

New therapies on the horizon for hepatitis C

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Abstract

Therapy of chronic HCV infection has greatly improved in recent years with the addition of ribavirin to alpha interferon and has further improved more with the use of PEG-interferons. However, more than half of patients do not achieve lasting benefits from these therapies. The future therapeutic developments may include one or more of the following approaches: understanding the HCV genomic organization, elucidating the viral life cycle and HCV replication strategy and understanding the immune mechanisms required for viral propagation or infectivity. The development of novel antiviral strategies and a preventive vaccine against HCV infection remains a major challenge for the future, and will depend on progress on both molecular biology as well as clinical studies. Unfortunately, the low replication of the virus in culture, the lack of convenient animal models, and the high genome variability present mayor challenges for drug development.

Key words: Chronic HCV infection, HCV, novel antiviral strategies.

The hepatitis C virus (HCV) is one of the leading causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. It is estimated that more than 200 million people worldwide are infected with HCV,¹ and this large reservoir of infected persons constitutes a daunting source of potential new infections. Therefore, there is a compelling need to develop an effective treatment.

Therapy of chronic HCV infection has greatly improved in recent years with the addition of ribavirin to alpha interferon and has further improved more with the use of PEG-interferons. However, more than half of patients do not achieve lasting benefits from these therapies.

Since the successful cloning of HCV in 1988, our knowledge of the molecular biology of the virus has increased rapidly and led to the identification of several potential targets for antiviral intervention. These future therapeutic developments may include one or more of the following approaches:

- 1) Understanding the HCV genomic organization.
- 2) Elucidating the viral life cycle and HCV replication strategy.
- 3) Understanding the immune mechanisms required for viral propagation or infectivity.

The purpose of this review is to up-date our current knowledge of the molecular virology of HCV and to speculate on potential avenues to manage this information in designing new antiviral agents.

- 1) Understanding the HCV genomic organization:

- *Structure of viral genome*

We understand the genomic organization of the virus, as well as the nature and function of a number of HCV gene products—this knowledge is contributing greatly to the development of new therapeutic targets.

The virus is the sole member of the *Hepacivirus* genus, family *Flaviviridae*.

The HCV genome is a enveloped, positive, single stranded RNA of 9.6 kilo bases in length, with a 5' noncoding region (NCR) of about 340 bases, followed by a single long open reading frame of more than 900 bases and a 3'NCR.²

The 5' NCR contains an internal ribosome entry site (IRES) that initiates translation of a 3,000 amino acid polypeptide, which is subsequently processed by viral and host proteinases into 10 mature structural (core, E1, E2, and p7) and nonstructural HCV proteins (NS2, NS3, NS4A, NS5A, and NS5B). Signal peptidase makes the primary cleavage in the Core-NS2 region. The NS2-3 auto protease, a zinc stimulated enzyme, makes a single cleavage at the NS2/3 junction. The NS3-4A serine protease is responsible for processing the four downstream sites (NS3/4A, 4A/4B, 4B/5A, 5A/5B). The IRES at the conserved 5' region of the viral genome serves as a landing pad for host ribosomes

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and cellular proteins to initiate translation of the viral polyprotein.

2) Elucidating the viral life cycle and HCV replication strategy: Implications for future drug development

• *The viral life cycle*

The replication cycle of HCV is incompletely understood due to the low viral titers found in sera and livers of HCV infected patients and the lack of an efficient cell culture system or small animal model permissive for HCV infection.

However, considerable progress has been made using heterologous expression systems, functional cDNA clones and more recently, sub genomic replicons.

It is currently thought that the HCV virion enters into the host cell binding to specific receptors on the hepatocytes surface, and then is deposited into cytosol by endocytosis.

The presumed life cycle of HCV is as follows: 1- binding to a cellular surface receptor and internalization into the host cell, 2- cytoplasmic release and uncoating of the viral RNA genome, 3- IRES-mediated translation, 4- polyprotein processing by cellular and viral proteases, 5- viral replication, 6- packaging and assembly, 7- virion maturation and 8- release from the host cell.

Following decapsidation, the genomic RNA is directly translated in the cytoplasm. Since the genome is not capped, translation is mediated by IRES and not by a cap-dependent mechanism. The HCV IRES resides between nucleotides 40 and 335 and forms four highly structured domains, allowing the direct binding of the 40S ribosome. Ribosome binding is thought to occur at or immediately upstream of the translation initiation codon. The very 3' end of the HCV genome interacts with the HCV IRES and enhances translation. Directed by the IRES, the polyprotein is translated at the rough endoplasmic reticulum and cleaved co- and post-translationally by host and two viral proteinases. The individual HCV proteins form a stable replicase complex associated with intracellular membranes. This allows the tight coupling of different viral functions as well as the production of viral proteins and RNA in a distinct compartment.

• *HCV RNA replication strategy*

Following attachment, penetration and uncoating the viral RNA is translated to produce a pool of replicase proteins required for genomic replication.

The positive strand is copied into a minus strand intermediate that serves for synthesis of the genomic strand. However, the individual steps underlying

RNA replication are largely unknown, even though the recent establishment of a tissue culture system for sub-genomic replicons will facilitate their study. In vitro, the RNA dependent RNA polymerase (RdRp) initiates replication by elongation of a primer hybridized to an RNA homopolymer or via a "copy-back" mechanism. In this latter case, sequences at the 3' end fold back intramolecularly and hybridize, generating a 3' end that can be used for elongation, resulting in a product about twice the length of the input template. However, in the presence of high GTP or ATP concentration, RdRp can initiate RNA synthesis de novo, and this probably occurs in vivo. Concerning the template specificity, NS5B seems to bind preferentially to a sequence in its own 3' coding region. Alternatively, the template specificity may be accomplished by the high local concentration of both NS5B and the viral RNA within the replicase complex. Efficient RNA replication very likely depends on additional viral and cellular factors, as the NS3 helicase, which unwinds stable RNA structures, or the phosphoprotein NS5A.³

Implications for future drug development

As is evident from the preceding data, each of the above mentioned steps of viral life cycle represent a future target for antiviral intervention.

• *Attachment and entry*

Although the mechanism of HCV entry into cells is unknown, the E2 glycoprotein is thought to play a major role in virus attachment to the target cell.

CD81, a member of the tetraspanin superfamily of cell surface molecules and expressed on virtually all cells, is the putative receptor for HCV entry into the host cell, since this molecule strongly interacts with E2 as well as virus particles in vitro. The virus may also be able to enter the cell by binding to low-density lipoprotein (LDL) receptors. But whether interaction with the LDL receptor or CD81 leads to internalization and a productive infection remains to be determined.

Based on comparison with fusion peptides of paramyxoviruses, E1 may be involved in membrane fusion. The low pH within the endosomal compartment may induce a major structural rearrangement of the E1, resulting in exposure of a fusion peptide which destabilizes membranes, leading to membrane fusion and particles entry into the cytoplasm.⁴

Recently, a new potential receptor has been proposed by two different groups of researches. Lozach and col., found that the HCV envelope glycoprotein E2 binds the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and the relat-

ed liver endothelial cell lectin L-SIGN through high-mannose N-glycans.⁵ Consequently, the authors hypothesized that high affinity interaction of viral glycoproteins with oligomeric lectins might represent a strategy by which HCV targets to and concentrates in the liver and infects dendritic cells.

Gardner and col., by using a virus-binding assay, demonstrate that L-SIGN and DC-SIGN specifically bind naturally occurring HCV present in the sera of infected individuals⁶ and speculate that envelope glycoprotein E2 might be blocked by specific inhibitors, including mannan, calcium chelators, and antibodies against the lectin domain of the SIGN molecules.

- *Inhibition of cell infection*

Interventions at this step may imply the use of potential neutralizing antibodies. Antibody binding could potentially interfere with viral entry into host cells and replication. Antibody binding could also opsonize virions for elimination by macrophages and transport to secondary lymphatic organs.

Preliminary observations suggest that boosting envelope glycoprotein (gp) antibodies by active or passive immunization may be beneficial. Active immunization may involve both HCV recombinant protein and DNA vaccines, as are being reviewed later.

- *Inhibition of viral replication and viral protein processing*

Several steps in the replication pathway of HCV have been identified as potential targets for selective intervention with antiviral agents.

In principle, every step in the viral cycle can be target for the development of an antiviral therapy as long as one condition is fulfilled: it must be indispensable for the production of infectious virus progeny.

Inhibition of viral enzymes critical to HCV replication could be used for selective antiviral intervention. This approach encompasses the use of small molecules inhibitors of HCV encoded enzymes such as: *protease, helicase and RNA polymerase as well as the use of gene silencing strategy.*

HCV Protease

Two virally encoded enzymes, NS2/3 and NS3 protease mediate the processing of HCV non-structural proteins. The first enzyme is a zinc-dependent, metalloprotease that cleaves the nonstructural proteins between the NS2 and NS3 polypeptides.

The NS3 protease encodes a multifunctional protein that contains a serine protease in the N-terminus, and catalyzes four or five processing events that take place dur-

ing the maturation of the nonstructural portion of the non-structural portion of the HCV polyprotein.

The both x-ray crystallography and NMR spectroscopy have been used to determine the three dimensional structure of the isolated NS3 proteinase domain, showing that the enzyme folds in a canonical chymotrypsin-like fold.^{7,8}

The NS3 protease is only marginally active on its own and requires interaction with a cofactor, the viral protein NS4A, to display optimal catalytic activity.

Another distinguishing structural feature of the NS3 proteinase is the presence of a zinc-binding site that was shown to have a structural role rather a catalytic role.

With the availability of *in vitro* test systems for drug design, at least three strategies for inhibitor development of HCV protease can be envisaged: inhibition of proteinase/cofactor interaction, interference with zinc binding in the NS3 domain and direct inhibition of catalysis.

All these features are being exploited to generate potent peptide inhibitors of the enzyme that will serve as scaffolds for the development of drug-like molecules.

HCV Helicase

The hepatitis C encodes an essential RNA helicase within non-structural protein 3 (NS3). The helicase functions to unwind the plus and minus strands of the RNA genome after polymerase replication, and therefore is probably part of the multienzyme replication complex as observed with other flavivirus.

This essential viral enzyme is involved in the modulation of RNA structure during viral replication, providing an excellent target for antiviral drug discovery. In addition, the helicase also catalyzes hydrolysis of nucleoside triphosphates, which provide the energy for unwinding of the duplex.

The three dimensional structure of the helicase has been reported and it indicates the presence of three interconnected functional domains, two $\beta\alpha\beta$ -sub domains and a third containing mostly α helices.⁹

Understanding the roles of helicases sub domains in RNA unwinding and the functional significance of linking activities within the NS3 polypeptide may provide a new data in favor of the development of a newer classes of helicase inhibitors.

NS5B RNA-dependent RNA polymerase

The NS5B region of the HCV genome encodes an RNA-dependent RNA polymerase. Crystallography studies of NS5B have shown a globular structure consisting on 3 closely interacting sub domains, termed fingers, palm and thumbs. NS5B is highly conserved across HCV genotypes and is virus specific.

Recently, a polymerase inhibitor has been tested in a phase I multicenter, placebo controlled study showing promising results.¹⁰

Gene silencing: Small interfering RNA's

A novel molecular strategy that holds promise is the use of small interfering RNA's (siRNA). RNA interference is a cellular process of gene silencing in which small duplexes of RNA specifically target a homologous sequence for cleavage by cellular ribonucleases.

Randal and col., have recently demonstrated that the introduction of siRNAs into cells with established HCV replication cured up to 98% of these cells of detectable HCV antigen and replication-competent HCV RNAs.¹¹

Koyota and coworkers designed the siRNAs to target the 5' untranslated region (5' UTR) of the HCV genome. The authors have identified an effective site in the 5' UTR at which approximately 80% suppression of HCV replication was achieved with concentrations of siRNA as low as 2.5 nM.¹²

These results support the feasibility of using siRNA-based gene therapy to inhibit HCV replication, which may prove to be valuable in the treatment of hepatitis C.

- *Translational control of HCV*

Conserved RNA elements (for example IRES and 3' untranslated region), which probably function via interaction with host and viral components, should also be considered in designing strategies to inhibit HCV replication. In the coming years our understanding of the mechanisms by which HCV proteins and RNA elements function will grow; with expanded knowledge additional targets will undoubtedly emerge.

Protein synthesis is initiated at the IRES within the 5' untranslated region. The RNA within this region has a high degree of secondary structure, which forms a pseudoknot containing a number of potential initiation codons. Antisense oligonucleotides and hammerhead ribozymes directed towards the IRES have shown to be useful in inhibiting protein synthesis *in vitro*.^{13,14}

Antisense oligonucleotides

Antisense oligonucleotides are synthetic fragments of ribo or deoxyribonucleic acids, which specifically binds to their complementary messenger RNA and then, block the corresponding protein translation.

Many problems are encountered when oligonucleotides are used in cellular systems and *in vivo*. In fact, oligonucleotides are rapidly degraded in biological fluids and in cells by exo- or endonucleases, which hydrolyze the phosphodiester linkage.

Genetic and biochemical studies have provided convincing evidence that the 5' noncoding region of hepatitis C virus is highly conserved among viral isolates worldwide and that translation of HCV is directed by an internal ribosome entry site (IRES) located within the 5' NCR. Hanecak

R and col. have investigated inhibition of HCV gene expression using antisense oligonucleotides complementary to the 5' NCR, translation initiation codon, and core protein coding sequences. Oligonucleotides were evaluated for activity after treatment of a human hepatocyte cell line expressing the HCV 5' NCR, core protein coding sequences, and the majority of the envelope gene E1.¹⁵

More than 50 oligonucleotide were evaluated, two of them, ISIS 6095, targeted to a stem-loop structure within the 5' NCR known to be important for IRES function, and ISIS 6547, targeted to sequences spanning the AUG used for initiation of HCV polyprotein translation, were found to be the most effective at inhibiting HCV gene expression.

Reduction of RNA levels, and subsequently protein levels, by these phosphorothioate oligonucleotides was consistent with RNase H cleavage of RNA at the site of oligonucleotide hybridization. Results of these studies show that HCV gene expression is reduced by antisense oligonucleotides and demonstrate that it is feasible to design antisense oligonucleotide inhibitors of translation that do not require RNase H activation.¹⁵

Other molecules recently used for the translational control of HCV are morpholino antisense oligonucleotides. These compounds have shown to be potent inhibitors of HCV IRES translation in a pre clinical mouse model,¹⁶ and are potential candidates for treating HCV and other viral infections.

Ribozymes

Ribozymes are synthetically catalytic RNA molecules engineered to act as "molecular scissors" capable of cleaving target RNA in a highly specific manner. The ribozymes are designed to cleave a highly conserved region of the HCV gene, one that the virus needs to survive its viral life cycle.

A hammerhead ribozyme consists of a conserved catalytic site that is flanked by engineered antisense sequences that mediate site-specific binding to the target RNA. In addition to the sequence specificity of ribozymes, they have the advantage of acting through "touch-and-go" mechanisms that allows a single ribozyme to cleave many target RNAs in succession.¹⁷

Ribozymes against hepatitis C are associated with significant reduction in HCV RNA production *in vitro*, and have a synergistic effect with interferon with a dose response effect of the ribozyme. This compound is in phase I, and preliminary results have shown that the drug has been well tolerated with no abnormalities in laboratory parameters and no major patient toxicity.

To investigate the potential use of synthetic stabilized ribozymes for the treatment of chronic hepatitis C virus (HCV) infection, Macejak and coworker designed and synthesized hammerhead ribozymes targeting 15 conserved sites in the 5' untranslated region of HCV RNA. The 15 synthetic ribozymes contained modified nucle-

otides and linkages that stabilize the molecules against nuclease degradation.¹⁸

3) Understanding the immune mechanisms required for viral propagation or infectivity.

The hepatitis C virus has remarkable ability to establish clinically persistent infections in individuals who are immunocompetent. As in other viral infections, the outcome of the disease is likely to depend on the kinetics of viral replication and infection of host cells on the one hand, and on quantitative and qualitative characteristics of the host's immune response on the other hand.

Several observations should be considered in order to understand these issues. First of all, it is presumed that the humoral immune response to HCV is targeted against epitopes within all viral proteins. However, the hypervariable region 1 (HVR1) of the HCV E2 protein has been identified as target for neutralizing antibodies. Second, early development of anti-E2 or anti-HVR 1 has been suggested to be associated with recovery from acute HCV infections in humans. Nevertheless, antibody responses to the envelope proteins develop slowly and achieve only modest titers during primary infection; as a result, neutralizing antibodies may emerge too late to prevent chronic infection. Finally, anti-envelope antibodies tend to be short-lived and disappear gradually after viral clearance.

On the whole, interventions in this approach may include: -HCV monoclonal antibodies, -hyperimmune HCV immunoglobulin, -therapeutic vaccination, -passive immunization and inosine monophosphate dehydrogenase inhibitors (IMPDH).

- *HCV monoclonal antibodies*

Antibodies have the potential to be immunotherapeutic agents, used either as stand-alone therapy or as an adjunct for managing chronic viral infection. In addition, antibodies may be used prophylactically in individuals who have been accidentally exposed to hepatitis B virus (HBV) or hepatitis C virus (HCV), or to prevent reinfection of the liver in patients who have undergone liver transplantation.¹⁹

Eren and colleagues from Israel presented the results of a phase IA clinical study to test the safety, tolerability and efficacy of a single infusion of human monoclonal antibody HCV-AB68 in 15 patients with chronic HCV infection.

Human monoclonal antibodies (HMAbs) to the HCV envelope protein (E2) were derived from peripheral B cells isolated from individuals with HCV genotype 1b, then tested *in vitro* for their binding, affinity and ability to immunoprecipitate viral particles from sera of patients infected with HCV of various genotypes. The

HMAbs with high affinities to E2 and broad immunoprecipitation ability were then selected and evaluated in a HCV-Trimera mouse model for *in vivo* screening of anti-HCV agents.

HMAbs inhibited the infection of human liver fragments by HCV as measured by reductions in mean viral load and in the percentage of HCV positive mice after exposure. The potential therapeutic role of HMAbs was shown by their ability to reduce mean viral load when administered to HCV-Trimera mice with already established hepatitis C viremia. One of the HMAbs (HCV-AB68) was then chosen for study in 15 patients with chronic HCV infection. HCV-AB68 was found to be safe and well tolerated. Significant reductions in HCV viral RNA levels, ranging from 2 to 100 fold, occurred in 8 out of 15 patients with HCV-AB68.²⁰

- *Hyperimmune HCV immunoglobulin*

Because passive immunization has been successfully used for the prevention of hepatitis A and hepatitis B infection, the efficacy of anti-HCV immunoglobulin to protect from HCV has been evaluated in several studies. Furthermore, early studies of post-transfusion non A-non B hepatitis showed that intravenous infusions of globulins, which might contain anti-HCV, reduced the incidence and severity of the disease. The existence of neutralizing anti-HCV antibodies was documented in experimental studies, thus it was hypothesized that the use of globulin preparations with high titered anti-HCV containing neutralizing antibodies could modify virus replication and the clinical course of the infection.²¹ These studies suggest that antibodies alone can prevent acute HCV infection and are even beneficial when administered in the chronic phase of HCV infection.

- *Therapeutic vaccination*

Houghton and Col.²² have shown in chimpanzee that vaccination with recombinant subunits of HCV gpE1 and gpE2 prevents the development of chronic infection following experimental challenge with homologous or heterologous HCV.

Other approaches include the use of vaccines that use recombinant HCV antigens combined with appropriate adjuvant, such as HCV core protein.²²

Therefore, complete protection of chimpanzees from experimental challenge of following vaccination gpE1/gpE2 encourages further investigation this vaccine strategy.

DNA vaccines, an emerging preventive tool, expose subjects to part of the viral genome, instead of extracts of whole dead viruses, as is the case with traditional vaccines. Nucleic acid immunization is the most recent approach in vaccine development, and the efficacy of

DNA vaccine to protect against challenge with pathogens has been demonstrated in animal models of influenza virus, malaria, mycobacterium, HIV and Ebola. A DNA-based vaccine usually consists of purified plasmid DNA carrying sequences encoding for an antigen of interest under the control of eukariotic promoter. After injection of the plasmid into the muscle or skin, the host cells take up the plasmid and express the antigen intracellularly. The expression of the encoded antigens by the host cells is one of the major advantages of this approach because mimics natural infection.

Another approach looked at the effectiveness of using a non-replicating canary poxvirus encoding the HCV gene to deliver the DNA vaccine to mice. This approach appears to diversify T-cell responses and enhance immune response to HCV proteins.

The DNA vaccines that are presently being constructed and tested usually consist of single or multiple genes of immunogenic proteins inserted into a commercially available DNA expression plasmid. When the DNA is taken up into cells, preferably antigen-presenting cells, the gene is transcribed and the mRNA translated in the cytoplasm of the cell.²²

• IMPDH: New Analogues of Ribavirin

Two new derivatives of ribavirin, Levovirin and Viramidine, are currently in development as HCV therapeutics. Both drugs retain ribavirin immunomodulatory properties but appear to be less toxic than the parent drug. Clinical evaluation of these drugs may aid in understanding the relevant mechanism of action of ribavirin itself, as well as the role of immunomodulators in HCV therapy.

Levovirin is the L enantiomer of ribavirin, which has a similar immunomodulatory activity as ribavirin, but without the associated toxicity. This drug has entered a phase I study at doses of between 200 and 1,200 mg and is well tolerated and orally absorbed.

The other compound, viramidine, is a prodrug of ribavirin, which is converted to the active drug by adenosine deaminase within the liver. Viramidine itself has no anti-HCV inhibitor effect, but when converted to ribavirin shows similar antiviral activity against DNA and RNA viruses. Within a monkey model a dose of 600 mg per kg had no significant hematological toxicity in males although it was associated with 10% reduction in red blood cells in the female.²⁴

In conclusion, the development of novel antiviral strategies and a preventive vaccine against HCV infection remains a major challenge for the future, and will depend on progress on both molecular biology as well as clinical studies.

Unfortunately, the low replication of the virus in culture, the lack of convenient animal models, and the

high genome variability present major challenges for drug development.

While new therapeutic agents for chronic HCV infection are on the horizon, currently available treatment regimens, including Peg-interferons in association with ribavirin, appear capable of eradicating HCV in up to 50% of treated patients. Novel delivery systems under development to improve the pharmacokinetic profile of interferons as well as alternative parenteral type I interferons are also at present being evaluated, and will supplement current therapies in the near future until newer antiviral agents demonstrate to be safe and be available for routine clinical use.

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