e. partial genomes of HCV encoding the non-structural proteins required for RNA replication, provided the first step towards this goal and became available in 1999 [19]. A few years later, the establishment of retrovirus-based pseudoparticle systems allowed studying the entry of HCV into susceptible human hepatoma cells [20, 21]. However, it was not before 2005 that a fully permissive cell culture model of HCV enabled to address viral assembly, egress and spread [22–24]. As those initial systems were based on a single genotype 2a viral genome with unprecedented capacity to replicate in cell culture (Japanese fulminant hepatitis, JFH1), researchers aimed at generating cell culture models for the remaining five major genotypes of HCV, in particular for genotype 1 strains, which are highly prevalent in the Americas and Europe. Recently, additional full-length cell culture infectious virus constructs for genotypes 1 and 2 were described and in addition chimeric genomes encoding genotypes 1 through 7 structural proteins in the context of the genotype 2a replication machinery allow studying certain aspects of the viral life cycle in a genotype-dependent manner [6, 25–27]. Lastly, trans-complementation systems [28], where subgenomic replicons are packed into single round infectious particles complete the toolbox of HCV cell culture models. Of note, it is possible that state-of-the-art stem cell technologies and primary hepatocyte culture systems may enable the analysis of HCV infection in various host genetic backgrounds. Here, we will describe each of the above-mentioned cell culture systems and critically evaluate their potential and limitations.

The first milestone in HCV research was the generation of replicating subgenomic HCV and the identification of a cell line allowing HCV genome amplification [19]. Subgenomic replicons encode the non-structural proteins NS3 to NS5B as well as the characteristic 5’- and 3’-non-translated regions (NTRs), thus showing that these elements are the minimal requirements for propagation of the HCV RNA in transfected cells (fig. 2b). Engineering a bicistronic system with a selectable marker under the control of a separate IRES allowed to select for viral genomes with replication-enhancing mutations (REMs) and for permissive clones of a human hepatoma cell line. Subsequent curing of the HCV replicating cell clones with IFN lead to the generation of the highly HCV-permissive Huh-7-derived cell lines Huh-7.5 and Huh-7-Lunet [29, 30]. To date, both cell lines are still the gold standard in HCV research as they support not only replication but also the other steps in the virus life cycle with high efficiency. Moreover, subgenomic replicon systems for the HCV genotypes 1 through 4 and with selection markers or luciferase reporters are available and used for testing the effect of DAAs and HTAs on HCV RNA genome replication. Limitations of subgenomic HCV replicons are that REMs do not seem to increase viral fitness...
in vivo and thus could have a limited predictive value when testing the efficacy of an antiviral compound.

Another in vitro system addressing an isolated step in the HCV life cycle is the pseudoparticle system (HCVpp). The most widely used HCVpp system is based on retroviral particles decorated with the HCV E1 and E2 glycoproteins. Such HCVpp are generated by triple transfection of (1) an E1E2 expression plasmid, (2) a plasmid encoding the retroviral polymerase and capsid protein (gagpol), and (3) a retroviral provirus into 293T cells. The proviral RNA is then packaged into retroviral particles, which display E1E2 on their surface and are released into the cell culture supernatant. Typically, the provirus contains the retroviral packaging signals and a reporter gene such as GFP or luciferase. The generated pseudoparticles rely on interactions of HCV E1E2 with HCV-specific host cell surface proteins, so-called entry factors and follow a similar route of entry as infectious full-length HCV. Upon entry into susceptible cells, e.g. Huh-7.5 cells, the proviral RNA is reverse transcribed and integrated into the host cell genome allowing the expression of a given reporter gene and thus the quantitation of successful entry events (fig. 2a). HCVpp systems paved the way for the identification of host proteins involved in HCV entry, e.g. the two tight junctional entry factors CLDN1 and OCLN. While many aspects of infectious HCV entry are reliably mimicked by HCVpp, the different architecture of lentiviral particles as compared to HCV particles results in differences in fusion properties. HCV particles are smaller in size (60 vs. 100 nm) and likely display a higher density of E1E2 dimers than HCVpp. This results in different membrane curvature, altered avidities and consequently differences in endocytosis and membrane fusion. Moreover, HCV is tightly associated with serum lipoproteins, which

Fig. 2. Important HCV cell culture systems. Cell-based HCV infection and replication models frequently utilized are depicted including retroviral HCV pseudotypes (HCVpp) (a), subgenomic HCV replicons (b), HCV trans-complemented particles (HCVTCP) (c), and cell culture-derived HCV (HCVcc) (d).
impacts on cell surface binding via LDL-R and SCARB1, a process that is poorly mimicked by HCVpp. Lastly, HCVpp with a lentiviral capsid preclude the analysis of HCV uncoating, i.e. the disassembly of the viral capsid and release of the RNA genome into the cytoplasm. In summary, HCVpp allow the isolated investigation of HCV entry and mimic well interactions of E2 with host entry factors; but the system cannot predict lipoprotein-dependent interactions and poorly mimics fusion and uncoating events.

While other systems to study aspects of HCV entry, including soluble E2 glycoprotein binding, membrane fusion and cell-to-cell spread assays, have been discussed elsewhere [31], we will here focus primarily on the intact particle assays, which provide a broader picture of the HCV infection process.

The biggest roadblock in HCV research was overcome in 2005 with the development of the first cell culture infectious clone. Despite the generation of HCV replicons and the identification of permissive cell lines, researchers failed for many years to generate infectious virus particles in these cells. In 2005, three independent laboratories finally described a recombinant genotype 2a HCV genome, which could replicate and assemble virus particles in Huh-7.5 cells [22–24]. Most of the non-structural regions of those chimeric genomes originated from HCV of a Japanese patient with fulminant hepatitis. Consequently, this HCV isolate was termed ‘Japanese fulminant hepatitis 1’ (JFH1). JFH1-based chimeric 2a genomes not only replicate without the need for adaptive mutations but also assemble and release viral particles, which are termed cell-culture infectious HCV (HCVcc) (fig. 2d). Such in vitro generated virions can infect chimpanzees and human liver chimeric mice, the two classical animal models for HCV. As the HCVcc system allows studying all aspects of the viral life cycle in vitro, it is still the most widely used system in HCV research. Although being the most robust and reliable HCV cell culture system, two major shortcomings still exist. First, HCVcc particles display a lower specific infectivity and higher buoyant density than serum-derived HCV [32] which may be due to impaired lipoprotein production in the context of Huh-7-derived cell clones. Second, standard Huh-7-based cultures are non-polarized and therefore poorly reflect the highly polarized hepatocytes in the liver. Importantly, recent work suggests that HCV can efficiently spread directly from cell to cell and that host factor requirements differ to some extent between cell-to-cell spread and cell-free infection [33]. While some studies try to address HCV infection of polarized cultures as well as cell-to-cell transmission, we still lack knowledge about the underlying mechanisms of HCV spread in polarized cell environments.

The first HCVcc systems were genotype 2a-based and thus precluded analysis of the clinically more relevant genotypes 1, 3 and 4. Two recent advances now allow studying the whole life cycle of other HCV isolates. First, researchers generated chimeric HCV genomes for all seven HCV genotypes [6]. These chimeric viruses encode the structural proteins of genotypes 1 through 7 and the non-structural proteins from JFH1 with certain adaptive mutations. Consequently, these intergenotypic HCV chimeras permit the analysis of genotype-specific entry and assembly events. Second, full-length infectious HCVcc was generated for genotypes 1 and 2, allowing the dissection of all life cycle steps [25–27]. Nonetheless, we still lack HCVcc systems for complete genotypes 3, 4, 5 and 6 and even for genotypes 1 and 2 only specific patient clones proliferate in culture. Thus a broad and patient isolate-specific analysis of particular life cycle steps, like e.g. assembly, is currently not possible.

An alternative system to study the whole life cycle of HCV are trans-complemented JFH1 particles (HCV TCP), which might overcome the hurdle of isolate specificity. HCV TCP generation requires (1) a JFH1 subgenomic replicon providing the replication machinery of the virus and (2) an expression system for the HCV structural proteins (core, E1, E2) and p7 and NS2 (fig. 2c) [28]. Theoretically, the in trans complemented structural proteins, p7 and NS2, can be derived from any patient isolate, thus providing isolate-specific information for HCV entry, replication and assembly. However, genetic incompatibility between the replication module and the packaging cassette is likely to limit efficacy of virus production when these units are derived from distinct genotypes. It remains to be shown if this hurdle can be overcome by use of specific adaptive mutations and/or custom-designed packaging systems for individual HCV genotypes.

With the technological advances in molecular biology including genome-wide association studies (GWAS), it became clear that host genetics is involved not only in hepatitis C disease progression but also in response to antiviral therapy. In particular a small nucleotide polymorphism upstream of a λ-IFN gene strongly correlates with disease and therapy outcome [7]. These and other findings underlined the need for primary cell culture systems in order to address the effect of host variance on HCV infection. To date, three systems for culturing patient-derived cells exist. Firstly, micropatterned co-cultures of human adult hepatocytes and non-parenchymal cells maintain hepatocyte characteristics for several
weeks and can be infected, although at low level, with HCVcc [34]. Secondly, fetal human hepatocytes remain hepatocyte-like for several days when cultured ex vivo and can be infected upon interference with antiviral innate immune responses [35]. Third, hepatocytes can be generated from induced pluripotent stem cells and are permissive to HCVcc at low level [14]. All these primary cell culture models are technically challenging, cost-intensive and require patient consent, precluding them from routine usage in a laboratory setting. Nonetheless, primary hepatocyte models hold the promise of understanding better the role of host genetics in HCV infection.

Despite the above-described major achievements in studying the cell biology of and thus the host factors involved in HCV infection, we are still unable to mimic certain aspects of HCV infection process. For instance, we currently lack robust polarized hepatoma cell systems, which would allow addressing cell entry, egress and cell-to-cell spread of HCV. Similarly, the culture of primary human hepatocytes and the maintenance of their differentiation status is highly challenging, limiting the study of host genetics during hepatitis C. Lastly, we are just starting to establish co-culture systems to characterize the intricate interplay of hepatocytes and other liver resident non-parenchymal cells, e.g. liver sinusoidal endothelial cells (LSECs). Taken together, the toolkit of HCV culture systems available to date primed the identification of several host targets for HCV therapy. Nonetheless, future developments, e.g. stem cell technologies, will further aid the design of HTAs and their host genotype-dependent validation.

**HCV Life Cycle**

HCV is a non-lytic virus, which infects target cells, proliferates inside them and then gets released into the extracellular space without disrupting the cell integrity. During its life cycle, HCV usurps host cell molecules, termed host factors, and various cell biological mechanisms ranging from endocytosis to the secretory pathway. Several host factors for HCV are expressed in a liver-specific manner thereby determining tissue tropism of the virus. Moreover, mammalian orthologs of host factors can vary from their human counterpart leading to the pronounced species tropism of HCV, which only infects humans and chimpanzees. The HCV life cycle can be separated into four steps: (1) virus entry; (2) genome translation and polyprotein processing; (3) genome replication, and (4) particle assembly and release from the host cell (fig. 3).

HCV, which is transmitted parenterally, enters the liver via the bloodstream. In the liver sinusoids, the virus can pass the fenestrated endothelium and contact the basolateral surface of hepatocytes, highly specialized parenchymal liver cells. HCV host cell entry is a complex multistep process that requires numerous host cell proteins. Among these, four so-called entry factors are indispensable for productive HCV uptake: scavenger receptor class B type I (SCARB1), the tetraspanin CD81 and the two tight junction molecules claudin-1 (CLDN1) and occludin (OCLN) [36–40]. While SCARB1 and CD81 bind the E2 glycoprotein on HCV particles [36, 37], evidence of E2 directly interacting with CLDN1 or OCLN is lacking. However, genetic studies underline that each of the four entry factors needs to be expressed on HCV-susceptible cells [39]. Kinetic and imaging studies suggest that HCV entry is a temporally and spatially tightly controlled mechanism, i.e. entry factors are likely used in a stepwise manner [38, 41–43] and in different membrane compartments [44, 45]. However, direct experimental evidence for this multistep and multidomain entry model is lacking. Without any doubt, CD81, SCARB1, CLDN1 and OCLN are indispensable for HCV entry in vitro and in vivo as demonstrated in human entry factor transgenic mice and human liver chimeric mouse models [46–49]. In addition to the four essential entry factors, other host molecules support HCV cell invasion. These include attachment factors like gangliosides [42, 50] and the low-density lipoprotein receptor (LDL-R) [51, 52], receptor tyrosine kinases [53], and Niemann-Pick C1-like 1 and transferrin receptor 1 [54, 55]. After cell surface binding and coordinated interaction with entry factors, HCV is taken up by clathrin-mediated endocytosis. Inside the low pH environment of early endosomes, the HCV envelope fuses with the endosomal membrane. This process is poorly characterized at the level of envelope protein refolding and fusion pore formation as we lack a high-resolution structural model of E1 and as E2 is structurally distinct from well-studied viral fusogens [118, 119]. In the cytosol the HCV capsid composed of the core protein disassembles and releases the HCV RNA genome into the cytosol thereby finalizing the invasion process.

The second step of the HCV life cycle takes place in the hepatocyte cytoplasm, where the plus-strand RNA genome is translated at the ER by host ribosomes generating a membrane-bound polyprotein. The polyprotein is then processed by host and viral proteases into the ten HCV structural (E1, E2, core) and non-structural proteins (p7, p30, p10, p8, p7, p6, p5, p4, p3, p2, p0) and non-structural proteins (p7, p30, p10, p8, p7, p6, p5, p4, p3, p2, p0).
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The HCV Life Cycle

1. Attachment
   - Attachment factors: NRP1, CD81, LDL-R, EphA2, EGFR, TFR1
   - Accessory factors: NS2, NS3, NS4A, NS4B, NS5A, NS5B

2. Entry
   - Essential entry factors: NS5A, lipid phosphorylation, CypA, processing, folding, ISG induction
   - Accessory factors: NS5A and lipid phosphorylation, E1/E2 dimers

3. Fusion
   - Essential entry factors: NS5A, lipid phosphorylation, E1/E2 dimers
   - Accessory factors: NS5A and lipid phosphorylation, E1/E2 dimers

4. Translation and polyprotein processing
   - Essential entry factors: NS5A, lipid phosphorylation, E1/E2 dimers
   - Accessory factors: NS5A and lipid phosphorylation, E1/E2 dimers

5. Membranous web
   - RNA replication
   - Accessory factors: NS5A and lipid phosphorylation, E1/E2 dimers

The viral RNA-dependent RNA polymerase NS5B subsequently amplifies the viral genome through an intermediate minus strand RNA. Replication takes place in a specialized cytosolic compartment, called the membranous web, which is induced by the virus [56–59]. Host factors aid in the formation of the ER-derived membranous web, at which multiple viral proteins including non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B and host factors assemble the HCV replication complex [60]. Among the most prominent HCV replication factors are cyclophilin A (CypA), phosphatidylinositol 4-kinase IIIα (PI4KIIIα) and microRNA-122 (miR-122), which will be discussed in more detail in the specific chapters below.

Once progeny HCV RNA genomes and the structural proteins E1, E2 and core are synthesized, the four components assemble to nascent virions. Initially, the RNA is packaged into capsids in close proximity to lipid droplets, which are cytosolic lipid storage organelles. Then, HCV capsids bud into the ER, thereby acquiring a host cell-derived envelope with E1E2 dimers embedded in the lipid bilayer. HCV egresses the cell usurping the secretory pathway and in close association with the very-low-density lipoprotein (VLDL) synthesis pathway. As a result, HCV virions circulate in the bloodstream in complex with host lipoproteins. Several host factors perform essential functions during HCV particle assembly and release. Two cellular lipid-modifying enzymes, namely diacylglycerol acyltransferase 1 (DGAT1) and the cytosolic phospholipase A2 (PLA2GA4), contribute to production of infectious HCV progeny [61, 62]. While DGAT1 directly interacts with HCV core and is important for load-

Fig. 3. HCV life cycle and host targets for therapy. Depicted are the first five steps of the HCV life cycle, namely (1) attachment; (2) entry; (3) uncoating; (4) translation and polyprotein processing, and (5) RNA replication. Each of these steps requires a set of host factors, some of which can be successfully targeted by the small molecule inhibitors, antibodies or antagonistic RNAs shown.
ing core protein onto lipid droplets, PLA2GA4 cleaves glycerophospholipids with arachidonic acid at the sn2 position, thereby affecting membrane fluidity and curvature conducive to virus production. In the ER, host glucosidases aid folding and maturation of HCV envelope glycoproteins. Lastly, apolipoproteins like apolipoprotein E (apoE) and proteins involved in VLDL generation like the microsomal triglyceride transfer protein (MTTP) support HCV particle production. With the release of infectious HCV lipoviroparticles into the bloodstream, the HCV life cycle is completed. Of note, in addition to infection of hepatocytes by free virions, HCV can, at least in vitro, spread directly from cell to cell. This direct cell-to-cell spread seems to require only a subset of the above-described entry host factors. However, to what extent cell-to-cell spread occurs in an infected individual remains to be clarified.

Taken together, HCV uses a multitude of host factors for each of its life cycle steps. Targeting such host factors with HTAs opens up unprecedented avenues in HCV therapy. Below we will report current HTA developments and highlight the three most advanced HTAs for HCV therapy.

**Prominent Host-Targeting Antiviral Strategies**

With the development of tissue culture model systems to study HCV and the discovery of host factors used by the virus, the targeted design of HTAs came into reach. Extensive studies on DAAs, i.e. agents that block viral proteins, clearly showed that DAA strategies have risks. In particular, fast emergence of resistance mutations and virus genotype dependency limits the use of DAAs to short-term combination therapy for mostly genotype 1 patients. HTAs now hold the promise of overcoming these caveats as the genetic barrier to resistance should be low and the diverse HCV genotypes seem to use the same set of host factors. Although no HTA is licensed to date, several compounds are in late-stage clinical development. Here we will summarize briefly the major HTAs, which are in preclinical and clinical stages and discuss their specific opportunities and caveats.

HCV entry is an attractive point of intervention in particular for preventive therapy. As 20% of HCV patients will develop severe liver disease 15–25 years after contraction of the disease, hepatitis C is still the number one indication for liver transplantation worldwide. Unfortunately, infection of the graft liver by HCV residing in peripheral reservoirs is almost universal and post-transplant patients oftentimes show an accelerated disease progression. Interference with HCV entry thus provides an attractive strategy to prevent infection of the transplanted liver. The most advanced entry HTA for HCV is ITX 5061, a small molecule inhibitor of the entry factor SCARB1 [63]. ITX 5061 successfully blocked entry of genotypes 1 through 6 in vitro and showed a good safety profile in clinical tests. When targeting host molecules a major concern is that interference with their endogenous function will result in adverse effects. Fortunately, the only major reported side effect of ITX 5061 is an elevated serum level of HDL. Although HTAs were initially thought not to suffer from resistance emergence, ITX 5061 long-term treatment in tissue culture resulted in an E2 (N415D) mutation [64]. Thus, HCV can in theory evade HTA therapy by mutating the viral binding partner of the targeted host factor. Whether such resistance can occur in vivo is unclear as the mutated virus had reduced fitness and might not emerge during short-term treatment. Clearly, the use of ITX 5061 will be limited to post-transplant settings, as a recent clinical phase 1 study in chronic non-transplant HCV patients showed low efficacy [65]. In addition to small molecule inhibitors of HCV entry factors, neutralizing antibodies are in preclinical development as HTAs. Antibodies targeting the ectodomains of SCARB1, CD81 and CLDN1 successfully block infection in vitro and – as shown for anti-SCARB1 and anti-CD81 – in mouse models for HCV. Future studies need to carefully evaluate whether short-term treatment with antibodies could prevent HCV infection of the liver graft in transplant patients without causing adverse effects.

HCV translation and polyprotein processing is a prerequisite for virus replication and thus a putative point of intervention in chronically infected individuals. While the translation machinery of the host cannot be targeted due to obvious adverse effects, polyprotein processing relies on viral proteases (NS3/4A) and HCV-specific host molecules, like CypA. HCV RNA stability and replication provides additional therapy options with antagonists of miR-122 and PI4KIIIα being the most advanced HTAs. We will discuss CypA, miR-122 and PI4KIIIα in detail in the following sections.

Lastly, host factors required for virion assembly and release could, similar to replication blockers, prevent disease progression in chronic patients. As cell-based assays to dissect the last steps of the HCV life cycle are relatively new [22–24], assembly and release HTAs are least advanced. The first assembly blockers described are iminosugars, which interfere with HCV glycoprotein folding.
and maturation [66–68]. Clinical trials were, however, terminated due to comparably modest efficacy [69]. Recently, several reports have highlighted additional cellular co-factors for virus assembly, which may be future targets for antiviral therapies. Among these are host factors of the VLDL pathway like apoE and MTTP. Inhibitors of MTTP show modest antiviral efficacy in preclinical tests. A small molecule inhibitor of intracellular apoE is currently not available. Other host assembly factors include the lipid-modifying enzymes DGAT1 and PLA2GA4 [61, 62]. Antagonists for both proteins exist and show promising anti-HCV activity in tissue culture systems. Importantly, the inhibitors are in development for other diseases (obesity and inflammatory disorders, respectively) and seem to show little adverse effects [70]. Thus, DGAT1 and PLA2GA4 inhibitors present a new avenue for assembly blockage and future preclinical tests could connect to the existing pipelines of anti-obesity and anti-inflammatory agents.

In summary, the identification of host lipoproteins and enzymes required for HCV assembly and release provides novel HTA development options. As we still have only limited knowledge on side effects of some compounds and efficacy of most assembly HTAs in vivo, future preclinical work needs to elucidate if assembly blockers merit further development as anti-HCV agents.

**Targeting HCV RNA Replication: Cyclophilin A**

Targeting HCV replication, i.e. amplification of the viral genome, is a particularly promising strategy, as it holds the promise of efficiently eradicating HCV from already infected tissue in chronic patients. CypA is a prominent HCV replication factor, which belongs to a highly conserved family of peptidyl-prolyl isomerases [71–74]. These enzymes isomerize peptide bonds at proline residues from trans to cis, thereby aiding protein folding or changing protein conformation [75]. In the case of HCV infection, CypA interacts with NS5A, which could promote viral protein folding, regulate polyprotein processing and thereby facilitate RNA replication [76, 77]. Inhibition of CypA by the cyclic polypeptide cyclosporine A (CsA) prevents the interaction of CypA and NS5A across all viral genotypes and has strong antiviral activity in vitro [78, 79]. As CsA is not only antiviral, but also immunosuppressive, several derivatives with exclusive antiviral functions were generated, including alisporivir (Debio 025), NIM811 and SCY-635. All three CypA-targeting antivirals are currently in clinical tests, with alisporivir being the most advanced [80]. Importantly, alisporivir efficiently reduced viral loads in genotype 1, 2, 3 and 4 patients without emergence of resistance to therapy [81–83]. Due to one fatal incidence in an alisporivir/IFN/ribavirin combination therapy phase 3 study, future clinical trials are limited to alisporivir monotherapy, which should resolve safety issues. Apart from its broad HCV genotype specificity and the high genetic barrier to resistance emergence in vivo, alisporivir seems to act independently of the host genetic background. A recent study investigated host variability of CypA and found that rare non-synonymous SNPs in CypA not only rendered cells largely resistant to HCV infection, but also residual replication was still sensitive to CypA inhibition [84].

The alternative CypA inhibitor SCY-635 disrupts the CypA-NS5A complex similarly to alisporivir, but might additionally stimulate antiviral innate immune responses. In a clinical phase 2a study, SCY-635 not only dose dependently repressed HCV load but also caused increased plasma levels of type I and III IFNs as well as 2′,5′-oligoadenylate synthase 1 (2′,5′-OAS-1), a key IFN-stimulated gene (ISG) [85]. Both transcriptional and post-translational mechanisms have been proposed to contribute to the activation of innate antiviral immunity by SCY-635 [86, 87]. To what extent these mechanisms contribute to the antiviral activity of CypA-targeting strategies and if they are shared by the different compounds targeting CypA remains to be clarified. Taken together, CypA-targeting HTAs could be attractive pan-genotypic therapeutics for IFN-free therapy, if concerns regarding their safety can be eliminated.

**Targeting HCV RNA Replication: Phosphatidylinositol 4-Kinase IIIα**

With the development of cell culture replication assays and full-length infectious HCVcc, genome-wide RNA interference screens allowed the identification of numerous additional host factors for HCV replication [88–96]. One of the most prominent and most consistently identified HCV replication factors is PI4KIIIα [90–95]. This lipid kinase resides at the plasma membrane and the ER, where it generates phosphoinositides at the cytosolic membrane leaflet. During HCV infection, PI4KIIIα additionally interacts with and phosphorylates NS5A [91, 94, 97]. Silencing of the kinase results in strongly reduced HCV replication and an aberrant structure of the membranous web [94]. Likely both the PI4KIIIα-dependent regulation of NS5A phosphorylation and local accumulation of phosphatidylinositol 4-phosphate pools are important for HCV replication. Two different classes of inhibitors targeting PI4KIIIα (AL-9 and compounds A and B) effi-
ciently block replication complex formation and consequently HCV genome amplification [98, 99]. Notably, PI4KIIIα HTA therapy resistance can emerge in vitro through mutation of NS5A and/or NS4B, but at a high fitness cost for the virus. Thus, it is questionable whether such resistant variants could develop in vivo. Both AL-9 and compounds A and B are in preclinical stages of development. Caution is, however, warranted for their therapeutic use, as PI4KIIIα is an essential host enzyme. This is stressed by the fact that PI4KIIIα knockout mice succumb to gastrointestinal disorders. Consequently, the expected strong adverse effects of targeting PI4KIIIα will limit further development of this class of inhibitors for future HCV therapy.

**Targeting HCV RNA Replication: MicroRNA-122**

In addition to host proteins, a host microRNA supports HCV replication. MicroRNAs (miRNAs) are non-coding nucleic acids of 20–22 nucleotide length. Typically, miRNAs bind to host mRNA and block translation or target mRNAs for degradation [100]. HCV, however, relies on the host miRNA-122 (miR-122) for degradation [101]. HCV, however, represses its replication, its high abundance is crucial for efficient replication [102]. Moreover, miR-122 is mainly expressed in the liver, thus miR-122 likely contributes to the hepatotropicism of HCV [103, 104]. MiR-122 gets efficiently inactivated by a complementary locked nucleic acid–modified oligonucleotide (miravirsen or SPC3649) and miravirsen treatment reduces HCV titers in vitro and in HCV-infected chimpanzees [105]. Moreover, broad HCV genotype specificity in vitro suggests a wide usage for a miravirsen-based therapy in patients [112]. Notably, a clinical phase 2a trial demonstrated efficacy of miravirsen monotherapy without any adverse effects or signs for the development of resistance mutations [113]. Therefore, the barrier to viral resistance to this drug seems high [114]. Additionally, miR-122 is required across all HCV genotypes suggesting that miravirsen is a promising pan-genotype HTA [111]. While miravirsen administration is currently only possible through the less attractive parenteral route, an advantage of miravirsen therapy could be the long-lasting effect, which might allow monthly administrations of miravirsen [115]. The long-lasting effect of miravirsen could, however, also pose a potential risk during therapy. In a mouse model, miR-122 deletion results in the development of steatohepatitis, fibrosis and hepatocellular carcinoma and the tumor development can be reversed upon reconstitution with miR-122 [116]. If miR-122 acts as a strong tumor suppressor in the liver, antagonizing this miRNA bears the risk of tumor induction. Further studies need to critically evaluate possible adverse effects during miravirsen treatment. If side effects can be kept in check, miravirsen is a candidate for future IFN-free regimen for the treatment of chronic hepatitis C.

**Conclusion: Current and Future HCV Therapy Targets**

Almost a quarter of a century ago, Michael Houghton and colleagues identified HCV as etiological agent causing non-A, non-B hepatitis. Since then, several milestones in HCV research have led to the diverse toolbox of HCV tissue culture systems available today. While initial studies centered around the delineation of the HCV genome and polyprotein organization, more recent achievements focused on the development of reliable cell culture models for HCV. Nowadays, we benefit from in vitro systems for all steps of the HCV life cycle, namely entry, translation and polyprotein processing, genome replication and assembly and release of virions. Ideally, such cell culture models should have a high predictive value for testing of antiviral drugs. Indeed, several virus- and host-targeting drugs stemmed from in vitro findings and are in clinical tests. Several direct acting antivirals targeting the viral protease NS3/4A or the NS5B polymerase are already approved for HCV therapy of genotype 1 patients. Nonetheless, there is a need for development of alternative treatment options for patients with genotypes other than genotype 1 and patients with counterindications for current therapies, e.g. HIV co-infected patients or patients with progressed liver disease. Moreover, viral resistance emergence can further limit DAA treatment. A promising new avenue presents HTAs which in general have a high genetic barrier for resistance development and are mostly pan-genotypic. Among the most advanced HTAs are inhibitors of CypA and PI4KIIIα and antagonims of miR-122. While these HTAs show high pan-genotypic efficacy in preclinical and clinical tests with no emergence of viral resistance variants, a risk of strong adverse effects remains. Future studies need to carefully monitor possible side effects of host-targeting drugs and evaluate optimal treatment duration.

Despite the fast advances in HCV therapy development, challenges still remain. Current in vitro systems...
cannot address host genotype-specific effects as primary hepatocytes are difficult to culture. Similarly, current hepatoma cell systems are non-polarized, thus poorly mimicking the architecture of hepatocytes in the liver. Stem cell technologies and improved culture systems for primary liver cells are under development and may in the future allow drug testing with increased predictive value. Taken together, intense research on DAAs and HTAs is needed to find the most effective drug combinations with the least adverse effects [9–12, 117]. Lastly, a better understanding of host and virus genetic diversity and their influence on drug efficacy could spur individualized therapy in the future. Such personalized medicine holds the promise of offering the best treatment options and therapy outcome for individual patients.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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