New Hepatitis C Virus Drug Discovery Strategies and Model Systems

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Abstract

Introduction: Hepatitis C virus is a major cause of liver disease worldwide and the leading indication for liver transplantation in the United States. Current treatment options are expensive, not effective in all patient groups and typically are associated with serious side effects. While pre-clinical anti-HCV drug screening is still hampered by the lack of readily infectable small animal models, the development of in vitro cell culture HCV experimental model systems has driven a promising new wave of HCV antiviral drug discovery.

Areas covered: This review contains a concise overview of current HCV treatment options and limitations with a subsequent more in depth focus on the available experimental models and novel strategies that have and continue to enable important advances in the anti-HCV drug development field.

Expert opinion:

With a large cohort of chronically HCV infected patients progressively developing liver disease that puts them at risk for hepatocellular carcinoma and hepatic decompensation, there is an urgent need to develop effective therapeutics that are affordable, well-tolerated and effective in all patients and against all genotypes. Significant advances in HCV experimental model development have expedited HCV drug discovery; however, additional progress is needed. Importantly, the current trends and momentum in the field suggests that we will continue to overcome critical experimental challenges to reach this end goal.

Keywords: Hepatitis C Virus (HCV), Experimental model systems, Antivirals, Replicons, HCV pseudoparticles (HCVpp), High throughput Screening (HTS).

1 Introduction

Approximately 170 million people or 3% of the world population, are chronically infected with hepatitis C virus (HCV)[1]. While new infection rates are difficult to determine, it is estimated that in 70 - 80% of infections, the virus is able to elude the host immune response and establish a chronic infection[1, 2]. The rate of disease progression in infected individuals is variable and many remain asymptomatic for 10-30 years; however, without therapeutic intervention chronic infection can lead to end-stage liver disease including steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma[2, 3]. As a result, in Europe and the United States, HCV is the most common cause of chronic infectious liver disease with HCV infection in the United States alone causing 8000–10,000 deaths annually[4, 5].

2 Current HCV Treatment and Obstacles

In the absence of a protective vaccine, combination therapy of pegylated interferon alpha (pegIFNα) and ribavirin (RBV) has been the mainstay of chronic HCV treatment for a decade. Although the pegIFNα/RBV combination therapy has been effective in up to 80% of HCV genotype 2 or 3 infected patients monitored in studies, sustained virological response rates (SVR) are much lower in patients with HCV genotype 1 and 4 infections (reviewed in [6, 7]) Success rates are also lower in particular patient populations such as African Americans and patients with high HCV RNA levels, high BMI, advanced liver disease, and those co-infected with HBV or HIV (reviewed in [7]). Notably, in 2011 two HCV NS3/4A protease inhibitors, Telaprevir and Boceprevir, were FDA-approved for use in combination with PEG-IFNa/RBV for patients infected with HCV genotype 1 because studies revealed that triple therapy in treatment-naïve patients increase SVR from ~ 40% to 70% or more (reviewed in [6, 7])

While there are still limitations associated with Telaprevir and Boceprevir, the recent advances in HCV molecular biology discussed below continue to facilitate the development of additional specifically-targeted HCV antivirals directed against not only viral proteins, but also viral-host interactions upon which successful infection is dependent. Development of additional HCV antivirals is critical not only in terms of identifying therapeutics that are more broadly effective against all the major HCV genotypes, but also to allow for effective treatment combination strategies designed to limit the emergence of drug resistant viral escape mutations, a concern that arises when RNA viruses are treated with compounds that directly target viral proteins and/or host interactions. Currently co-administration of PegIFNα/RBV has proven relatively effective at preventing rapid viral escape from Telaprevir and Boceprevir, but due to the limited efficacy of IFN in many patients and its associated side effects the goal is to eventually eliminate the use of IFN when sufficient direct-acting antiviral are clinically available..

3 Hepatitis C Virus Biology

HCV is an enveloped, positive-strand RNA virus belonging to family Flaviviridae. The viral genome consists of a single uncapped RNA of approximately 9600 nucleotides containing one large open-reading-frame, flanked by highly structured 5′ and 3′-nontranslated regions (NTRs) which play important regulatory roles in cap-independent translation of the viral proteins and viral RNA genome replication[8, 9]. The HCV virion consists of the genomic RNA encased in an icosahedral nucleocapsid formed by the core protein surrounded by a lipid bilayer containing the viral E1/E2 glycoproteins[10] . These virions exist in the serum of patients associated with trigryceride-enriched low and very low density lipoproteins[11, 12] and exhibit a wide range of densities with the more lipid-enriched lower density particles tending to exhibit the highest infectivity[13-15]. HCV viral entry into cells requires sequential interaction between virion

components and cellular factors[16-18]. The receptor engagement as well as acidic pH of endosomes triggers the fusion of the virus envelope and endosomal membrane, leading to release of the viral genome into the cytoplasm[15, 19-23] . In the cytoplasm, internal ribosome entry site (IRES)-dependent translation of viral mRNA by the host cell translation machinery produces a single polyprotein of ~3000 amino acid, which is co- and post-translationally cleaved by cellular and viral proteases to yield at least 10 mature viral proteins (Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B)[24]. Based on a functional HCV replicon system, it has been demonstrated that NS3, NS4A, NS4B, NS5A and NS5B as essential for viral RNA replication[25] . The replication complex uses the viral genome as template to generate negative-strand viral RNA intermediates, which in turn are used to synthesize new positive-strand genomic RNAs. These newly synthesized HCV genomic RNAs then in theory can serve as template for translation of more viral protein, as template for replication by the NS5B polymerase, or be encapsidated to produce new progeny[26] . Newly formed nucleocapsids acquire their envelop by budding through the ER membrane containing envelope glycoproteins and secreted from the cell through exoctyosis[9]. Differences in the densities of intracellular assembled virion particles compared to extracellular secreted virion particles are consistent with post-assembly lipidation of virion particles during and/or after secretion through ER/post-ER compartment [27]. Secretion of infectious HCV particles is influenced by components of the VLDL pathways such as micosomal triglyceride transfer protein (MTP), apolipoprotein B (ApoB), apolipoprotein E (ApoE)[11, 28] suggesting that HCV uses the lipoprotein/cholesterol export system, a concept consistent with the use of lipoprotein receptors such as LDLR, SR-BI, and NPC1L1 as cellular entry factors.

4 HCV drug discovery tools: Experimental Model Systems

4.1 Biochemical assays

The successful cloning of HCV in 1989 [29]provided the first insight into the viral genome as well as the ability to expression and characterization the viral proteins making the development of conventional biochemical assays feasible. Biochemical assays using purified protein and defined buffers can be quite useful for elucidating the mechanistic requirements of enzyme function (e.g. substrate specificity, co-factor requirements, etc…) and for identifying direct small molecule inhibitors. Hence, early HCV drug discovery included these types of standard well-documented in vitro approaches. Using purified NS3 protein, fluoregenic, chromogenic and radioactive assays for NS3 protease and helicase activity were developed and used to analyze NS3 activity and screen for potential inhibitors[30-33]. However, biochemical assays designed to assess the enzymatic activity of purified NS5B have been more challenging and controversial as polymerase activity levels achieved were lower than observed with analogous viral polymerases and substrate specificity varied among reports. Some of these issues likely reflect that while biochemical assays can be extremely useful that they can also be limited due to their inherently isolation away from the relevant host cell environment. Hence, even when biochemical approaches are successful, it is important to validate the outcome in a more physiologically relevant system that can additionally assess practical issues of cell-permeability and off-target effects.

4.2 Cell based assays

The development of cell based HCV assays was a major step forward in the arena of HCV drug development. At a minimum, cell-based HCV models for assessing the efficacy of specific antivirals depend on the existence of cell lines that support the viral activity under investigation and virus components/strains that can perform that activity in cell culture. The problem is that these types of tools have not always been readily available for HCV research perhaps because HCV is a hepatotropic virus that primarily infects fully differentiated hepatocytes in the liver of infected patients, a cellular phenotype not maintained when these cells are removed from their in vivo environment. As a result, HCV historically has not grown well in cell culture and therefore several surrogate model systems such as stable transfection of partial or full length HCV genomes (replicons)[25] and pseudotyped retroviral particles (HCVpp)[22, 34, 35] were developed. While these simple and robust systems are still used extensively to investigate specific aspects of HCV biology and assess HCV inhibitors, in 2005 an infectious HCV cell culture system supporting the entire HCV life cycle also finally became available[13, 36, 37]. The advantages and limitations of these various experimental systems are discussed below.

4.2.1 HCV Replicons.

While the cloning of HCV in 1989 allowed for the classification of the HCV into a reasonably well understood family of related viruses, specific understanding of HCV replication remained elusive for more than a decade until the development of the HCV replicon system[25], which recapitulates the intracellular steps of HCV RNA replication. The first replicons were self-replicating recombinant viral RNA in which the viral structural genes were replaced with the neomycinephosphotransferase (Neo) selection marker[25]. The Neo gene is translated from HCV internal ribosomal entry site located in the HCV 5'NTR, whereas the HCV replication proteins (NS3 – NS5B) are translated from an inserted encephalomyocarditis virus (ECMV) internal

ribosomal entry site. Because the encoded viral proteins are able to replicate the template replicon RNA from which they are expressed, cells that support replicon replication maintain high levels of the HCV replicon RNA and can be selected based on their constitutive Neo expression. These cells can then be expanded to create HCV replicon cell lines, which serve as a model of autonomous HCV replication.

The HCV replicon system has proven to be very useful for HCV drug discovery efforts. Not only did the replicon system provide an HCV-specific, robust assay to screen for HCV NS5B polymerase inhibitors, but it allowed for the first time non-target-specific screens that could identify inhibitors of HCV replication that interfered with any aspect of replication (i.e. host or viral factors). The ability to create non-infectious replicon systems from multiple different HCV genotypes also is advantageous in terms of assessing whether an inhibitor is broadly effective against multiple HCV genotypes. Some of the more widely used replicons reported to date include numerous genotype 1b replicons (e.g. Con1, HCV-N), the H77 clone genotype 1a replicon and the JFH-1 genotype 2a replicon[25, 38-44]. Notably, as replication-enhancing cell-culture adaptive mutations within different replicon clones were identified[42, 45, 46] and cell line more permissive for HCV replication were created[47], the requirement for active selection was reduced and replicons expressing various reporter genes (e.g. GFP, beta-lactamase and luciferase) instead of selection markers were generated for more convenient mix-and-read high throughput monitoring of replicon replication[38, 48-50]. While cell line restriction is still a limitation of the more recently established cell culture HCV infection system, another advantage of the replicon system is that replication has been established in multiple liver derived cell lines (e.g. HepG2 and Huh6) as well as non-liver cell lines (e.g. 293 and HeLa) and rodent cell lines (e.g. NIH3T3, AML12, MMHD3)[51-55].

The most obvious limitation of the replicon system is that it cannot be used to study the entire HCV infection process, particularly the early steps of virus life cycle before viral replication (e.g. viral attachment, entry and un-coating) or the late steps of viral infection after genome replication (e.g. virion assembly, maturation and release). Additionally, in some cases caution may be warranted because all replicons, except for the genotype 2a JFH-1 clone, contain cell culture adaptive mutations which when introduced back into viral genome, render it non-infectious in chimpanzees[56]. Hence, like many in vitro viral systems, HCV replicons may not accurately mimic which viral-host interactions are most critical in vivo.

4.2.2 HCV Pseudotyped Particles.

A relatively convenient approach to study the entry of enveloped viruses is the use of pseudotyped retroviral particles harboring the envelop glycoproteins of the virus of interest. HCV pseudoparticles (HCVpp) were first generated in 2003[22, 34, 57]. Analogous to other viral pseudotyped particles this was accomplished in 293 cells by co-transfecting a plasmid encoding an envelope deficient, packaging competent retroviral genome (MMLV or HIV) engineered to express a reporter gene (e.g. GFP or Luciferase) along with a plasmid encoding the viral glycoproteins of interest, in this case HCV E1 and E2. Entry of the recombinant viral particles in to cells is then dictated by the envelop glycoproteins and efficiency of the entry process can be measured by a simple reporter gene assay. Importantly, functional analyses by many research groups have demonstrated that the requirements for HCVpp cell entry closely mimic HCV virus in terms of antibody neutralization and it has proven to be a useful method to study the entry of various HCV genotypes, the factors required for HCV entry, and the tropism restrictions associated with HCV entry[18, 35, 58-60]. As such, HCVpp systems are safe, robust and easy to manipulate to for drug discovery efforts focused specifically on specific steps of the viral entry

process. As a result, HCVpp systems have been used to screen libraries of small molecule inhibitors and several promising entry inhibitors are in early clinical testing[61-64]. One caveat, however, is that these particles are not identical to authentic HCV particles in terms of lipid composition and this does result in some function differences in entry factor requirements and kinetics. For example, the LDL receptor and NPC1L1 cholesterol uptake receptor are required for the entry of infectious HCV particles such that HCV infection is blocked by antibodies or other inhibitors directed against these cell surface proteins, while HCVpp entry is much faster and independent of these cellular factors[17, 22, 34, 65]. It is interesting to speculate that perhaps the production of HCVpp in VLDL-synthesizing cells might lead to the assembly of lipoprotein-associated HCVpp; however, the envelopment of retroviral particles at the plasma membrane compared to the natural maturation of HCV particles through the ER/Golgi and might prevent the association of HCVpp with lipoproteins regardless of producer cell type.

4.2.3 HCV cell culture infection system.

The advance that finally allowed for the study and screening of all aspects of the viral life cycle is the HCV cell culture (HCVcc) infection system. The HCVcc infection system was made possible by the identification of the HCV genotype 2a isolate, JFH-1, which was cloned from a Japanese patient suffering from fulminant hepatitis[66]. This HCV clone was unique because replicons derived from this consensus genome did not require "infection-limiting" adaptive mutations in order to replicate efficiently in cell culture [40]. Based on this phenotype, the JFH-1 clone [36, 37] and a chimeric genotype 2a derivative (J6/JFH-1)[13] were tested and found to initiate productive infection in Huh7 human hepatoma cells. Specifically, electroporation of genomic HCV RNA in vitro transcribed from this construct into Huh7 cells resulted in efficient replication and rapid spread of HCV to non-transfected cells. Additionally, filtered medium from

the transfected cell culture was able to transmit infection to naïve cell cultures indicating the presence of secreted infectious virus particles. Cell culture produced virus was confirmed to be infectious in chimpanzees [37] and chimeric SCID-uPA mice transplanted with primary human hepatocytes [67]. Likewise virus recovered from infected mice was infectious in Huh7 cells in vitro [67]. Cell culture produced virus is antigenically similar to HCV clinical isolates being neutralized by anti-CD81 antibodies as well as by antibodies isolated from chronically HCV infected patients [36, 37, 68] and it demonstrates similar sensitivity to antivirals that directly target the viral proteins [13, 36, 37, 68].

The obvious advantage of the HCVcc system is that it encompasses the complete viral life cycle and allows for production and analysis of infectious HCV particles. It also offers additional experimental flexibility in terms of time of inhibitor treatment relative to viral inoculation, the ability to alter viral dose, and the opportunity to measure of multiple different viral parameters (i.e protein expression, replication, infectious virus production) which allows for more informative screens tailored to specific questions. However, in line with the historically slow progress in the HCV research field, six years since the development of the HCVcc system there are still major drawbacks to the system in terms of drug development. A continuing technical challenge is that even under ideal conditions HCVcc grows to relative low titer making typical high MOI drug screening approaches less feasible. Low MOI longer term assays have been successfully established (discussed below), but these by nature are more challenging, expensive, and more prone to cell culture related variability. That being said, a more fundamental limitation of the system is its dependence on the HCV JFH-1 clone. Being restricted to a single viral clone is problematic because different HCV genotypes typically exhibit differential sensitivity towards individual antivirals, thus screening compounds against the available JFH-1 genotype 2a clone

often identifies genotype-specific inhibitors that are less effective against other more clinically prevalent and challenging genotypes (Yu and Uprichard, unpublished data). To address this limitation, JFH-1 based chimeric viruses have been created by substituting discrete regions of different HCV genotype clones into a JFH-1 backbone[69-73]. These chimeric viruses have proven quite useful in assessing difference in viral entry among genotypes (i.e. chimeric viruses containing the structural region of different HCV genotypes fused to the non-structural region of the JFH-1 clone), but in terms of drug discovery they are limited as they often grow to lower titers and to large extent conceptually negate the major advantage of the infection system because once again the focus becomes restricted to only a pre-designed limited aspect of the viral life cycle. While efforts are being made to propagate other HCV clones in Huh7 cells with varying success[74-76], another ongoing strategy is to try and develop more physiologically relevant hepatocyte cell culture systems that better mimic natural in vivo virus-host interaction and thus might allow for infection with other HCV genotype as well as a better understanding of how HCV infection impacts normal hepatocyte function. Many have confirmed that primary human hepatocytes are permissive to different HCV genotypes and clinical isolates [65, 77-79], but these cells can be difficult to obtain and are notoriously difficult to maintain in cell culture which in general limits their practical use for drug screen, though examples certainly exist where groups have managed to overcome the challenges and successfully grow primary hepatocytes in a reasonably high throughput 96-well format [80].

4.3 HCV animal models

While typically large scale antiviral screening approaches begin in vitro, later stage drug discovery confirmation efforts are often performed in small animal model systems. However, in the case of HCV, the restricted species tropism of the virus has been a significant obstacle.

Although chimpanzees are susceptible to HCV infection, their usage is limited due to ethical concerns including their endangered status as well as their exceptionally high costs. Consequently, a main focus in the field has been development of HCV mouse models[81].

To overcome the known and unknown species barriers that prevent HCV infection of mice, chimeric xenograft mouse model approaches have been developed. The first and most commonly used model is based on severe combined immune deficiency (SCID) mice carrying a lethal urokinase-type plasminogen activator transgene expressed in their hepatocytes by the albumin promoter. While the endogenous hepatocytes in these mice are destine to die, these uPA-SCID mice can be rescued by transplantation with human hepatocytes which repopulate the liver. The human hepatocytes in these mice remain permissive to human hepatotropic viruses such as HBV and HCV[82, 83]. These xenotransplanted mice are a great tool to assess the efficacy of HCV antivirals and several groups have demonstrated that in vivo HCV infection can be inhibited by blocking either the viral E1/E2 glycoproteins or one of the host cell HCV entry factors as well as a variety of other known HCV inhibitors (e.g. interferon, NS3 protease inhibitors, etc.)[84-86].While the use of this model is not widespread due to technical as well as physiological limitations of the system, chimeric mice with humanize livers are currently the most relevant small animal model for testing inhibitors of HCV infection in vivo.

Importantly, sensitive luciferase reporter strategies indicate that transgenic mice expressing the human HCV entry factors CD81, Occludin, SRB1, and Claudin 1 are permissive for low level of HCVcc entry[87]. However, the inability to directly detect infection in these animals (i.e. HCV RNA and/or protein expression) suggests other blocks to infection exist and/or that additional factors are required for efficient infection of mice with HCV. Notably, Sainz et al., (2012) recently identified the cholesterol transporter NPC1L1, as new factor required for infectious

HCV entry[17]. Considering that NPC1L1 is expressed on hepatocytes only in humans and chimpanzees, it will be interesting to see if the additional expression of exogenous NPC1L1 on the hepatocytes of these mice will confer more robust permissiveness to infectious HCV entry. This combined with the recent demonstration that over expression of human miR122 significantly enhances HCV RNA replication in mouse cells [88] adds renewed hope that a mouse model of HCV infection may soon be a reality.

5 HCV drug discovery: Approaches and Methodologies

5.1 Reporter viruses / Cell Lines

Some of the more interesting recent advances made in HCV drug discovery have been related to the development of novel strategies for simple yet sensitive detection of HCV in cell based assays. RT-qPCR detection of the viral genome and ELISA-based detection of viral antigen are two common ways for detecting HCV. However, by nature these assays are cumbersome and time consuming not lending themselves well for high throughput analysis. As mentioned above, after identification of cell culture adaptive mutations required for robust replication of HCV genomes in cell culture, HCV replicon were modified to express various reporter genes such as GFP, luciferase and β-lactamase for convenient detection of HCV replication in cell culture [38, 49, 50, 89, 90] and similar approaches have now also been used by inserting analogous reporter genes into the full length HCVcc genome[72, 91-94]. While such modified viruses are in theory better suited for high throughput screening, insertion of foreign genes into viral genome often results in impaired viral replication, raising other technical hurdles. To facilitate the rapid detection of HCV infection without compromising the replication efficiency of the virus, an alterative approach has been to develop reporter cell lines that transmit a detectable signal in response to HCV infection. One example is a cell line engineered by Iroa et al to express EGFP attached in-

frame with the secreted alkaline phosphatase (SEAP) gene via a linker region that contains the recognition sequence of the HCV NS3/4A protease. While the cells constitutive make this fusion protein, it is only upon HCV infection that the viral NS3/4A is available to cleave the linker region and release SEAP from the fusion protein triggering secretion into the extracellular culture medium [95]. To monitor HCV infection on a single cell level, Jones et al, created a stable Huh 7 cell line expressing a fluorescent protein with a nuclear localization signal fused to the mitochrondrial bound IPS proteins (e.g. RFP-NLS-IPS)[96]. In uninfected cells this mitochondrial tethered fusion protein exhibits a standard punctuate IPS localization pattern. However because the cellular IPS protein contains an NS3/4A cleave site, HCV infection of these cells results in NS3 cleaves of the fusion protein from the mitochondrial membrane resulting in relocalization of RFP fluorescence from the cytoplasm to the cell nucleus[96]. These types of reporter cell lines are particularly useful for monitoring initial infection events, but perhaps could also be adapted for monitoring the inhibition of steady-state (i.e. chronic) infection depending on the stability of the signal molecule used.

5.2 High throughput compound screening.

As noted above, well before cell based HCV model systems were available, efforts were made to produce active forms of the HCV encoded NS3 protease and NS5B polymerase to screen for inhibitors. Due to the success in expressing active NS3 and the ease of creating suitable substrates, biochemical screens for inhibitors of this critical viral protein are still being performed[97]. Likewise, using a double-stranded DNA (dsDNA) with a 5'-fluorescent-dye (BODIPY FL)-labeled strand hybridized with a complementary strand, the 3'-end of which has guanine bases, Tani et al recently set up a screen to identify inhibitors of the NS3 helicase activity [98]. However, since the development of cell based HCV model systems, the bulk of HCV

drug discovery has turned to cell-based high throughput screening (HTS). Traditionally HTS involves large collections (i.e. libraries) of compounds to identify those that exhibit antiviral activity and thus could potentially be developed into a clinically useful therapeutic drugs. Notably, all the cell based HCV experimental systems described above have been adapted and successfully used for high throughput compound screening

5.3 Reporter Gene Based Screening.

Not surprisingly, high throughput luciferase-based HCVpp entry screens[63, 64, 99] as well as replicon replication screens have been reported[89, 100, 101] and clearly provide a robust approach for identifying inhibitors against the specific aspects of HCV infection recapitulated by these systems. However, this approach is more problematic in the context of the full infection system due to the significantly lower titers that can be achieved with viral constructs after the insertion of the reporter gene, which significantly limits the ability to create the large high titer viral stocks required. Additionally, with all reporter gene assays, one has to be cognizant of false positive hits that result when compounds directly inhibit the report gene assay.

5.4 HCV NS3 as a reporter gene.

As an alternative HTS approach, other groups have opted to use the viral NS3 protease as an innately encoded reporter gene. This has the advantage of not requiring deleterious manipulation of the viral genome (i.e. insertion of foreign reporter gene sequences) and avoids the issue of identifying irrelevant reporter gene inhibitors. As described above, two groups stably integrated NS3 cleavable reporter gene fusions into Huh7 cells such that a detectable signal is released from the cell or relocalizes to the nucleus upon infection of the cell. Boyle et al,[102] and Yu et al[103] developed more traditional mix-and-measure fluorescence resonance energy transfer (FRET)-based assays that uses NS3 protease activity as quantitative read out for HCV levels in the

replicon system and HCVcc infection system, respectively. The approach consists of using a peptide substrate containing the HCV NS3 peptide cleavage sequence flanked on either side with fluorphores that quench each other when they are in close proximity on the intact peptide. Replicon cells or infected cells are first incubated in the presence or absence of compounds from a library. After a period of time is allotted for potential inhibitory activity, the cells are lysed. The level of HCV in the cells is then quantitatively detected by adding the quenched NS3 substrate peptide to the cell lysate and measuring the fluorescence generated when NS3 cleavages the substrates.

5.5 High throughput siRNA library screens

Compound library screening has long been a staple of drug discovery, but the same high throughput approaches are now also routinely used to screen genomic siRNA libraries to identify host cell factors that are required for viral infection. In terms of drug discovery, the concept behind functional siRNA screening is the identification of useful drug targets rather than screening directly for potential inhibitors. An advantage of this approach is that it is likely that all HCV genotypes interact with the same host cell factors and thus cellular targets would be more likely to be broadly effective. Additionally, host cell targets typically exhibit a higher barrier to the development of resistance. The challenge is to then either design an inhibitor that specifically blocks the viral interaction with the host cell protein or focus on host factors that are transiently dispensable for host cell function but absolutely critical for virus life cycle. One recent example of potential HCV antiviral targets originally identified via siRNA screening are EGFR and EphA2, which highlights the utility of the approach in general[104]. One curiosity associated with these high throughput siRNA screens however is the variability in the genes identified by different groups. Presumably at least some of the differences are due to diverse

siRNA chemistries, efficacies, and off target effects combined with different assay endpoints, cell types, virus preps, and filtering parameters, but it is still surprising how little overlap (~<10%) at individual genes level has been observed. Future comparisons of results across studies with a focus on biological process /pathways might help to provide more insight into the large amount of information being generated by these studies.

6 Expert Opinion:

Although acute HCV infection rates have declined over the years, a large chronically HCV infected cohort is progressing to cirrhosis and is at risk for hepatocellular carcinoma and hepatic decompensation. Hence, more than ever, there is an urgent need to develop effective therapeutics that can clear chronic HCV infection. For decades, slow progress in the HCV research field has left us clinically dependent on suboptimally effective and difficult to tolerate interferon and ribavirin based therapies. Hence, while significant advances in HCV experimental model development (e.g. HCV replicons, HCVpp, the HCVcc infection system) and innovations in high throughput screening have ushered in a new era of discovery that has expedited HCV drug development and provided new hope that chronic HCV will ultimately be a "curable" disease, it is imperative that we rapidly reach the goal of developing interferon-free, broad spectrum HCV treatment regimens that are affordable, well-tolerated and effective in all patients and against all genotypes.

A number of advances are needed to facilitate HCV drug discovery. Perhaps at the top of that list would be a robust cell culture system permissive to infection by all HCV genotypes as this would allow for the identification of more broadly effective inhibitors or at least a panel of inhibitors optimized for specific genotypes. Closer to the clinic, the ability to study the drug sensitivity and viral escape patterns of clinical isolates in vitro might additionally allow for

preliminary efficacy testing, providing means to individualize therapy in more difficult to treat patients. Notably, while such ideal systems may be years in the making, it is important to realize that the insights about key viral-host interactions gained during the process of trying to develop more physiologically relevant in vitro and in vivo model systems will continue to bring us closer to achieving our drug development goals.

While this review focuses on the tangible bench top tools and screening strategies that have lead to and continue to feed the current richly populated HCV drug development pipeline, it is relevant to note that systems biology approaches will also continue to be central in promoting the rapid creation and translation of new knowledge into practical use in the clinic. For example, mathematical modeling of HCV inhibition kinetics under treatment has played a major role in evaluating, understanding and even directing HCV treatment in the clinic. Current in vitro and in vivo modeling efforts are further elucidating the complex mechanism of action of different HCV antiviral agents and in the future the use of in silico simulation modeling should prove useful for efficiently predicting optimal synergistic therapeutic combinations that minimize viral escape. Likewise, advances in sequencing and genomic analysis are allowing in depth characterization of host and viral genomic differences which should also allow for a more informed understanding of the antiviral potential of specific drugs in different clinical situations. Of course ultimately, the final challenge will become accessibility and affordability of care, but this issue should be facilitated proportionate to the level of successful we achieve in developing an effective array of clinical therapeutics options.

7 Article highlights box

- New HCV infection rates have decreased, but there is a rapidly growing public health burden of liver disease, including hepatocellular carcinoma, from those who are chronically infected. Hence, there is an urgent need to develop broad spectrum HCV treatment regimens that are affordable, well-tolerated and effective in all patients and against all genotypes.

- Although research efforts have been hindered in the past due to the lack of suitable HCV experimental models, the development of several robust cell-based system, particularly the first HCV cell culture infection system (HCVcc) has expedited HCV drug discovery.

- Despite the incredible advances in HCV model development, critical limitations of the current experimental systems exist and continued efforts to attain more physiologically relevant in vitro and in vivo models permissive to infection by all HCV genotypes and clinical isolates are needed.

- Creative innovations in simple, rapid, and sensitive HCV detection methods have contributed to the effective adaptation of emerging HCV experimental models for high throughput antiviral screening.

- With the advent of RNAi technology, high throughput screening of host factors involved in HCV infection has become a new and effective means of identifying potential host cell antiviral targets that may prove useful for broadly inhibiting HCV infection of all genotypes while additionally minimizing the rapid viral escape that can occur when drugs are targeted to specific residues within individual viral proteins.

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