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New targets for treatment against HCV infection

Qiuwei Pan, PhD, Assistant Professor a,*, Luc J.W. van der Laan, PhD, Associate Professor b

aDepartment of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Room L-462, ’s Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands
bDepartment of Surgery and Laboratory of Experimental Transplantation and Intestinal Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

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Abstract

Owing to the tremendous effort from both academia and industry, drug development for hepatitis C virus (HCV) infection has been flourishing, with a range of pipeline compounds at various stages of development. Although combination of the recently launched serine protease inhibitors will further improve the response rate of current interferon-based therapy, some intrinsic limitations of these compounds and the tendency of resistance development by the virus, urge the development of alternative or additional therapeutic strategies. In this article we provide an overview of different host and viral factors which have emerged as new potential targets for therapeutic intervention using state-of-the-art technologies.

Introduction

Despite the identification of the causative agent, hepatitis C remains a tremendous global health problem. Since the discovery of the hepatitis C virus (HCV) in 1989 [1], research has been initially hampered by the lack of good culture and animal model systems. However, owing to the development of various cell culture models in the past decade [2], HCV research has been flourishing both in academia and industry. This progress has enabled both the further improvement of the current interferon-based standard antiviral therapy and the development of novel antiviral compounds. With increasing knowledge of the structure and function of the viral proteins, it came to the development of a range of directly acting antivirals (DAAs), culminating in the launch of telaprevir and boceprevir in...
In addition to viral protein-targeted compounds, the improved understanding of virus–host interactions and the tremendous evolutions of biotechnology have provided the basis for further exploration of new therapeutic avenues. In this article, we provide a summary of recent literature on new therapeutic strategies targeting the HCV RNA genome or host factors involved in viral entry and replication. The potential strength and weaknesses of each approach will be discussed.

**Targeting viral entry**

**Biology of HCV entry**

Viral entry is the important first step of establishing infection in the host cell. Though HCV can be internalized by several cell types, including cells of the immune system, its primary tropism is the liver, in particular the hepatocytes. HCV infection of hepatocytes involves the interaction of envelope proteins of the virus with specific membrane proteins expressed on the surface of the host cell. Virions contain two envelope glycoproteins: E1 (up to six glycosylation sites) with largely unknown function and E2 (11 glycosylation sites) that is important for entry receptor binding [4]. In patient serum, HCV is often associated with lipoproteins, which are thought to play an important chaperone function for viral entry. The association with lipoproteins results in heterogeneity and complexity of the viral structure, which in turn influences viral entry [4].

The development of HCV pseudoparticles (HCVpp) models has greatly facilitated the study of viral entry. This led to the discovery of multiple hepatocyte surface receptors involved in the distinct steps of viral entry. First interaction between virus and hepatocytes involves both glucosaminoglycans (GAGs) [5] and low density lipoprotein receptors (LDLRs) [6]. Following this initial binding, the virion will engage with scavenger receptor-B type I (SR-BI) [7] and the tetraspanin, CD81 [8]. Following this event, the virus particle can interact with tight-junction proteins, claudin-1 [9] and occludin [10], which are essential for initiating viral entry into the cell. Recently, two receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2), were found to be also involved in HCV entry. These receptors are thought to regulate CD81-claudin-1 associations and viral glycoprotein-dependent membrane fusion [11]. In addition, the Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) was identified as a new host factor involved in entry, though the exact mechanism of action remains largely elusive [12].

Viral entry represents a potential multifaceted target for both prevention and treatment of HCV infection. Blocking of entry could potentially limit de novo infection of hepatocytes and is particularly relevant for prevention of HCV re-infection after liver transplantation. Liver transplantation is currently the best treatment for hepatitis C patients with end-stage liver disease. However, long-term success of transplantation is still compromised by HCV re-infection of the graft and recurrence of the disease. Recurrence often results in accelerated liver fibrosis and early development of cirrhosis [13]. In general, peg-interferon/ribavirin is much less effective in transplant patients with sustained virologic response (SVR) rates of only approx. 20% as well as poor tolerability [14]. Therefore, blocking viral entry into the new liver graft is an attractive approach for prevention of infection in these patients. Interestingly, a recent study has shown a correlation between the viral load decay during the first 24 hours after graft reperfusion and the SR-BI expression level in the donor liver. In addition, HCV recurrence was associated with increased levels of claudin-1 and occludin in the hepatocyte cell membrane, although their localization was not changed within the tight junctions [15]. Thus, experimental research have been actively exploring this aspect [16], although there is still no FDA-approved entry inhibitors available.

**Antibody-mediated inhibition of viral entry**

The HCV envelope glycoproteins E1 and E2, involved in viral entry, are primary targets of neutralizing antibodies. A number of monoclonal antibodies against conserved regions of E2 have been demonstrated effective neutralizing capabilities against HCV infection in experimental models [16,17]. However in the clinical setting, neutralizing antibody treatment did not prevent HCV recurrence and had only limited effects on HCV viral load in patients after liver transplantation [18,19]. Mechanisms, such as heterogeneity of the virus, viral protein associated with lipoproteins or glycans, interfering antibodies or cell-to-cell transmission, have been proposed to explain the unsuccessful outcome of
antibody treatment in patients [16,17]. Despite the hampering of further clinical development, experimental research is still continued [20]. Recent studies reported the identification of human broadly neutralizing antibodies to the E1-E2 complex [21,22]. In particular, the monoclonal antibody AR4A, recognizing a discontinuous epitope outside the CD81-binding site on the E1-E2 complex, has an exceptionally broad neutralizing activity towards diverse HCV genotypes and protects against heterologous HCV challenge in a small animal model [21].

The host factor SR-BI is an integral membrane protein found in numerous cell types/tissues, including the liver and adrenal gland. It is best known for its role in facilitating the uptake of cholesteryl esters from high-density lipoproteins in the liver, although the mechanism of SR-BI-mediated HCV entry is still not clearly understood. Rat polyclonal antibodies against the extracellular loop of SR-BI were shown to specifically inhibit infection by cell-culture-derived HCV (HCVcc) in vitro [23]. Two mouse monoclonal antibodies against SR-BI, 3D5 and C167, were also reported to inhibit the interaction of HCV E2 protein with SR-BI and efficiently blocked HCVcc infection in vitro. In addition, a human SR-BI monoclonal antibody (mAb16-71) was described to efficiently prevent HCVcc infection in vitro [24]. Using the uPA-SCID human liver-chimeric mouse model, it was further demonstrated that mAb16-71 prevents infection and viral spread of multiple HCV genotypes [24]. Of note, mAb16-71 also interfered with direct cell-to-cell transmission shown in a cell co-culture system [24].

Other host cell targets involved in entry are CD81 and Claudin-1. CD81 is a 25-kilodalton transmembrane protein belonging to the tetraspanin family. CD81 is one of the earliest and best characterized HCV entry receptor [25]. The extracellular loop of CD81 can bind to HCV E2 protein with high affinity [26]. Knockdown of CD81 expression in cell lines profoundly reduce HCV infection [27,28]. CD81 is involved in multiple steps of viral entry, including actin-dependent lateral diffusion within the cell membrane, interactions with claudin-1 and subsequent internalization [25]. Prevention of HCV infection by anti-CD81 antibodies has been demonstrated in vitro as well as in the uPA-SCID human liver-chimeric mouse model [29]. In addition, one in vitro study has demonstrated the feasibility of using anti-claudin-1 antibodies for prevention of HCV entry [30].

Small-molecule inhibitors of viral entry

The requirement for sequential interactions between the viral envelope and key host receptors provides potential new drug targets by small-molecule inhibitors. ITX 5061, an arylketoamide, is an SR-BI inhibitor that has been shown to inhibit viral entry of all major genotypes of HCV in cell culture [31]. Kinetic studies suggest that the compounds act at an early post-binding step [31]. Although ITX 5061 was initially developed as a p38 MAP kinase inhibitor for inflammatory disease, it was latterly discovered to unexpectedly elevate high density lipoprotein levels in experimental animals and humans mediated via SR-BI [32]. This compound exhibits a good safety profile in previous clinical studies and therefore has been initiated for evaluation in chronic HCV patients and patients undergoing liver transplantation (www.itxpharma.com), although the progress is still ongoing [33]. Pharmacological inhibitors erlotinib (an EGFR inhibitor) and dasatinib (an EphA2 inhibitor) impaired HCV entry and infection possibly by interfering with the CD81-claudin-1 co-receptor association [11]. The clinically available FDA-approved NPC1L1 antagonist ezetimibe was shown to block HCV uptake via a virion cholesterol–dependent step before virion-cell membrane fusion [12].

The application of high throughput screening approaches has further stimulated the discovery of new inhibitors. The screen of a proprietary small-molecule compound library using HCVpp model, a series of 1, 3, 5-triazine compounds were identified as potent, selective and non-cytotoxic inhibitors of HCV entry [34]. A screening of a plant-derived small molecules library has discovered a flavonoid, ladanein (BJ486K) that has oral bioavailability and interferes with entry of HCV into cultured human hepatocytes [35].

Targeting host cellular factors

Intracellular lifecycle of HCV

Following HCV entry, the positive-stranded genomic RNA is released into the cytoplasm and there acts both as a template for the production of the negative-stranded RNA replication intermediate and for the
synthesis of the viral polyprotein. Translation of the viral polyprotein is mediated by an internal ribosome entry site (IRES) located within the highly conserved 5′ non-coding region (NCR). The synthesized polyprotein is subsequently cleaved into four structural (core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [36]. Processing of structural proteins, including the p7/NS2 junction, is carried out by at least two host signal peptidases and non-structural proteins are matured by two viral proteases, NS2 and NS3/4A [36,37]. De novo generated viral proteins and RNA genomes will be assembled and released to infect other cells. The 5′ NCR, in particular the IRES sequence, plays an important function in ribosomal assembly. The function of the non-structural proteins is essential for replication [38]. NS5B is the active subunit of the replication complex known as an RNA-dependent RNA polymerase for the de novo generation of positive and negative stranded RNA [39].

Recently, new light has been shed on the interplay between cellular membranes, lipid metabolism and HCV infection [40]. The primary location for the assembly of HCV virions in hepatocytes are shown to be the small lipid droplets located in close proximity of the endoplasmatic reticulum [41]. The HCV core protein initiates the recruitment of nonstructural proteins and replication complexes to lipid droplet-associated membranes. Autophagy (‘self eating’), a process for catabolizing cytoplasmic components through the lysosomal machinery, has been implicated in the modulation of interactions between RNA viruses and their host. HCV infection has been shown to induce autophagy [42] and facilitate the viral infection by damping the host innate immunity [43], such as interferon response [44].

The early knowledge of host factors involved in viral replication actually came from studies with immunosuppressive drugs, particular cyclosporine A (CsA) [45,46]. The anti-HCV activity of CsA revealed the function of cyclophilins, in particular cyclophilin A, as cellular cofactors for NS5B mediated replication [47,48]. Other studies on virus host interaction showed that HCV NS5B and NS5A proteins bind to two different domains of a vesicle membrane protein hVAP-33 [49]. Knockdown of hVAP-33 was shown to inhibit HCV RNA replication [50]. Other host cellular proteins were also identified as binding partners of HCV viral proteins, including RNA Helicase p68 [51], Ras-GTPase–activating protein binding protein 1 [52] and polypyrimidine-tract-binding protein [53]. Recent development of high-throughput technologies, in particular RNA interference (RNAi) libraries, allowed genome-wide screening of HCV host factors. Using the HCV infectious cell culture model, Randall and coworkers re-evaluated the significance of 62 host genes, which physically interact with HCV RNA or proteins or belong to cellular pathways thought to modulate HCV infection [54]. Dicer was found to be one the factors required for viral replication, however, this was not consistent with an earlier study [55]. By using an RNAi library that targets approximately 4000 human genes in an HCV subgenomic replicon cell culture model, screening revealed nine cellular genes that potentially regulate viral replication. Silencing of these genes resulted in inhibition of viral replication by more than 60%. These genes included those that encoded a G-protein-coupled receptor (TBXA2R), a membrane protein (LTbeta), an adaptor protein (TRAF2), two transcription factors (RelA and NFKappaB2), two closely related transporter proteins (SLC12A4 and SLC12A5), and two protein kinases (MKK7 and SNARK) [56]. Another study identified three other kinases (Csk, Jak1, and Vrk1) [57]. Inconsistency in findings reported by different RNAi library studies could be explained by technical limitations and differences in HCV models used. Therefore most of these host factors still need further evaluation using other techniques.

Arguably the most suppressing host cellular factor identified for HCV replication is a microRNA, miR-122. MicroRNAs (miRNAs) are endogenous noncoding RNAs with approximately 22 nucleotides in length that are generally known to suppress gene expression by blocking translational of specific mRNAs and triggering their degradation [58]. For HCV, it was the first time reported that miR-122, the most abundant miRNA expressed in hepatocytes [59], in fact can support translation and protect viral RNA from degradation [60,61]. This discovery has opened up a new avenue of therapeutic interventions targeting miRNAs for the treatment of HCV as this will be further discussed in the section below.

Targeting of cyclophilins

Cyclophilins are a family of proteins from vertebrates and other organisms that bind to the immunosuppressant CsA. These proteins have peptidyl prolyl isomerase activity, which catalyses the isomerization of peptide bonds from trans form to cis form at proline residues and facilitates protein folding. Viruses, including HIV, herpes simplex virus, vaccinia virus, vesicular stomatitis virus and
coronaviruses, take advantage of cyclophilins for their replication [62,63]. For HCV, it is well established that cyclophilins have an essential role in viral replication and de novo virus production. Early studies suggest that HCV replication is dependent on the interaction between cyclophilin B and NS5B to stimulate its RNA binding activity and thereby promote the de novo synthesis of positive and negative stranded RNA [45,46]. CsA blocks the binding of cyclophilin B to NS5B and thereby inhibits viral replication [45,46,64]. Later studies showed also cyclophilin A directly interacts with NS5B [47] and is involved in modulating the polyprotein cleavage activity of NS2 [48]. In addition, cyclophilin A interacts with NS5A and stimulates its binding with HCV RNA [47,65].

Despite the fact that a series of in vitro studies have shown potent anti-HCV effects of CsA [45,64,66], clinical evidence to support these findings is still limited [67,68]. Explanation to this in vivo and in vitro discrepancy is that hampering of the host immune system by CsA probably masks its direct antiviral effects. This has sparked the generation of CsA derivatives which lack the immunosuppressive properties but retain the antiviral activity. Three cyclophilin inhibitors are currently being evaluated in preclinical and clinical studies: Debio-025, NIM811 and SCY-635 [69]. These compounds are developed by structural modifications of CsA to reduce the immunosuppression capacity by abolishing calcineurin affinity. Debio-025, now named alisporivir, has been the first to enter clinical trials and the first results are encouraging. In a cohort infected with HCV and HIV, two weeks of treatment with Debio-025 decreased HCV viral load by more than 3-log, including in patients infected with HCV genotypes 1, 3 and 4. In contrast, the anti-HIV effects were much less pronounced (1-log reduction of viral load) [70]. In treatment-naive patients infected with just HCV, treatment with Debio-025 alone resulted in a more than 2-log reduction of viral load in those with genotypes 1 and 4, and a more than 4-log reduction in those with genotypes 2 and 3 [71]. When combined with peg-interferon/ribavirin, additive antiviral effects were demonstrated [71]. Since side effects, such as severe pancreatitis, have occurred, the future role of alisporivir in the treatment of hepatitis C patients remains to be determined [72].

**Targeting of miRNA**

The important role of miR-122 in supporting HCV infection is well-established [60,61]. Two putative binding sites located in the 5′ NCR are considered for miR-122 targeting, and binding to both sites was found to be necessary for viral replication. The two sites are adjacent and are separated by a short spacer, which is largely conserved between HCV genotypes [73]. Although the exact mechanism by which miR-122 regulates HCV has proved difficult to establish [74], therapeutic targeting miR-122 for anti-HCV therapy is already being explored in patients.

Inhibition of miR-122 function can be achieved through the use of conventional antisense technology. This antisense approach can act at multiple levels to affect miR-122, such as by binding to its precursor or its mature form and block their function. However, the low efficacy of the conventional antisense oligonucleotides (ASO) often requires additional modification to improve their biological activity. The first report on the successful use of 2′-O-methyl (OMe) ASO was to knockdown let-7 function in Drosophila [75]. Subsequently, ASO were developed to inhibit miR-122 in mice [76], which were termed as ‘antagomiRs’. Transfection of OMe antagomiRs against miR-122 was shown to reduce HCV replication up to 84% [77]. Another approach, MOE (2′-O-methoxyethyl phosphorothioate) modification, has also shown to effectively inhibit miR-122 activity in the liver [78].

Locked nucleic acid (LNA) technology is probably the most successful approach of ASO modification. LNA is a class of ASO with the ribose ring ‘locked’ by a methylene bridge connecting the 2′-O atom with the 4′-C atom. By ‘locking’ the molecule with the methylene bridge, LNA ASO is constrained in the ideal conformation for Watson-Crick binding, making the pairing with a complementary nucleotide strand more rapid and stable [79]. A simple systemic delivery of an unconjugated, PBS-formulated LNA-antimiR, was shown to effectively antagonize miR-122 in non-human primates. This resulted in effective uptake of the LNA probe in the cytoplasm of primate hepatocytes and formation of stable heteroduplexes between the LNA probe and miR-122 [80]. As miR-122 was shown to be a key regulator of cholesterol and fatty-acid metabolism in the adult liver [78], this treatment led to a long-lasting and reversible decrease in total plasma cholesterol without clear evidence for LNA-associated toxicities or histopathological changes in these animals [80]. Treatment of chronically infected chimpanzees with this LNA probe led to long-lasting suppression of HCV viraemia [81]. Most excitingly, in a recent Phase
2a trial, five injections of this LNA antisense probe has demonstrated continuous and prolonged antiviral activity without evident side effect in HCV patients [82]. In the high dose group (7 mg/kg body weight), approx. 3 log reduction of viral load was achieved [82], therefore holding promise for further clinical development.

**Targeting the viral genome**

**HCV genome**

HCV genome, a single positive-stranded RNA, consists of about 9,600 nucleotides in length and belongs to the *Flaviviridae* family. To date, six major genotypes and over 70 subtypes of HCV have been identified that differ by 31%–34% between genotypes and 20%–25% between subtypes in their nucleotide sequence [1,83]. The whole genome composes of a 5′ and 3′ NCR flanking a single open reading frame encoding a polyprotein precursor of approximately 3,000 amino acids. Within the viral genome, the IRES sequence is most conserved compared with other regions [84].

**Targeting viral genome by RNAi**

RNA interference (RNAi) is a sequence-specific inhibition of gene expression at posttranscriptional level [85]. It is triggered by small interfering RNA (siRNA), which can be introduced into cells directly as synthetic siRNA or indirectly as vector expressed short-hairpin RNA (shRNA) precursor [86]. Encoded shRNA can be exported to the cytoplasm and cleaved into active siRNA by a cellular enzyme, Dicer. These siRNAs are assembled into a multicomponent complex, known as the RNA-induced silencing complex (RISC), which incorporates a single strand of the siRNA serving as a guide sequence to target and silence homologous messenger RNA (mRNA) [87]. Both RNAi and miRNA shares the same cellular gene silencing machinery [86].

As the HCV genome functions both as a template for the viral messenger RNA and viral replication, the HCV genome appears to be a sensitive target for RNAi. A multitude of in vitro studies have been published showing the suppression of HCV infection or replication by RNAi targeting different regions of the virus, including IRES, NS3, NS4B, NS5A and NS5B [88–93]. Despite the apparent simplicity from a technical view, it has become clear that the therapeutic application is far from straightforward.

If RNAi is to be utilized as an effective treatment of chronic HCV infection or prevention of re-infection after liver transplantation, long-term and stable gene silencing needs to be achieved in the liver. Therefore, efficient RNAi delivery is the first challenge towards clinical application. Nanoparticles are favoured for efficient delivery of chemically-synthesized siRNAs [94,95]. However, synthetic siRNA-induced gene silencing is transient and therefore it requires repeated administration. Viral vector delivered RNAi can potentially circumvent this limitation [96]. Viral vectors derived from adeno-associated viruses (AAV) represent the most advanced approach for RNAi delivery in the context of developing anti-HCV therapy [97]. Investigators have pioneered the development of an AAV-based anti-HCV RNAi regimen, termed ‘TT-033’. It contains three shRNA cassettes with one targeting the IRES and two targeting NS5b regions and these targets are conserved across multiple viral genotypes. TT-033 was shown to have potent activity against HCV in a replicon model and demonstrated almost complete penetration of hepatic tissues following intravenous injection in nonhuman primates [93].

Potential induction of off-target effect is the second hurdle surrounding the development of RNAi therapeutics. Off-target effects are mainly caused by the convergence of the siRNA and miRNA pathways. Therapeutic delivery of RNAi potentially affects the biogenesis of miRNAs in the target cells [98]. Because of the broad regulatory function of miRNAs on gene expression, disturbance of miRNA biogenesis may fundamentally change cell physiology. Vector expressed shRNA requires export from the nucleus to the cytoplasm and processing into functional siRNA. Thus, it is likely to have a bigger impact on miRNA machinery than raw synthetic siRNA. AAV-mediated over-expression of shRNA has been shown to evoke liver toxicity in mice, ultimately even causing death [99]. Here, cellular RNAi factors including exportin-5 and four Argonaute proteins were likely saturated [100]. Saturation of the miRNA pathway was also observed using some lentiviral RNAi constructs as a consequence of over-expression of shRNA, although no significant cell toxicity was observed [98]. The lethal toxicity that
was observed [99] could have been caused by the combination of AAV vector and over-expressed shRNA.

Administration of TT-033 into nonhuman primates confirmed the induction of liver toxicity in a dose-dependent manner. This was associated with the high-expression levels of shRNA driven by the wild-type Pol III promoters [93]. To reduce the transcriptional activity by exchanging a less active regulatory element into each promoter, second generation of vectors have been developed (TT-034). Despite reduced levels of shRNA, the construct retained comparable anti-HCV efficacy but avoided liver toxicity in nonhuman primates [93]. Other issues, including resistance development, mutational escape and viral suppression of RNAi, remain challenges for large scale application of this approach [101]. However, the field is still moving forward towards initiating the first clinical trials [93].

Summary

An overwhelming number of pipeline compounds at various clinical stages are being developed for HCV, with the most advanced being the recently approved DAAs. However, some intrinsic limitations of DAAs/other compounds and potential resistance development and mutational escape by the virus may still urge the development of additional therapeutics, which should ideally act on distinct mechanisms. These new compounds shall be applicable in patients that are non-responders or not eligible to current antiviral therapies. Extensive studies have demonstrated the feasibilities of targeting HCV receptors by either neutralizing antibodies or small molecule inhibitors. These approaches are attractive for both treatment and prevention of HCV infection, in particular for the prevention of HCV recurrence after liver transplantation, since there is still no specific treatment available for HCV recurrence. Targeting cellular factors, including cyclophilins and miR-122, have already moved into clinical stages. One of the most exciting achievements, from basic and clinical research point of view, is the blockage of miR-122 by LNA probes, which has led to continuous and prolonged antiviral activity without evident side effect in HCV patients. However, the safety profile regarding this approach still needs to be further investigated, as recent study demonstrated that deletion of mouse miR-122 resulted in hepatosteatosis, hepatitis, and the development of tumours resembling hepatocellular carcinoma [102]. Targeting HCV genome by RNAi remains at preclinical stages. The power of this innovative technology allows flexible screening and testing of potential targets within the viral genome and the genome can be simultaneously targeted by multiple antiviral siRNAs. Thus, with the rapid improvement of gene delivery technology, RNAi therapeutics hold potential for future clinical development. With the future launch of various antivirals with distinct mechanisms, more optimal combinations will be available for treating HCV. As being expected, the dream of achieving high SVR rates with all-oral, interferon-free regimens will be no longer far from clinical reality.

Practice points

1. Targeting viral entry is particular attractive for prevention of HCV recurrence after liver transplantation
2. Cyclophilin inhibitor, alisporivir, has a significant anti-viral effect in HCV patients but this was associated with signs of toxicity and upcoming resistance
3. A preliminary study shows proof-of-concept in HCV patients that LNA antimiR against miR-122 can have potent antiviral activity without evident side effect.

Research points

1. The combination of LNA antimiRs against miR-122 with interferon and/or DAAs
2. The long-term safety profile of LNA anti-miR-122 probe, in particular the effect on hepatosteatosis, hepatitis and hepatocellular carcinoma
3. The initiation of clinical study of AAV delivered anti-HCV RNAi (TT-034)
Disclosure

No conflict of interest to report.

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