Targets for Antiviral Therapy of Hepatitis C

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Abstract

Presently, interferon- (IFN-) containing treatment regimens are the standard of care for patients with hepatitis C virus (HCV) infections. Although this therapy eliminates the virus in a substantial proportion of patients, it has numerous side effects and contraindications. Recent approval of telaprevir and boceprevir, targeting the protease residing in nonstructural protein 3 (NS3) of the HCV genome, increased therapy success when given in combination with pegylated IFN and ribavirin, but side effects are more frequent and the management of treatment is complex. This situation will change soon with the introduction of new highly potent direct-acting antivirals. They target, in addition to the NS3 protease, NS5A, which is required for RNA replication and virion assembly and the NS5B RNA-dependent RNA polymerase. Moreover, host-cell factors such as cyclophilin A or microRNA-122, essential for HCV replication, have been pursued as therapeutic targets. In this review, the authors briefly summarize the main features of viral and cellular factors involved in HCV replication that are utilized as therapy targets for chronic hepatitis C.

Keywords

- ▶ NS3 protease
- ▶ NS5A
- ▶ NS5B
- ▶ miR-122
- ▶ cyclophilin A

Hepatitis C virus (HCV) infection still is a major health burden affecting 130 to 170 million people worldwide. Most infections persist and persistently infected individuals have a high risk to develop liver cirrhosis and hepatocellular carcinoma after 10 to 30 years of infection.¹ The first effective therapy regimen that became the standard of care (SOC) for many years, was the combination of interferon α (IFN α) and ribavirin (RBV).^{2,3} However, sustained virological response (SVR) rates strongly depended on the HCV genotype.^{3,4} In addition, severe side effects such as hemolytic anemia, changes in blood cell counts and psychiatric disorders such as depression are major limitations of this therapy. Because of that, many patients are not eligible for this treatment.^{4,5}

To overcome these problems, intensive research efforts have been undertaken to develop HCV-specific direct-acting antivirals (DAAs). Direct-acting antivirals target viral proteins required for the HCV lifecycle, whereas host-targeted agents (HTAs) target host cell factors needed by the virus for productive replication (▶ **Table 1**). The first DAAs approved in 2011 were inhibitors of the HCV protease residing in

nonstructural protein 3 (NS3): boceprevir (BOC) and telaprevir (TVR), which are now implemented into SOC protocols for HCV genotype 1 infections.^{6–10} These drugs increased SVR rates in genotype 1 infections from 40% to around 75%.^{6–10} However, these DAAs are very selective for HCV genotype 1 and increase the frequency of side effects as compared with SOC.^{7–10} In addition, the barrier to resistance for these drugs is low. In fact, single-nucleotide substitutions are sufficient to render HCV resistant while retaining replication fitness.^{11,12} Thus, research efforts aimed to develop new DAAs targeting the NS3 protease with higher potency or additional viral proteins as well as HTAs. These efforts are beginning to pay off and second-wave, first-generation, and second-generation NS3 protease inhibitors as well as drugs targeting the NS5B RNA-dependent RNA polymerase (RdRp) or the NS5A protein are in late-stage clinical development. In this brief review, we will summarize the major structural and functional features of the prime targets of HCV-specific DAAs. We will include short descriptions of host factors that are considered as targets for antiviral therapy as well as alternative viral targets.

Table 1 Targets for hepatitis C virus (HCV) specific antiviral therapy. Viral and cellular factors involved in the viral life cycle and their respective functions are listed. Drug classes and their mode of action are also given.

Targets for antiviral therapy		
<i>Viral factor</i>	<i>Virological function</i>	<i>Drug classes/ mode-of-action</i>
E1/E2	Envelope glycoproteins, cell attachment and cell entry	Neutralizing antibodies
p7	Viroporin; involved in assembly and release of infectious virions	Amantadine, imino sugars
NS3/4A	Serine protease; processing of viral polyprotein; interference with innate immunity	Covalently, but reversibly binding linear peptidomimetics; noncovalently binding linear inhibitors; macrocyclic inhibitors <i>Preventing proper polyprotein processing; restoring interferon response</i>
NS4B	Membrane remodeling	Various small-molecule inhibitors; silibinin <i>Interference with ns4b-RNA interaction; membrane association; ns3/4a-ns4b interaction</i>
NS5A	RNA replication, assembly of virus particles, induction of double membrane vesicles	Ns5a-inhibitors <i>Interference with membranous web formation, impairing virus production</i>
NS5B	RNA-dependent RNA-polymerase	Nucleosidic inhibitors (NI); Nonnucleosidic inhibitors (NNI) <i>Chain termination; inhibiting RNA template binding and initiation of elongation</i>
<i>Host factor</i>	<i>Virological function</i>	
CD81	Essential entry factor	Various antibodies and small molecule inhibitors <i>Interference with cell-attachment, virus entry and cell-to-cell-transmission</i>
SCARB1	Essential entry factor	
OCLN	Essential entry factor	
CLDN1	Essential entry factor	
Cyclophilin A	NS5A cofactor	(Non-) immunosuppressive cyclosporine A derivatives; sanglifehrin <i>Interference with membranous web formation and RNA replication</i>
miR122	Stabilizing viral RNA, promoting RNA translation and replication	Antagomirs <i>Sequestering mir-122</i>

HCV Genome Organization and Replication Cycle

HCV is grouped in the family *Flaviviridae* in the genus *Hepaciviruses*.¹³ These viruses have in common a single-stranded RNA of positive polarity (►Fig. 1A). The RNA has a single open reading frame that is flanked by 5'- and 3'-nontranslated regions (NTRs), respectively.

The HCV replication cycle is tightly linked to the lipid metabolism of the host cell (►Fig. 1B). Virions are closely associated with apolipoproteins and circulate in the bloodstream of patients as lipovirions.^{14,15} Trapping of virus particles on the surface of the host cell is mediated by interaction with glycosaminoglycans (GAGs) and the low-density lipoprotein receptor (LDL-R). Thereafter, the viral envelope glycoproteins E1 and E2 interact with four different cellular receptor molecules: scavenger receptor class B type 1 (SCARB1), the tetraspanin CD81, and the tight junction components occludin (OCLN) and claudin 1 (CLDN1) (reviewed in¹⁶)

(►Fig. 1B). In addition, entry is facilitated by interaction of HCV with the epidermal growth factor receptor (EGFR)¹⁷ or the Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1),¹⁸ which likely contributes to virus uptake and liberation of the viral RNA genome into the cytoplasm. Upon release, this RNA is translated at the rough endoplasmic reticulum (ER) giving rise to a single polyprotein, which is co- and posttranslationally processed into 10 proteins (reviewed in¹⁹) (►Fig. 1A): the structural proteins—core and envelope glycoproteins E1 and E2; the viroporin p7 required for virus particle formation²⁰; nonstructural protein 2 (NS2) that mediates cleavage between NS2 and NS3 and is also required for virion assembly; NS3, composed of protease and helicase domains that are required for polyprotein processing and RNA replication, respectively; NS4A that acts as a cofactor of NS3 and activates NS3 protease activity; NS4B that induces membrane alterations; the multifunctional NS5A, required for replication and assembly; and the NS5B RdRp. The structural proteins as well as p7 and NS2 are processed by host cell signal

Fig. 1 Genomic organization and replication cycle of the hepatitis C virus (HCV). (A) The single-stranded RNA genome forms highly structured nontranslated regions (NTR) at the 5' and 3' ends; the 5'-NTR contains an internal ribosome entry site (IRES). The open reading frame encodes for a single polypeptide that is processed by cellular signal peptidases and signal peptide peptidases (arrows below the schematic), as well as the viral proteases NS2 and NS3 (curved arrows above the schematic). The polypeptide can be functionally divided into the assembly module, comprising core to NS2 (grey boxes) and facilitating assembly and release of new virions,²⁰ and the replication module consisting of NS3 to NS5B (green boxes). The latter are required for biogenesis of the membranous web (MW) and replication of the viral RNA. (B) Entry of HCV particles requires successive interactions with CD81, SCARB1, CLDN1, and OCLN (step 1). The viral RNA is released into the cytoplasm and translated at the rough endoplasmic reticulum (ER; step 2). The polypeptide is proteolytically processed and membranes are remodeled giving rise to the membranous web that is composed primarily of double membrane vesicles in close proximity to lipid droplets (step 3). Within the MW, viral RNA is amplified via a negative-strand intermediate (step 4). Virus particles are assembled at the ER in close vicinity of lipid droplets (LDs) and released via the secretory pathway (step 5). Steps of the viral life cycle addressed by direct-acting antivirals and their respective targets are indicated.

peptidase and signal peptide peptidase,¹⁹ whereas the remainder of the polypeptide is processed primarily by the NS3 protease (►Fig. 1A).

HCV replication occurs in the cytoplasm in a specialized membranous compartment that has been designated “membranous web” (►Fig. 1B).^{21,22} This web is induced by a concerted action of the “replicase factors” NS3, NS4A, NS4B, NS5A, and NS5B.²³ The NS5B RdRp is responsible for amplifying the positive-strand RNA genome via a negative-strand

copy that in turn serves as a template for the synthesis of large amounts of new positive-strand RNA molecules. Virion assembly occurs in close vicinity to lipid droplets where core protein and NS5A localize.^{24,25} Newly formed nucleocapsids acquire their envelope via budding through the ER membrane in close association with the host cell machinery that is responsible for the synthesis of very-low-density lipoproteins (VLDL). In this way, newly assembled virions associate with VLDL/LDL components and are secreted as lipovirions, presumably along the constitutive secretory pathway.^{14,26–28}

Released particles infect new host cells either via the extracellular route or by direct cell-to-cell-transmission. The latter is thought to be the major route of HCV propagation in vivo. This route appears to be less susceptible to neutralizing antibodies and has somewhat different requirements for entry molecules as compared with infection by cell-free virions.¹⁶ For instance, it has been reported that cell-to-cell transmission occurs independent from CD81.^{29,30} Moreover, SCARB1 appears to play a more prominent role in cell-to-cell spread as compared with infection with cell-free virus.³¹

Viral Targets for Therapy of Chronic Hepatitis C

NS3 Protease

NS3 is a bifunctional molecule (►Fig. 2A). The amino-terminal domain comprises a serine-type protease, whereas the carboxy-terminal domain possesses ATP-dependent helicase activity.¹⁹ Although the helicase function is likely involved in unwinding viral RNA and supporting RNA replication, the protease domain is responsible for cleavage between NS3-4A, NS4A-4B, NS4B-5A, and NS5A-5B. In addition to processing of the viral polypeptide, host-cell factors that play important roles for the activation of the innate immune response are proteolytically inactivated by NS3. Best-known examples are mitochondrial antiviral-signaling protein (MAVS) and TIR-domain-containing adapter-inducing IFN- β (TRIF) that are essential for the activation of the IFN system.^{32,33} In this way, HCV subverts proper innate immune response that otherwise efficiently blocks viral replication.

The NS3 protease has several unique properties.³⁴ It has a rather low substrate specificity that is defined by an acidic amino acid residue at the P6 position (i.e., six residues N-terminal of the cleavage site) and a cysteine residue at the P1 position. High protease activity requires NS4A as a cofactor that serves several purposes: First, it tethers NS3 to the outer leaflet of the ER-membrane (►Fig. 2A) and stabilizes the protein that is otherwise rapidly degraded when NS4A is missing³⁵; second, the NS4A cofactor enhances catalytic activity of the protease as well as its interaction with the substrate³⁶; third, NS4A forms an integral part of the protease.^{37,38} The NS3 protease is composed of two domains that are separated by a deep cleft that contains the active site. The amino-terminal domain consists of eight β -strands. Seven of them are formed by NS3 itself, whereas one is formed by the central region of NS4A, which thus constitutes an integral part of the protease (►Fig. 2A). The structural role of NS4A is

Fig. 2 Three-dimensional structures of hepatitis C virus (HCV) proteins that are prime targets for antiviral therapy. (A) Membrane topology of the NS3 protease/helicase in complex with the NS4A cofactor.⁴¹ The amino-terminal protease domain is indicated in blue, the carboxy-terminal helicase domain in green. The NS4A cofactor that tethers NS3 to intracellular membranes is indicated in brown. Note that the central region of NS4A forms an integral part of the protease domain. Amino acid residues forming the active site of this enzyme are shown in red. (B) Structures of membrane-associated NS5A dimers according to Love and colleagues⁶² (back-to-back structure model) and Tellinghuisen and coworkers⁶¹ (claw-like structure model). Each subunit (light blue and pink, respectively) consists of the amino-terminal AH, (PDB accession 1R7E), the highly structured D1 (PDB accession 3FQO and 1ZH1 for left and right structures, respectively) and the intrinsically unfolded D2 and D3 (only representative conformers are shown; F. Penin, unpublished). Note that the relative positions of the different structure elements are speculative, especially the position of D1 relative to the membrane and the AH helices. The only consistent positioning is that of AH relative to the membrane that was simulated by using molecular dynamics (F. Penin, unpublished). (C) Structure of membrane-bound NS5B. Ribbon diagram of NS5B (PDB accession 1GX6) and association with the membrane via the carboxy-terminal transmembrane tail (bronze). Finger (F), palm (P), and thumb (T) subdomains (light blue, yellow, light green, respectively) are indicated. The “closed” conformation of the proposed membrane topology of NS5B is shown in the upper panel. This structure is thought to represent the initiation state of the RdRp. The active site of the enzyme is highlighted by two priming nucleotides (red). Note the stacking of the NS5B ectodomain to the membrane in this proposed conformation. A model of NS5B in a hypothetical elongation model is shown in the lower panel. In this structure, the RNA-binding groove would be released and could accommodate a double-stranded RNA replication intermediate. This intermediate was modeled into the active site according to a recent publication.⁸⁵ According to this model, the catalytic core of NS5B would be dislocated away from the membrane as a result of “stretching” of the linker sequence (F. Penin, unpublished). Such a conformational change might occur during switching from initiation to elongation of RNA synthesis. In all panels, the membrane is represented as a simulated model in which polar heads and hydrophobic tails of phospholipids (stick structures) are colored light gray and light yellow, respectively.

underscored by the fact that in the absence of this cofactor, the 30 amino-terminal residues of NS3 are loosely oriented and do not form a rigid structure.

The carboxy-terminal NS3 protease domain forms a β -barrel structure stabilized by a zinc atom that is complexed by three highly conserved cysteine residues and one histidine residue.³⁹ This zinc atom plays a structural role and is not involved in the catalytic activity of the protease. The active site composed of the catalytic triad (H57, D81, and S139 in case of genotype 1) of the NS3/4A protease is rather flat and lacks prominent surface loops that usually surround the active site of other serine-type proteases. Given this shallow substrate-binding pocket, the NS3 protease requires relatively long substrates for efficient binding. Optimal substrate recognition occurs with substrates that are 10 amino acid

residues long interacting with the enzyme by a series of weak molecular interactions distributed across the substrate surface (reviewed in⁴⁰). It is hypothesized that the low substrate specificity is compensated by the tethering of the NS3/4A complex to the ER membrane, thus providing spatial proximity between the substrate and the protease.⁴¹

Different approaches have been pursued to develop NS3 protease inhibitors (PIs). These are competitive inhibitors preventing substrates to enter the active site or allosteric inhibitors interfering with crucial conformational changes that are required for substrate cleavage. Up to now, three different classes of competitive NS3 PIs have been developed: (1) covalently, but reversibly binding linear peptidomimetics (e.g., telaprevir, boceprevir); (2) noncovalently binding linear inhibitors (e.g., BMS-650032, BI201335); (3) macrocyclic

inhibitors (e.g., MK-5172, vaniprevir, danoprevir, simeprevir).^{40,42,43} Development of these compounds was based on the observation that the NS3 protease undergoes end product inhibition.^{44,45} This means that after cleavage of its substrate, the P-side cleavage product remains tightly bound to the active site, thus competing with the binding of new substrates.^{44,45} By using P-side peptides as lead structures and intensive derivatization, highly efficient NS3 protease inhibitors have been developed.

First proof-of-concept was achieved with the macrocyclic inhibitor ciluprevir (BILN 2016).⁴⁶ However, during subsequent trials with other PIs, it became rapidly clear that monotherapy would not be successful with these drugs because of the rapid selection for drug resistance. Indeed, for many PIs single-nucleotide substitutions are sufficient to render the NS3 protease resistant; unfortunately, many of these mutations have little effect on replication fitness and thus, are retained even after cessation of therapy.^{11,47,48} Moreover, many of these mutations confer resistance against multiple PI-classes.⁴⁹ For instance, mutations affecting amino acid residues 41, 43, 54, 155, and 156 of NS3 confer resistance against linear peptidomimetics and macrocyclic inhibitors, thereby causing a profound loss of therapeutic options.⁵⁰ Moreover, natural polymorphisms affecting these sites contribute to poor antiviral efficacy against HCV genotypes other than 1,^{50,51} which is most pronounced for “first generation” PIs that have been developed and optimized by using genotype 1-based replicon systems. More recently developed PIs, such as MK-5172, overcome this limitation.⁴³ Apart from its pan-genotypic activity, this compound is substantially less affected by mutations that confer resistance to first-generation drugs, which is likely due to the very close interaction of MK-5172 with the catalytic triad of the enzyme that has not been observed for other PIs.^{43,52,53}

One alternative concept to inhibit the NS3 protease is interference with the binding site of the zinc atom, thus inducing misfolding of the protein.⁵⁴ Another possibility is a block of the interaction of NS3 with its cofactor NS4A, as exemplified with the compound ACH-806 (halted development).^{55,56} Finally, recent crystallography studies revealed a region residing between the NS3 protease and the helicase domain (→Fig. 2A) that can be targeted by antiviral compounds. It is proposed that the NS3/4A complex exists in two distinct conformations: an open conformation with catalytic activity and a closed one, the autoinhibited form. The newly identified compounds block protease activity by keeping the enzyme in the autoinhibited conformation, that is a state where the carboxy-terminal region of NS3 itself is located close to the active site of the amino-terminal NS3 protease domain,⁵⁷ thus preventing substrates to enter. Interestingly, resistance mutations affect amino acid residues located in the putative binding site of the compound. This site has only moderate intergenotypic conservation, explaining the limited antiviral activity of this compound class across different HCV genotypes.^{50,51}

NS5A

NS5A is a multifunctional factor with no known enzymatic activity.¹⁹ It is required for RNA replication, formation of the

membranous web, and assembly of infectious HCV particles. In addition, it has been reported that NS5A might affect the antiviral response of the host cell and contribute to pathogenesis.⁵⁸ NS5A is composed of an amino-terminal amphipathic α -helix (AH) required for membrane attachment of the protein⁵⁹ and recruitment to lipid droplets²⁵ and three domains (D1–3) that are separated by two low complexity sequences (→Fig. 2B).⁶⁰ Although D1 forms well-structured homodimers,^{61,62} D2 and D3 are intrinsically unfolded and thus capable to interact with multiple viral and cellular factors.^{63,64} It is thought that NS5A exerts its multiple functions by interacting with various viral factors, such as the RNA genome, NS4B, or NS5B, as well as with host-cell proteins like human vesicle-associated membrane protein-associated protein A (hVAP-A), phosphatidylinositol-4-phosphate kinase III α (PI4KIII- α), or cyclophilin A (CypA). Although D1 and parts of D2 are required for RNA replication, most of D3 is dispensable for this process, but essential for assembly of infectious virus particles,^{65,66} presumably by mediating an interaction with the core protein during virion formation.⁶⁷ D2 and especially D3 are also very tolerant to insertion of large heterologous sequences, such as green fluorescent proteins, thus allowing the set-up of systems that monitor NS5A-containing structures in live cells.^{68–70}

NS5A is phosphorylated at several sites, giving rise to a basal and a hyperphosphorylated form (p56 and p58, respectively).⁷¹ Phosphorylation is mediated by cellular kinases, most notably the α -isoform of casein kinase 1 and casein kinase 2.^{66,72} It is assumed that additional host-cell kinases are involved in NS5A phosphorylation, but this is not definitively proven. Interestingly, mutations enhancing RNA replication in cell culture frequently affect putative phosphorylation sites in NS5A, but reduce virus particle formation,⁷³ arguing that the phosphorylation status might regulate different steps of the HCV life cycle.

Two alternative NS5A homodimers have been identified by X-ray crystallography of highly purified recombinant NS5A D1 proteins (→Fig. 2B).^{61,62} Although the structures of the monomers are identical, they can self-interact in different ways, giving rise to two alternative dimer structures designated the “claw-like” structure and the “back-to-back” structure. Moreover, by using in silico studies, it has been proposed that the monomers and/or dimers might form large multimeric complexes.⁶² Although experimental proof is lacking, it is tempting to speculate that multimerization might be regulated by the phosphorylation status of NS5A.

Owing to the lack of enzymatic activity, NS5A has been considered “nondruggable.” However, this view changed with the discovery of compounds that suppress HCV replication with high potency in cell-culture systems and select resistant viral variants with mutations in NS5A. By using random screening of a large compound library and intensive subsequent optimization of the identified lead structure, Gao and colleagues were able to develop the antiviral compound daclatasvir (formerly BMS 790052), suppressing HCV RNA replication with unprecedented potency.⁷⁴ The exact mode-of-action of this compound is not known, but several phenotypes have been reported. These include a change of the p56/p58 ratio in favor of the basal

phosphorylated NS5A form, as well as redistribution of NS5A from the ER to LDs.^{75–77} However, these phenotypes are pleiotropic and can be induced by several other means such as treatment with a NS3 protease inhibitor or knockdown of PI4kIII- α expression.⁷⁸ Thus, a more plausible hypothesis that takes into account the high potency of this drug class is the disruption or “fragmentation” of NS5A multimers. In this model, biological activity of NS5A would be exerted by large multimeric complexes, forming “RNA railways” that could bind the viral RNA genome (especially in case of multimers composed of the claw-like structure). Binding of only a few inhibitor molecules might fragment these multimers, rendering them nonfunctional. This is an attractive hypothesis, but confirmation will require assays that allow discrimination between NS5A dimers and oligomers. For instance, we found that highly potent NS5A inhibitors do not disrupt NS5A self-interaction as determined by pulldown (C. Berger and R. Bartenschlager, unpublished). However, pulldown assays do not discriminate between dimers and multimers. Thus, more sophisticated biochemical approaches are required.

Whatever the impact of Daclatasvir-like drugs on NS5A structure is, we found that these inhibitors potently block biogenesis of the membranous web, and thus RNA replication (C. Berger and R. Bartenschlager, unpublished). This result corroborates the important role of NS5A in inducing membrane alterations, most notably formation of double membrane vesicles (DMVs), which are the likely sites of HCV RNA replication.^{23,79}

A hallmark of highly active NS5A inhibitors is their symmetric structure. They are thought to bind to NS5A across the dimer interface.⁸⁰ This binding mode would fit with the position of the most frequently found resistance mutations affecting amino acid residue 93, which resides near the dimer interface in each of the dimer structures. Interestingly, several additional resistance mutations affect residues that are located close to the amino-terminal end of D1, arguing that they might affect positioning and/or folding of the linker segment connecting D1 with the amino-terminal membrane-anchoring α -helix (►Fig. 2B). How such alterations of NS5A structure would affect its ability to induce MW formation remains to be determined.

Apart from affecting RNA replication, recent results obtained with mathematical modeling based on HCV replication kinetics in infected patients suggest that production and release of HCV particles is also affected by NS5A inhibitors.⁸¹ Consistent with the model, comparison of HCV-infected cells treated with daclatasvir or a NS5B RdRp inhibitor revealed comparable kinetics of inhibition of RNA replication with both drugs in vitro. However, daclatasvir induced a much faster decline of HCV titers as compared with the polymerase inhibitor, arguing that this highly potent NS5A inhibitor has a dual mode of action: blocking RNA replication and virus production. Whether this is due to interference with two structurally distinct NS5A complexes remains to be determined.

NS5B

The core of the replication machinery responsible for amplification of the HCV genome is the NS5B RdRp. This protein is

tethered to intracellular membranes posttranslationally by a membrane anchor located in the carboxy-terminal region (►Fig. 2C).¹⁹ NS5B displays the typical “right-hand” conformation with finger, palm, and thumb subdomains.^{82–84} The active site of the polymerase is located in the palm subdomain and includes a GDD motif that is involved, via complexed Mg^{2+} ions, in binding of nucleotide substrates and nucleotide polymerization. A hallmark of the enzyme is the rather closed conformation of the active site that is due to intensive interactions between fingers and thumb subdomains. Moreover, the active site is occluded by a short segment of the thumb domain, designated the β -flap. This closed conformation likely reflects the enzyme at the stage of de novo initiation of RNA synthesis, which is thought to be the mechanism by which NS5B initiates HCV RNA replication in vivo. Transition into the elongate state requires removal of the β -flap.⁸⁵ Apart from conformational changes around the active site, NS5B likely has to undergo major structural rearrangements to allow binding of the RNA template (►Fig. 2C, lower panel). Molecular modeling suggests that the RNA-binding groove is stacked toward the membrane surface, thus precluding binding of the RNA template.¹⁹ RNA binding might be achieved by a “stretching” of the linker connecting the carboxy-terminal membrane anchor with the RdRp domain, thus liberating the RNA binding groove (►Fig. 2C).

Inhibition of NS5B can be achieved by two different strategies: first, nucleoside or nucleotide inhibitors (NIs) that mimic the natural substrate and are incorporated into the newly synthesized RNA strand finally leading to termination of elongation⁸⁶; second, nonnucleoside inhibitors (NNIs) that bind to the enzyme and act as allosteric inhibitors by inducing conformational changes that impair polymerase activity.^{40,87,88}

Nucleotide inhibitors have pangenotypic activity, which is likely due to the high conservation of the active site. Nucleotide inhibitors are given as ribonucleosides to allow membrane permeation, but need to be converted into the corresponding 5′-triphosphate by host cell kinases. After phosphorylation, NIs are accepted as substrates of the viral RdRp and incorporated into the growing RNA chain, thus causing “chain termination.” However, most NIs developed for hepatitis C therapy retain a free 3′-hydroxyl group, but contain modifications at the ribose 2′-position because 3′-deoxy-modified nucleosides are inefficiently phosphorylated and thus, poorly active in cell culture.⁸⁹ For 2′-modified NIs, chain termination is indirect and induced by steric hindrance resulting from the modifications at the ribose 2′-position, which is at variance to classical chain terminators such as acyclovir or tenofovir that are used in other viral infections. Moreover, NIs have to be given as prodrugs, most often as nucleosides to allow membrane penetration. Upon uptake, these NIs have to be converted first into the 5′-monophosphate, which is a rate-limiting step, and then into the di- and triphosphate. To overcome this rate limitation, NIs such as sofosbuvir have been designed as nucleotide analogues, that correspond to 5′-monophosphates.⁹⁰ To allow efficient uptake into cells, these poorly membrane-permeable

5'-monophosphates are extensively derivatized. These chemical modifications have to be removed by cellular enzymes, thus liberating the corresponding 5'-monophosphate in the cell.

Given the structural similarity of HCV-specific NIs, a single amino acid substitution in NS5B (S282T) confers resistance to all 2'-modified NIs.⁹¹ However, only low-level resistance is conferred by this mutation that causes dramatic reduction of replication fitness. This explains why the S282T mutation has been rarely observed at clinically meaningful levels in NI-treated patients.⁹²

Nonnucleoside inhibitors comprise a chemically heterogeneous group of compounds. There are at least four distinct allosteric NNI-binding sites designated according to their position within NS5B⁴⁰: thumb-1 and thumb-2 are located on the polymerase thumb domain whereas palm-1 and palm-2 reside in the palm domain and close to the active site. The NNI-binding sites are not well conserved among HCV genotypes, thus explaining profound genotype specificity of antiviral activity. For instance, thumb-1 NNIs show good antiviral activity against genotype 1 and genotype 3 isolates, whereas activity against genotype 2 isolates is rather poor.⁹³ Because NS5B has two major conformational states, a closed one to initiate RNA-synthesis and a more open one for elongation of the RNA chain (see above), NNIs can either induce unfavorable conformational changes like a hyperclosed active site in which template binding is not possible, or limit the overall mobility of the enzyme that is necessary for initiating RNA synthesis.^{94,95}

Due to the different binding sites of NNIs, these compounds can be used for combination therapy. In case of thumb-1 and thumb-2 NNIs, resistance mutations at one binding site do not affect the other binding site. Only palm-1 and palm-2 are physically overlapping; therefore, cross-resistance occurs. By using cell-based assays, Delang and colleagues⁴⁰ demonstrated rapid selection for resistance against single NNIs that did not affect susceptibility of HCV to other NNI classes. Emergence of resistance could be delayed when drug combinations were used and selection for resistance against all classes of NNIs was only achieved in triple therapy with low inhibitor concentrations. Subsequent mutation analyses revealed no emergence of novel mutations, but combinations of resistance mutations against the single classes of NNIs.

Host Targets for Therapy of Chronic Hepatitis C

MicroRNA-122

Micro-RNAs (miRNAs) are small noncoding RNAs that are involved in controlling RNA translation. They bind to complementary target sequences in the 3'-UTR of mRNAs and cause translational arrest or degradation of the mRNA, depending on the degree of homology between the miRNA and the target sequence. In this respect, miRNAs are negative regulators of host-cell protein synthesis. Surprisingly, the liver-specific miRNA-122 (miR-122) was found to be a crucial host dependency factor for HCV by promoting viral RNA

translation and/or replication.⁹⁶ Two binding sites for miR-122 reside within the 5'-NTR of the HCV RNA genome.^{96–98} Single nucleotide substitutions affecting the complementarity between miR-122 and the “seed sequence” in the HCV-RNA abrogate viral replication, but these mutants can be rescued by expression of an artificial miR-122 containing the compensatory mutation. A third miR122 binding site is located in the 3'-NTR of HCV, but seems to be dispensable for RNA translation and replication.

The exact mechanism by which miR-122 contributes to HCV replication is under investigation. Recent studies argue for a stabilizing effect of miR-122 on HCV RNA and protection from degradation by the exonuclease Xrn1,^{99,100} stimulation of HCV-RNA translation,¹⁰¹ or enhanced RNA replication.⁹⁶ Whether only one of these mechanisms is responsible for promoting the HCV life cycle or whether they act in concert is not completely clear. In any case, the dependency of HCV replication on miR-122 provides an explanation for an initially puzzling observation: The knockdown of factors of the miRNA-pathway, such as Dicer or Drosha—two components of the RNA-induced silencing complex, efficiently impairs HCV replication.¹⁰² These manipulations likely lower miR-122 amounts, thus interfering with viral replication.

Given the important role of miR-122 for HCV replication, therapeutic approaches based on sequestration and degradation of miR-122 have been pursued. Proof-of-concept studies confirmed that depletion of miR-122 by chemically modified antisense oligonucleotides (antagomirs) potently blocks HCV replication in cell culture and in experimentally infected chimpanzees.¹⁰³ Based on these results, clinical trials with the miR-122 targeting candidate Miravirsin have been conducted and were found to reduce viral load or even lead to SVR after short-term treatment without evidence of antiviral resistance.¹⁰⁴

Despite of these encouraging results, more recently concerns have emerged because it was found that miR-122 knockout mice display major lipid disorders like steatohepatitis and can develop fibrosis and even tumors, arguing that miR-122 is a tumor suppressor.^{105,106} Indeed, preclinical and clinical evaluation of miR-122-specific antagomirs revealed alterations of lipid metabolism by these antagomirs resulting in reduced serum cholesterol levels, but these aberrations resolved completely after cessation of therapy and no long-term adverse effects have been reported.^{103,104}

The Host-Cell-Chaperone Cyclophilin A

Cyclophilins comprise one of three protein families having peptidyl-prolyl cis-trans isomerase activity; they alter the conformation of proteins by interconverting the cis and trans isomers of peptide bonds with the amino acid proline. Cyclophilin A (CypA) is expressed in almost all tissues and is inhibited by cyclosporin A (CsA) that binds to and sequesters CypA (reviewed in¹⁰⁷). This complex binds to the phosphatase calcineurin and blocks its enzymatic activity. Because calcineurin regulates activity of the T cell transcription factor NF-AT (nuclear factor activating T cell), T-cell activation is suppressed, thus inducing an immunosuppressive state in which allograft tolerance is significantly improved. Hence, CsA is used in transplantation medicine.

By using self-replicating HCV RNAs (replicons) in human hepatoma cells, Watashi and coworkers made the surprising observation that CsA efficiently suppressed viral replication.¹⁰⁸ Subsequent studies identified cyclophilins as targets for the replication-suppressive effect, most notably CypA.^{109–111} It was found that enzymatic activity of CypA is essential for promotion of HCV replication because active-site mutants of CypA were unable to rescue viral replication in CypA knock-down cell lines.^{111–113} However, caution should be taken because active-site mutants of CypA no longer bind the substrate; therefore, it is unclear whether the lack of rescue is indeed due to requirement for isomerase activity or simply to loss of substrate binding. Nevertheless, biochemical studies have demonstrated that CypA interacts with NS5A. The interaction site resides in D2 of NS5A and comprises a cluster of proline residues.^{63,64} The mechanism by which CypA contributes to HCV RNA replication is not clear, but it is thought that the chaperone induces conformational changes in NS5A, thus enhancing replicase activity. This might be due to enhanced RNA binding of NS5A,¹¹⁴ or stimulation of NS5B RdRp activity via interaction with (properly folded) NS5A or efficient recruitment of NS5B to membranous replication complexes.^{113,115} Apart from enhancing replicase activity, evidence has been presented that NS2 also might be affected by CypA because replication of HCV RNAs containing NS2 were much more sensitive to CypA inhibitors as compared with viral RNAs lacking NS2.^{111,116} However, subsequent studies showed that sensitivity to CypA inhibitors correlates inversely with replication fitness, which is impaired by NS2.¹¹⁷ We hypothesize that impairment of replication fitness by NS2 might be due to a rate-limiting cleavage at the NS2–3 site. Importantly, increased sensitivity of HCV replication to CypA inhibitors was only found with JFH-1-, but not with genotype 1-derived replicons.¹¹⁷ Thus, NS5A most likely is the only HCV target of CypA.

Based on the initial observation that CsA efficiently inhibits HCV in replicon systems¹⁰⁸ and in vivo in liver transplant recipients,¹¹⁸ CsA derivatives have been developed lacking immunosuppressive activity, but retaining antiviral activity.¹¹⁹ These compounds retain interaction with CypA, but have greatly reduced binding to calcineurin. Best clinical evaluation is available for alisporivir (formerly Debio025). In combination therapy with SOC, alisporivir enhanced SVR rates significantly as compared with SOC, and even in monotherapy promising antiviral potency has been observed.¹¹⁹ Interestingly, CypA is also utilized by other viruses such as coronaviruses,¹²⁰ raising the possibility that nonimmunosuppressive CypA antagonists might have the potential to be a broad-spectrum antiviral.

As expected, inhibitors targeting CypA have a high barrier for drug resistance. Nevertheless, at least in cell culture, viruses can be selected that are able to replicate in cells in the presence of CypA antagonists. Two “classes” of such mutations have been found: (1) mutations residing close to the NS5A-B cleavage site that slow down polyprotein processing,¹¹¹ and (2) mutations within D2 of NS5A affecting residues D316 and Y317 (isolate JFH-1) that are located in a proline-rich region, which has been implicated in CypA

binding.^{121–124} Importantly, resistance mutants still bind CypA^{122,125} suggesting that these mutations render NS5A folding less dependent on CypA.

Alternative Targets for HCV-Specific Therapy

Inhibition of HCV Entry

As described above, HCV utilizes several molecules for cell entry (►Fig. 1B). Especially in the setting of liver transplantation where reinfection of the allograft occurs without exception, inhibition of viral entry is an attractive therapeutic approach. One possibility to achieve this goal is neutralizing antibodies directed against the viral E1/E2 envelope glycoprotein complex.¹⁶ In fact, highly potent neutralizing antibodies directed against E2 have been developed and were shown to block HCV infection in vitro.^{126,127} Moreover, cross-neutralizing antibodies conferring some level of protection to challenge with heterologous HCV strains in xenograft mouse models have been reported.¹²⁸ However, antiviral efficacy is limited, which might be due to the high variability of HCV envelope proteins and the tight association of virus particles with lipoproteins. Another reason appears to be the efficient cell-to-cell transmission of HCV, thus providing limited access of virus particles to neutralizing antibodies. Nevertheless, more potent antibodies have been developed that are currently under investigation.^{129,130}

An alternative approach that overcomes some of these limitations is to target host cell entry factors and pathways. Antibodies directed against CD81 and SCARB1 have been developed and evaluated both in cell culture and in vivo. Moreover, inhibitors of kinases and host cell pathways involved in HCV entry such as the EGFR¹⁷ or NPC1L1¹⁸ have been evaluated in vitro and promising results have been obtained in mouse models.

The Viroporin p7

The viral protein p7 is composed of two transmembrane segments that are connected by a cytoplasmic loop. P7 resides in the ER and is part of a multiprotein complex containing E1, E2, and NS2.²⁷ Cryo electron microscopy and more recently, nuclear magnetic resonance-based studies revealed that p7 forms hexameric complexes.^{131–133} Owing to this property and its ion channel activity, p7 is classified as a viroporin together with influenza virus M2 and HIV-1 vpu.¹³⁴ Although p7 is crucial for assembly and release of infectious virions in cell culture and is indispensable for formation of infectious particles in the chimpanzee animal model, its exact role in the HCV life cycle is not completely clear.^{135,136} However, a recent study provided evidence that p7 is required for assembly of nucleocapsids and their envelopment.²⁰

Based on the fact that M2 of influenza virus can be blocked by amantadine and rimantadine, antiviral efficacy of these compounds against HCV has been tested. However, results are conflicting. Although some studies reported antiviral efficacy of amantadine and rimantadine against HCV, others did not observe this effect, at least when using concentrations that did not exert cytotoxicity.¹³⁴ In agreement with the limited antiviral activity observed in vitro, clinical trials revealed

little, if any, benefit of amantadine given with SOC as compared with SOC alone.^{137–139}

NS4B

NS4B displays a complex structure with two amphipathic helices in the amino-terminal region, four predicted transmembrane segments and a putative palmitoylation site at the carboxy-terminus.¹⁹ Interestingly, NS4B most likely undergoes a complex posttranslational conformational change by which the amino-terminal helices translocate through the membrane, giving rise to a protein that has an amino-terminal helix in the ER lumen and a total of five transmembrane segments. This membrane slippage appears to be regulated by NS5A.¹⁴⁰ The complex membrane topology might allow NS4B to form multiple in-membrane interactions including self-interactions that are required for membrane remodeling and thus, RNA replication.^{141,142} For a long time it was thought that NS4B is the main driver of the MW. However, recent studies have shown that DMVs are induced by NS5A and a concerted action of all HCV replicase factors is required for web formation.²³ Thus, NS4B appears to be one of several viral components contributing to MW biogenesis. Besides its membrane remodeling capacity, NS4B can also bind to the 3'-NTR of negative strand HCV RNA.¹⁴³ Whether NS4B is directly involved in HCV RNA replication is not yet known, but it likely has important roles in assembly and release of infectious particles.^{142,144}

Inhibition of NS4B function with respect to RNA replication has been achieved by two classes of compounds. The first one is represented by clemizole hydrochloride that interferes with NS4B-RNA interaction and might impair NS4B function in RNA-replication and virus assembly/release. Resistance mutations map to NS4B, but interestingly also to the 5'-NTR.¹⁴³ The second class of NS4B inhibitors comprises small molecules preventing proper membrane association and thus, MW formation and/or induce NS4B aggregation.^{145–147}

Silibinin is a substance that is primarily given to patients with intoxications of amatoxins, but has also been used for treatment of chronic hepatitis C. Interestingly, in some cases virus elimination has been achieved even with silibinin.¹⁴⁸ Especially difficult to treat patients showed improved virological response upon Silibinin therapy in combination with SOC.^{149,150} Numerous mechanisms underlying the antiviral effect of silibinin have been described, including interference with HCV entry,^{151,152} destabilization of core and NS5A,¹⁵³ blockade of virus production, or interference with cell-to-cell spread¹⁵¹ (reviewed in¹⁵⁴). Moreover, it was reported that silibinin A, silibinin B and the related compound Legalon SIL inhibit RdRp activity of NS5B.¹⁵⁵ Interestingly, a recent study described mutations in NS4B that confer partial resistance to Legalon SIL in vitro.¹⁵⁶ Moreover, mutations residing in NS4B were also detected in a patient with viral breakthrough under silibinin therapy, thus supporting the in vitro data. To identify the molecular mechanism, biochemical studies were conducted; they suggest that silibinin might target an interaction between the NS3/4A complex and NS4B. Consistent with the important role of NS4B for MW formation, silibinin treatment induced aberrations of remodeled membranes, which was

not the case with a silibinin-resistant isolate.¹⁵⁶ These results suggest that NS4B is a target of silibinin and they are consistent with mathematical modeling studies showing potent interference of silibinin with virus production and only moderate effects on HCV entry.¹⁵⁷

Conclusions

Although we are not yet at the end of the road, therapy of chronic hepatitis C can now be considered a success story. Since the original description of the virus around 25 years ago, major progress has been made in our understanding of the viral life cycle, which ultimately defined the prime targets for antiviral therapy and laid the ground for development of highly potent DAAs. The high rate of virus elimination that can be achieved in clinical trials with the most recent generation of DAAs even in IFN-sparing regimens is unprecedented and on one hand the result of hard work to develop the most potent antiviral, but on the other hand also due to the biology of this virus. Key features facilitating virus clearance under therapy are the very short half-life of HCV, which has to be compensated by high reproduction rate and the absence of stable "latency" reservoirs. As much as we know, HCV can only persist when sustaining high-level replication and is unable to stably "archive" drug-resistant variants, which makes the virus particularly vulnerable to highly active DAAs. As we have learned from HIV, drug resistance can be overcome by combination therapy and in the case of HCV, an amazing arsenal of DAAs with different targets is in late stage clinical development or has just been approved. These include NIs targeting NS5B, second-generation protease and highly potent NS5A inhibitors as well as cyclophilin blockers. Many of these DAAs have pan genotypic activity and moderate to very high barrier to resistance. Moreover, drug administration is improved by more favorable pharmacokinetics and side effects are significantly reduced. These developments will revolutionize the therapy of chronic hepatitis C to the benefit of patients.

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