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HCV drug discovery aimed at viral eradication

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SUMMARY

Hepatitis C virus (HCV) causes significant morbidity and mortality worldwide with nearly 3% of the world population infected by this virus. Fortunately, this virus does not establish latency, and hence it may be possible to eradicate it. HCV is strongly associated with liver cirrhosis and hepatocellular carcinoma and is currently treated with pegylated interferon- α (peg-IFN- α) and ribavirin. Unfortunately, these limited treatment options often produce significant side effects, and currently, complete eradication of virus with combined drug modalities has not yet been achieved for the majority of chronically HCV-infected individuals. Restricted treatment options, lack of a universal cure for HCV and the link between chronic infection, liver cirrhosis and hepatocellular carcinoma necessitate design of novel drugs and treatment options. Understanding the relationship between the immune response, viral clearance and inhibition of viral replication with pharmacology-based design can ultimately allow for complete eradication of HCV. This review focuses upon significant novel preclinical and clinical specifically targeted antiviral therapy (STAT-C) drugs under development, highlights their mechanism of action, and discusses their impact on systemic viral loads and permanent clearance of infection.

Keywords

antiviral; drug; HCV; hepatitis C; inhibitors; therapy

INTRODUCTION

Hepatitis C affects approximately 170 million individuals worldwide (World Health Organization, 2008), and 4 million in the United States are chronically infected with hepatitis C virus (HCV) (WHO, 2008). Although the current standard of care for chronic HCV infection, combination peg-IFN- α and ribavirin treatment, displays efficacy in ~80% of individuals infected with genotype 2 or 3, a blanket cure that is efficacious across all genotypes does not exist. In particular, less than 50% of individuals with genotype 1 – the most aggressive and prevalent strain in Americas, Europe, China and Japan – present with sustained virological response (SVR) [1–4].

HCV is an enveloped RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family and is comprised of approximately 10 000 nucleotides, displaying significant heterogeneity, with six main genotypes and more than 50 subtypes [5,6]. HCV persists asymptotically in the majority of infected individuals and is strongly associated with chronic hepatitis, hepatic steatosis and cirrhosis and hepatocellular carcinoma [7–9].

The high prevalence of HCV variants within HCV-infected individuals represents a significant problem preventing complete elimination of virus and is a function of the error-prone proofreading capability of the RNA polymerase, conferring a high rate of mutations [10,11]. As a result of marked heterogeneity in intra-patient viral quasispecies, complete elimination of systemic virus by the adaptive immune response does not always occur (Reviewed in [12,13]). In an attempt to circumvent this problem, treatments with cytokines that modulate the innate immune response, most notably pegylated IFN- α , have become a primary therapy in management and treatment of HCV infection. Unfortunately, the two existing treatments for HCV infection, IFN- α and ribavirin, can produce unfavourable side effects, including alopecia, rash/itching, thyroid dysfunction, nausea, leucopenia, thrombocytopenia and anaemia [14].

The development of novel therapeutics designed to completely eliminate HCV infection across all genotypes and subtypes remains an attractive area of research. Because latency cannot be established with HCV, eradication is possible even with the current standard of care for certain persons. To date, most studies focus upon the inhibition of various stages of the viral replication cycle. Drugs targeting modulation of the immune response, inhibition of viral fusion with the host cell membrane, viral helicase, polymerase or protease are under clinical investigation [15]. Therapies designed to stimulate the innate and adaptive immune responses in favour of rapid elimination of virions are underway [13,16–18], and, together with traditional drug-based therapies, provide a foundation for systemic eradication of HCV in infected persons.

Upon definition of the multiple mechanisms that the host uses to effectively control and eventually eliminate HCV replication, aggressive therapies designed to amplify the host immune response in tandem with traditional pharmacology-based treatment options can be employed. Whether the current therapies IFN- α and ribavirin will remain cornerstones of future treatment options is uncertain, however, co-administration of the traditional therapies in concert with novel therapies could provide an improved approach towards eradication. Upon identification of novel treatment options with diminished side effects, administration of IFN- α and ribavirin could be reduced below those associated with significant side effects, by combination with novel, potent, multi-target inhibitors. This review highlights the recent progress in developing anti-HCV drugs (Figure 1) and their mechanisms of action.

ENTRY INHIBITORS

Inhibition of viral entry into HCV-permissive cells could provide an efficacious mechanism for reduction or elimination of productive infection. Specific factors necessary for virus/host cell fusion are incompletely understood, which, until recently, represented an insurmountable obstacle in design of specific fusion inhibitors. Studies have defined surface

receptor CD81 and scavenger receptor class B Type 1 binding lectins (SCARB1), in concert with claudin 1 protein, as necessary factors for HCV fusion and entry [19–22]. These reports provide the foundation for design of inhibitors targeting one or more essential steps of HCV entry into host cells.

To date, numerous entry inhibitors are in various stages of preclinical development, including SP-30 (Samaritan Pharmaceuticals, Las Vegas, NV, USA), PRO 206 (Progenics, Tarrytown, NY, USA), and REP 9C (REPLICor, Laval, QC, Canada) and multiple early compounds identified by large-scale screening. Takebe *et al.* [23] recently performed an evaluation of 8,000 compounds for anti-HCV activity within the JFH-1 system and reported EC₅₀ values ranging from 53 to 113 nM across the top four hits, with encouraging toxicology data demonstrating CC₅₀ values of >35 μM [23]. Although the mechanism of action is not fully defined, it is believed that the anti-HCV activity is linked to interference with viral binding to the CD81 receptor. Although this study is in the early stages of development, it establishes the importance of CD81 in HCV infection and provides an excellent foundation for further studies designed to exploit the CD81 receptor as a target to inhibit viral replication.

Building upon the recently established mechanisms of entry represents an attractive area of research that can further define additional potential entry inhibitors (Figure 2A). Gastaminza *et al.* [24] identified the antiviral activity of D,L-αpeptides using the JFH-1 system: these compounds were active in genotypes 1a, 1b and 2a. Polymerization into nanotubes was associated with the ability to inhibit viral replication, and EC₅₀ values ranged from 5 to 10 μM, with CC₅₀ values greater than 100 μM. Although the specific mechanisms are not fully defined, these peptides interfere with viral entry and could serve as parent compounds from which further drugs can be designed.

Inhibition of entry and fusion of HCV with host cells represents an emerging field of study, and further elucidating the relationship between CD81, claudin and SCARB1 will allow for design of specific inhibitors which interfere with each facet of viral entry. As the interplay between CD81 inhibitors and viral replication is only narrowly understood, it is important that future efforts classify the activity of anti-CD81mAb or inhibitors of virus/CD81 interaction, against both acute and chronic infection, while simultaneously relating these data to SVR and viral clearance. To this end, Meuleman *et al.* [22] reported that anti-CD81mAb does not inhibit replication for previously established infection in a murine model with humanized liver.

These data emphasize the importance of the timing of initiation of therapy: there was lack of antiviral activity during chronic infection. These types of approaches may find utility in prophylactic treatment for high-risk populations. In addition, further assessment of entry-based inhibition of HCV could provide a useful tandem mechanism for use with other inhibitors of viral replication, ultimately leading to a multifaceted approach to eradication.

HELICASE INHIBITORS

The NS3 region of the HCV genome contains a serine protease that possesses NTPase and helicase activity. Together, the coupled NTPase and helicase function to unwind duplex

RNA, and is an essential mechanism in the viral replication cycle (Reviewed in [25,26]). Identification of critical steps in the viral replication cycle provides a unique opportunity to design compounds targeting one or more of these mechanisms.

Krawczyk *et al.* [27] recently reported EC₅₀ values ranging from 32 to 35.5 μM for two tropolone derivatives, BTN10 and BTN11, using the HCV replicon system. In addition, Huang *et al.* [28] report an EC₅₀ of 0.48 μM for trixalen, an NS3 helicase inhibitor in early phases of investigation. These findings represent studies in early stages of development, and antiviral activities in the context of the JFH-1 system or antiviral and toxicological profiles *in vivo* are undefined. These studies provide a basis for future design of combination therapy simultaneously targeting multiple stages of viral replication.

INTERNAL RIBOSOME ENTRY SITE (IRES) INHIBITORS

The internal ribosome entry site (IRES) of HCV serves as a direct regulator for assembly of initiation of translation complexes on viral mRNA (Reviewed in [29,30]). As this mechanism is distinct from those observed in prototypical eukaryotic translation machineries [29–31], the inhibition of viral IRES could provide a virus-specific target for antiviral compounds.

RNA molecules designed to interfere with formation of a translationally active complex, ultimately inhibiting particle formation, have recently been identified as novel anti-HCV compounds [15]. Romero-Lopez *et al.* [15] characterized the anti-HCV activity of chimeric RNA molecule HH363-50, HH363-10 and HH363 designed to concomitantly target position 363 of the HCV genome and an essential domain IV stem-loop structure within IRES containing the translational start codon. Antiviral activities ranging from 0.15 to 3.46 μM were reported in Huh-7 cells, establishing the potential role of RNA molecules as inhibitors of HCV replication by interference with IRES [15].

An alternate strategy to RNA-mediated inhibition of IRES function is the use of DNazymes. DNzyme molecules specifically targeting conserved regions of HCV IRES consistent across genotypes, as well as regions unique to genotype 1b demonstrated target-specific cleavage and subsequent inhibition of IRES-mediated translation [32]. These data were reported in a cell-free system, and thus their ability to display antiviral activity in either the replicon or JFH-1 systems and their potential in man are not defined. Despite these current gaps in knowledge, inhibition of IRES by either RNA- or DNA-mediated mechanisms provides a unique and specific tool for future design of novel compounds.

POLYMERASE INHIBITORS

Inhibitors of HCV NS5B RdRp are divided into two classes, namely the nucleoside and non-nucleoside inhibitors (NI and NNI, respectively). In their active triphosphate (TP) forms, NI metabolites act as nonobligate chain terminators while competing with the natural substrate nucleotides [i.e. for $\beta\text{-D-}2'\text{-deoxy-}2'\text{-fluoro-}2'\text{-C-methycytidine}$ (PSI-6130), CTP or $2'\text{-C-methyl-}7\text{-deaza-adenosine}$, ATP] for the HCV NS5B RdRp, thereby reducing the efficiency of further RNA elongation through steric resistance. To date, the primary NI are the $2'\text{-C-}$

methyl (containing a hydroxy or fluorine function also at the 2' position) and 4'-C-azido nucleosides.

Nucleosides inhibitors (NI)

The cytidine NI PSI-6130 demonstrated potent and specific *in vitro* anti-HCV activity, with no apparent cytotoxicity [33–36] and functions synergistically with IFN- α 2b and ribavirin to inhibit HCV RNA replication using the replicon system [36]. In addition, PSI-6130 displays greater potency against the S282T mutant *vs* 2'-C-methyl nucleoside analogues in both the HCV replicon and HCV-Cp7 systems [35,37]. In a 14-day Phase I monotherapy study, the tri-isobutyl ester prodrug of PSI-6130, named R-7128, demonstrated high oral bioavailability, significant potency and conferred a substantial drop in levels of HCV RNA, with a 2.7-log reduction in the viral load for individuals infected with genotype 1 who had failed prior IFN therapy [38,39]. In addition, co-administration of R-7128 with peg-IFN- α 2b and ribavirin resulted in a 5.0-log viral load reduction for patients infected with genotype 2 and 3 who were previously classified as nonresponders [40]. R-7128 has advanced to an expanded Phase 2b study, for the investigation of co-administration of R-7128 with IFN- α 2b and ribavirin in genotype 1 or 4 treatment-naïve infected individuals. Such studies provide a strong virological foundation for using 2'-C-methyl (or 2'-C-fluorine) nucleoside analogues together with the standard of care (peg-IFN α 2b with or without ribavirin) treatment for HCV infection. In addition, a recent late breaker abstract presented at European Association for the Study of the Liver (EASL) 2009 reported that co-administration of R-7128 plus ITMN-191 over the course of a 14-day study reduced HCV viral loads to below the limit of quantification for 63% of subjects enrolled [41]. Of interest is that PS-6130 in its monophosphate form can be deaminated intracellularly to the uridine analogue. The U analogue like PSI-6130 can be phosphorylated to the 5'-triphosphate, which is a potent inhibitor of HCV polymerase [42]. Thus, delivery of the active form of the uridine plus cytidine 5'-triphosphate analogue intracellular provides potential for synergy and a high genetic barrier towards selection of resistant virus because of this combination. This led to the realization that the uridine analogue in a prodrug form could itself be a potent inhibitor of HCV. PSI-7851 represents such a promising nontoxic uridine nucleoside 5'-monophosphate prodrug under Phase 1 clinical evaluation. The mechanism of action is linked to deamination of PSI-6130 in hepatic cells, and *in vitro* studies demonstrated superior anti-HCV activity of PSI-7851, with 10- to 20-fold greater potency *vs* first generation Phase 2b inhibitor of NS5B RdRp, R-7128 [43].

PSI-938 was recently reported as a novel proprietary nucleotide purine analogue for the treatment of HCV [44]. PSI-938 confers a resistance profile that differs from the pyrimidine analogues R-7128 and PSI-7851 and is metabolized by a phosphorylation pathway that is distinct from both R-7128 and PSI-7851 [44]. Together, R-7128, PSI-7851 and PSI-938 provide a multifaceted approach for combination therapy, wherein utilization of various combinations of C or U pyrimidine analogues with a purine analogue can be administered for maximized inhibition of viral replication. This strategy has proven to be very successful for the treatment of HIV infections (e.g. Truvada or Combivir).

The 4'-C-azido nucleoside inhibitor R-1626, another tri-isobutyl ester prodrug of R-1479, provided a robust antiviral activity while inducing 2.6 log₁₀ reduction in plasma HCV RNA levels of genotype 1-infected individuals in a 14-day monotherapy, with no appearance of resistance to R-1479 [45]. Nevertheless, severe adverse haematological effects were reported with the highest dose of 4500 mg R1626, leading its termination of further development [45].

Valopicitabine (NM283), the 3'-valine ester of β-D-2'-C-methylcytidine (2'-C-MeC; NM107), was the first prodrug nucleoside under clinical investigation by Idenix Pharmaceuticals (Cambridge, MA, USA). NM107-TP inhibited the activity of HCV NS5B RdRp by competing with the natural substrate CTP and terminates the RNA elongation step and demonstrated potent *in vitro* antiviral activity against both genotype 1 (HCV replicons), [36,46] and genotype 2 (JFH-1-based system, Cp7 wild type). In addition, *in vitro* synergistic activity has been demonstrated when combined with IFN-α2b or triple combination with IFN-α2b and ribavirin, with no evidence of cytotoxicity [36]. Although valopicitabine was evaluated in clinical trials, and co-administration with peg-IFN-α has led to successful viral RNA suppression in numerous HCV-infected persons [47], this drug was discontinued in its present form, largely as a function of gastrointestinal toxicity and pancreatitis [48]. Learning from that failure, IDX184 was developed as a novel liver-targeted nucleotide HCV NI, which demonstrated potent antiviral activity against genotype-1-infected chimpanzees (>3.0 log₁₀ HCV viral reduction for 4 days), with no emergence of the S282T mutation. IDX184 was generally well tolerated in healthy subjects at single dose up to 100 mg in a Phase 1 clinical trial [49]. Although extensive additional studies must be conducted to determine the long-term impact of IDX184 on viral loads, viral clearance and toxicity, these data are promising.

Nonnucleosides inhibitors (NNI)

The primary mechanism of action for non-nucleoside inhibitors (NNI) is specific targeting of different and less conserved allosteric sites of the HCV NS5B polymerase. NNI present with differences when comparing the mechanism of action of NI, which exert their inhibitory effect by binding the active site of HCV. Multiple NS5B HCV inhibitors are under clinical investigation, and extensive data exist for numerous compounds. To date, many other NS5B polymerase inhibitors are under early stages of clinical investigation and present with promising results thus far including GS-9190, ABT-333 and PF-00868554. GS-9190 is currently the most advanced NS5B polymerase NNI (Phase 2) and reported encouraging antiviral data when administered in a combination therapy with peg-IFN-α and ribavirin. ABT-333 has demonstrated a promising *in vitro* antiviral profile, with enzyme inhibition IC₅₀ levels of 2.2 nM against HCV genotypes 1 and 2 and EC₅₀ values of 0.5–0.8 nM in the context of the replicon system against HCV genotypes 1a and 1b (EASL 2009).

PF-00868554 is a novel NNI of HCV NS5B with potent and selective inhibition of HCV polymerase, which demonstrates an encouraging pharmacokinetic profile in rats and monkeys [50]. *In vitro*, PF-00868554 displayed potent antiviral activity against genotypes 1a and 1b (EC₅₀ of 0.06 μM) without apparent toxicity. M423T is the primary mutation associated with PF-00868554 resistance, but no cross-resistance to other polymerase

inhibitors was identified in replicons containing this mutation [51]. Similar levels of potency have been observed with NNI Phase 1 antiviral ANA598 (Anadys Pharmaceuticals, San Diego, CA, USA). Four days of monotherapy resulted in a 2- \log_{10} decrease in plasma HCV RNA levels. In quadruple combination with IFN- α , telaprevir [protease inhibitors (PI)], and PSI-6130 (NI), ANA598 demonstrated a favourable pharmacokinetic and tolerability profile *in vivo* [52]. IDX375 (Idenix Pharmaceuticals) is a novel preclinical HCV NNI candidate targeting the palm pocket of NS5B polymerase. IDX375 demonstrated strong inhibition of HCV replication ($EC_{50} = 2.3$ nM) in the subgenomic replicon system, with no *in vitro* cytotoxicity in rat, mouse, monkey and human hepatocytes, and no apparent *in vivo* adverse events in monkeys [53,54].

INHIBITORS OF NS5A

The function of HCV NS5A is not fully defined. Despite the current gaps in knowledge, NS5A has been implicated as a modulator of critical viral functions including facilitation of viral RNA replication, virus assembly and regulator of the antiviral interferon response. Together, these roles, combined with traditional chemistry-based drug discovery, represent a unique platform for NS5A inhibition.

Two potent NS5A specifically targeted antiviral therapy (STAT)-C compounds have been evaluated in clinical trials, including compounds **A-832** (Phase 1) (AstraZeneca, Boston, MA, USA) and **BMS-790052** (Phase 2) (Bristol-Myers Squibb, Wallingford, CT, USA). The latter has shown strong activity against several genotypes [1–5] in both replicon and JFH-1 systems. *In vivo*, BMS-790052 demonstrated a rapid and robust HCV RNA decline ($\sim 3.6 \log_{10}$), with no signs of adverse effects [55]. These studies with novel agents provide promising data that may allow further advancement through clinical investigation, while simultaneously providing a foundation for design of second-generation NS5A polymerase inhibitors.

PROTEASE INHIBITORS

The HCV NS3/NS4a serine protease is responsible for poly-protein processing that is essential for the production of infectious virions [26,56] and therefore provides an attractive target for inhibition of viral replication. To date, multiple HCV PI are in various stages of clinical development, including telaprevir/VX-950 (Phase 3, Vertex, San Diego, CA, USA), VX-500 (Phase 1, Vertex), VX-813 (Phase 1, Vertex), boceprevir/SCH503034 (Phase 3, Schering-Plough, Whitehouse Station, NJ, USA), ITMN-191/R7227 (Phase 1b, Intermune/Roche, Brisbane, CA, USA), TMC435 (Phase 2, Medivir/Tibotec, Yardley, PA, USA), MK-7009 (Phase 2, Merck, Whitehouse Station, NJ, USA) and PHX1766 (Phase 1, Phenomix, San Diego, CA, USA).

Of the PI under clinical investigation, MK-7009 demonstrates noncovalent competitive inhibition of HCV NS3/4A and has recently advanced to phase 2 clinical trials. Telaprevir and boceprevir are currently in Phase 3. Phase 2a studies reveal that MK-7009 demonstrated a rapid virological response (RVR) of 71–83% against HCV genotype 1 when co-administered with peg-IFN- α [57]. Telaprevir is a reversible peptidomimetic inhibitor of HCV NS3/NS4a protease and produced synergistic potency in triple combination with

ribavirin and IFN- α , with viral breakthrough reported for some patients in telaprevir monotherapy cohorts [58]. Subjects receiving telaprevir/IFN- α combination therapy were less likely to experience viral suppression, coupled with an increased risk of relapse vs persons receiving telaprevir/IFN- α /ribavirin triple therapy [59–61]. Boceprevir is a peptidomimetic HCV NS3 PI that reduces viral loads when administered as a monotherapy, and triple combination with IFN- α and ribavirin confers a further reduction in viral loads [62]. These data suggest that telaprevir and boceprevir may provide a tandem approach for triple combination with IFN- α and ribavirin, although their use as a monotherapy is unlikely to result in any long-term clinical benefits because of the low genetic barrier to resistance of PIs in general.

Recent efforts have employed the construction of derivatives or analogues of existing compounds to further refine antiviral activity [63–65]. Pompei *et al.* and Avolio *et al.* [11,64] recently synthesized a series of analogues derived from P2–P4 macrocycle inhibitors of the NS3/NS4a HCV protease, wherein the carboxylic acid residue is replaced with phosphorus acid functionalities. These analogues demonstrated potency against HCV within the context of the HCV genotype 1b replicon system in Huh-7 cells, with multiple analogues conferring EC₅₀ values in the low nanomolar range, and CC₅₀ values ranging from 50 to 100 μ M. Unfortunately, the lead compound demonstrated poor protein kinase (PK) values in rat studies, necessitating further study to define compounds with more favourable PK parameters.

Barros *et al.* [63] described the synthesis of a series of pseudo-peptides derived from isomannide 28–48 which resulted in one compound that demonstrated anti-HCV genotype 1b antiviral activity of 20 μ M in the context of the replicon system. Unfortunately, these were also associated with a toxic profile, necessitating further investigation to refine this compound.

Other studies have utilized alternative medicine as a basis for the discovery of novel anti-HCV compounds. Phuong *et al.* explored the antiviral activity of specific components of the maleic and succinic acid antrotoxins of the *Antrodia cinnamomea* fungus. *Antrodia cinnamomea* is an alternative medicine that has been administered for treatment of HCV-induced hepatocellular carcinomas, and specific components of this fungus displayed functionalities that were explored for anti-HCV activity. Phuong *et al.* [66] report an IC₅₀ of 0.9 μ g/mL in a cell-free assay, presenting encouraging preliminary data from which to design more advanced study.

HCV NS3/NS4a protease presents a unique target for treatment of HCV infection. PIs in advanced stages of clinical development coupled with fundamental studies elucidating novel compounds and exploration of distinct mechanism of HCV protease inhibition provides an optimistic outlook for current and future therapies targeting NS3/NS4a protease.

CYCLOPHILIN INHIBITORS

Administration of the immunosuppressive agent cyclosporine A (CsA) has been associated with a reduction in HCV viral loads, and the mechanism has been localized to specific inhibition of cyclophilins [67,68]. CsA is administered as an immunosuppressive agent,

most notably for control of organ rejection or advanced autoimmune disease, and its mechanism is defined by the CsA/cyclophilin complex, which inhibits calcineurin, conferring a dramatic reduction in IL-2-mediated adaptive immunity [69]. Immunosuppressive agent tacrolimus, which inhibits calcineurin by a cyclophilin-independent mechanism, does not exert anti-HCV activity [70], implicating the cyclophilins as primary mediators of the observed CsA anti-HCV activity.

Debio 025 inhibits cyclophilins, but does not confer the inhibition of calcineurin activity: it results in the inhibition of HCV without immunosuppression [71–73]. Debio 025 is under phase 2 clinical investigation and has demonstrated a sustained 4.6-log reduction in viral loads when administered in combination with IFN- α [71]. Debio 025 is the first non-immunosuppressive, cell-specific inhibitor that confers substantial reductions in viral loads. It provides a promising foundation for further design of cell-based inhibition of HCV.

NOVEL IMMUNE-BASED INHIBITORS

Manipulating the immune response to enhance adaptive immunity against HCV-specific epitopes, with concomitant alteration of the cytokine milieu, affords multiple opportunities for targeted drug design. Together, this approach could confer antigen-specific clonal expansion and antibody-specific responses, allowing for rapid identification and elimination of both infected cells and circulating free virus. Many classes of immunomodulators are under various stages of clinical investigation, including polyclonal antibodies, interleukin therapy, broad-spectrum anti-inflammatory agents and immune enhancers [71–75].

Resiquimod (S-28463, R-848, VML600; Graceway Pharmaceuticals, Exton, PA, USA) explores a unique means of amplifying the anti-HCV immune response and targets toll-like receptors 7 and 8 (TLR7, TLR8) [75]. Signalling through TLRs is often conferred as a nonspecific mechanism of activation by foreign pathogens, orchestrating induction of IFN- α , IL-12 and TNF- α [76]. Together, these cytokines can function in an autocrine fashion to stimulate the release of cytokines, while concomitantly activating antigen presenting cells including macrophages and dendritic cells, resulting in an improved ability to mount an adaptive immune response (Figure 2B). Levels of IFN- α , IL-12 and TNF- α are not elevated under normal physiological conditions; therefore, therapies designed to manipulate the immune response indirectly by increasing levels of circulating cytokines can result in nausea, fever, malaise, headache, shivering and lymphopenia [14].

Understanding the interplay between HCV and the immune response, and subsequent targeted alteration of these tightly regulated systems represents a delicate balance. Previous hypotheses suggested that HCV may escape the antiviral properties of IFN- α by inhibition of IFN- α -mediated activation of the Janus activated kinase and signal transducer [77] and activators of transcription (JAK-STAT) pathway [78–80]. The JAK-STAT pathway is a critical transducer of IFN- α -mediated signalling and serves as a global director of the IFN- α -driven innate immune response. Recent reports have built on this previously established hypothesis [77,79,80] and defined specific HCV proteins as direct inhibitors of IFN- α -mediated JAK-STAT activation. Heim *et al.* [79] reported HCV proteins derived from the entire open reading frame of HCV genotype 1a as inhibitors of IFN- α -induced activation of

the JAK-STAT pathway. These data are complemented by reports by Luquin *et al.* [80], which establish that structural proteins expressed in the context of a genomic HCV replicon inhibited IFN- α -mediated STAT phosphorylation, and that the IFN- α -directed antiviral effect was significantly lower in these cells, suggesting that HCV structural proteins may play a sentinel role in viral escape from IFN- α antiviral-mediated antiviral activity. Complementary to these data, Melen *et al.* demonstrated that HCV core proteins inhibit IFN- α -induced nuclear import of phosphorylated activated STATs [77]. As activated, phosphorylated STATs bind to specific transcription sites and promote transcription and translation of multiple factors involved in innate and subsequent adaptive anti-HCV activity, these data provide an upstream mechanism that, in part, explains how inhibition of functional immunity may be achieved. Together, these findings demonstrate the complex interplay between the immune response and HCV infection and present multiple targets from which to develop immunomodulator-based therapies designed to reduce or eliminate HCV infection.

THIAZOLIDINE SMALL MOLECULE MODULATORS

Targeting host factors independent of the HCV replication cycle represents a unique opportunity to design small molecule inhibitors with a high threshold for resistance, as selective pressure for emergence of resistant virus is significantly attenuated when targeting a host factor *vs* the rapidly replicating HCV [10,12,81–84].

Thiazolidines represent a class of inhibitors that specifically target a pathway involved in modulation of the host cellular antiviral response [74,85]. Nitazoxanide (Romark Laboratories, Morristown, NJ, USA) is a thiazolidine that exploits the host's protein kinase-mediated antiviral pathway. Activation of the protein kinase R (PKR) results in phosphorylation and subsequent activation of its endogenous substrate, eukaryotic initiation factor α (eIF2- α). eIF2- α in turn modulates a complex network of signalling cascades that orchestrates the host antiviral response to HCV [74,85]. Nitazoxanide confers activation of PKR, resulting in a robust antiviral response. In phase 2 clinical studies examining HCV genotype 4, nitazoxanide demonstrated a SVR of 80% when administered in combination with peg-IFN- α , *vs* 50% for peg-IFN- α alone [74], underscoring the potential value of host cell-directed inhibitors of HCV. Advanced studies are underway to determine the long-term implications of nitazoxanide, and, together with current Phase 2 findings, facilitate a pronounced understanding between the relationship of host cell pathways and reduction or elimination of virus.

GLUCOSIDASE INHIBITORS

α -1 glucosidase I inhibitors possess a potential for broad range antiviral application, and their mechanism of action is correlated with inhibition of proper folding of envelope proteins found on the surface of many viruses [86,87]; however, because they can also target cellular functions, specificity can be an issue. Celgosivir (Imigenix Pharmaceuticals) is under Phase 2a clinical investigation and has demonstrated potency against both HCV and HBV (Reviewed in [87]). Inhibition of proper protein folding by interference with glucosidase provides a unique mechanism to inhibit the production of infectious virions, and

further studies elucidating novel mechanisms to interfere with protein folding and envelope structure provide an attractive area of research.

OTHER DRUG DISCOVER INITIATIVES

Defining the structure–activity relationship (SAR) for compounds with their biological targets can elucidate valuable chemical relationships that can be utilized to design novel therapeutics. Understanding the specific residues responsible for the potent effects of compounds and subsequent modification of existing lead compounds has become an expanding area of research [88–94]. SAR can also be employed to define residues that may become the site of specific resistance mutations, while concomitantly predicting the potency of novel compounds against resistant HCV [91]. Together, these capabilities provide valuable information when determining mechanisms responsible for emergence of resistant mutations.

Design of novel HCV PI could significantly reduce viral loads and provides an attractive, virus-specific target. Velasquez *et al.*, recently employed SAR to identify and refine L-serine- and allo-threonine-derived macrocyclic compounds with EC₉₀ values of 30 nM as potent inhibitors of HCV NS3 protease [94]. Chen *et al.* took a similar approach and defined sultam and cyclic sulfonyl urea P3-capping groups as critical modulators of increased potency for NS3 PI. Using a step-wise system, bicyclic and thiophene-sultam or phenylsultam cappings were subjected to further SAR analysis, allowing for optimization of specific residues to confer increased potency and selectivity. Together, this approach facilitated the discovery of a compound with a K_i profile of 5.3 nM and EC₉₀ of 80 nM in the context of the replicon system [90]. Arassapan *et al.* sought to define a second-generation HCV NS3 PI and reported that specific hydrogen bonds with Cys-159 and additional hydrophobic interactions with the S4 enzymatic pocket as critical modulators of increased potency. Together, these data resulted in EC₉₀ values of 70 nM in the replicon system and favourable oral absorption in rats [88].

Bosse *et al.* modified the carbon at the 1-position of the B-ring of novel gem-dialkyl naphthalenones, resulting in potent inhibition of HCV polymerase. Further SAR defined an optimized compound with 75-fold greater potency than the original parent compound, underscoring the importance of SAR in design of compounds with increased antiviral activity [89].

Manfroni *et al.* evaluated the SAR between HCV helicase and potential inhibitors and reported acridone-based inhibitors as the most potent inhibitors of bovine viral diarrhoea virus (BVDV), a virus often employed *in vitro* as a surrogate system for assessment of anti-HCV activity of test compounds [93]. These data provide a platform for further design of helicase inhibitors and emphasize the role of SAR as a tool for identification of potent compounds.

SAR provides an important tool in design and assessment of the potency of novel compounds, while simultaneously predicting the responsiveness of resistant HCV to potential inhibitors and defining critical residues that confer anti-HCV activity. Together

with *in vitro* and *in vivo* analyses, SAR represents a unique tool that can elucidate novel drug targets and define novel anti-HCV compounds.

MICRO-RNAs AND MICROARRAYS AS THERAPEUTIC TARGETS OR PREDICTIVE BIOMARKERS

Micro-RNAs (miRNAs) represent a class of small, noncoding RNAs that possess a limited ability to recognize viral gene transcripts and subsequently alter viral gene expression [95]. Recent reports have defined miRNA, mR-122, as a positive modulator of HCV replication and report high mR-122 levels exclusively in hepatic cells [96]. High levels of mR-122 are associated with a favourable response to IFN- α and ribavirin [96], implicating a role for predictive screening of mR-122 levels prior to administration of therapy. Use of predictive, microarray-based screens for evaluation of therapy response or progression towards HCV-associated hepatocellular carcinomas could provide a unique mechanism to design customized therapy regimens for the individual. Recent data have begun to explore this field and attempt to define a role for microarray analysis as a potential tool for analysis of genes associated with HCV-mediated liver disease.

DRUG DISCOVERY INITIATIVES: ADVANCES WITH THE JFH-1 SYSTEM

One decade after the discovery of HCV [97], the development of selectable subgenomic HCV RNAs ('replicons') and genome-length RNAs capable of supporting HCV propagation provided for the first time, highly valuable tools for studying HCV replication mechanisms, virus-host cell interactions, and developing novel antiviral agents *in vitro*, especially compounds that may inhibit viral entry.

These model systems have been used for a number of HCV isolates. A genotype 2a full-length genome (JFH-1) isolated from a patient with fulminated hepatitis C [98–100] gave efficient replication in Huh7 cells without adaptive mutations. In addition, chimeric replicons of JFH-1 replicase and its nontranslated regions produce high levels of infectious virions *in vitro* [101]. The new HCV infection systems allow for the exploration of multiple phases of the viral replication cycle including entry, assembly and release. These allow the identification of antiviral agents targeting novel steps in the viral replication cycle that could not be addressed within the traditional replicon system.

CYTOTOXICITY ASSAYS FOR PREDICTION IN HUMANS

Before an antiviral agent becomes a drug, advanced toxicity testing and pharmacological, antiviral combination and drug-interaction studies are needed. The use of novel cell-based assays that can predict mitochondrial toxicity, development of lactic acidosis, peripheral neuropathy, anaemia, hypersensitivity, lipodystrophy and other potential side effects can minimize these hurdles [102]. Mitochondria-associated toxicities, such as pancreatitis are frequently demonstrated in HIV/HCV co-infected individuals and may significantly influence treatment options [103]. Animal models have now been developed to predict nucleoside-induced pancreatitis. Recently, an association of HCV replication with mitochondrial DNA depletion in human lymphocytes of HIV/HCV co-infected individuals

under concomitant administration of HCV and HIV medications was demonstrated by De Mendoza *et al.*, 2007 [104]. The authors claim that the use of HCV medication together with certain antiretroviral agents seems to enhance mitochondrial damage because of a synergistic deleterious interaction between the HCV and HIV drugs. In contrast, an improvement in mitochondrial DNA content with effective anti-HCV therapy was confirmed by this group [104].

This rigorous process has led to the discovery of numerous anti-HCV hits and leads. Three STAT-C compounds that were in Phase 1 or 2 clinical trials, including NM283 (Idenix), BILN2061 (Boehringer Ingelheim, Ridgefield, CT, USA) and R1626 (Roche) are no longer in development because of serious toxicity issues [105]. More biologically stable nucleosides with improved pharmacological profiles are being evaluated, including the two potent and selective nucleoside analogues, which are in clinical trials (R-7128 and PSI-7851).

Current data have defined the necessity for *in vivo*-based toxicology studies designed to assess the systemic and long-term impact of novel antiviral agents. Although most studies to this end are not fully defined, *in vitro* studies, in combination with current reports of detrimental side effects among drugs under Phase 1 to 3 clinical investigation, provide potential areas of interest for anti-HCV-associated toxicity.

DRUG–DRUG INTERACTION AND GUIDELINES FOR SUSTAINED VIROLOGICAL RESPONSE

Similar to HIV-1, HCV replicates very rapidly, with approximately 10^{12} virions produced per day *in vivo* and presents with high levels of heterogeneity, largely as a function of the lack of proofreading ability of the RdRp (Reviewed in [7,26]).

Because of the high genetic variability and high efficiency of replication of HCV, resistance mutations rapidly arise, especially for PIs and non-nucleoside small molecules [10,81,106]. These obstacles, together with the inability of standard IFN- α and ribavirin to eliminate emergence of resistant virus, underscore the importance of launching drug combination therapies of STAT-C compounds.

HCV RNA rebound in clinical trials is most likely associated with the development of resistance to STAT-C compounds including protease and polymerase inhibitors. Thus, combination of anti-HCV targets with nonoverlapping resistance including protease plus nucleoside and non-nucleoside polymerase inhibitors should be strongly encouraged to achieve a RVR and long-term HCV clearance (\geq 6 months after end-of-treatment or SVR).

NOVEL HCV INHIBITORS: RECENT PROGRESS AND FUTURE DESIGN

The promise of novel and potent inhibitors of HCV without detrimental side effects is beginning to become a reality. Shorter therapy is possible for many infected persons and is associated with SVR. Ribavirin and IFN- α , although often poorly tolerated, remain the cornerstone for the treatment of HCV infection [1,2]. Novel nucleoside analogues under clinical investigation provide hope for significant reduction in viral loads and demonstrate

results that mirror the marked reduction in systemic virus reported for protease inhibitor treatment of HIV-1 infected individuals [105,107,108]. In addition, this class of drug appears to be genotype independent making them highly valuable for combination modalities. Tandem targeting of host mechanisms that mediate viral entry and replication, coupled with targeted modulation of specific facets of the adaptive immune response, provides a dual approach that could improve the rate of HCV eradication (Reviewed in [109]).

Although recent progress provides optimism for success, the manifestation of copious side effects associated with prolonged treatment present a formidable obstacle that must be underscored. Pharmacology-based drug design faces a delicate balance between inhibition of viral replication and simultaneous circumvention of cross inhibition of essential host functions. Immunomodulators present with similar obstacles as interference with the adaptive or innate immune response through targeted inhibition of essential pathways and cytokine milieus can alter sentinel host dynamics, potentially resulting in toxicity or clonal anergy [110]. A concern of equal importance is the emergence of resistant mutations, conferring cross-resistance to multiple therapies and significantly diminishing treatment options. This obstacle emphasizes the importance of elucidating multiple mechanisms to inhibit HCV and conferring several methods for concomitant inhibition of viral replication.

These hurdles are greatest in subjects classified as nonresponders and must be addressed in full when designing studies. Ultimate design of multifaceted, multi-mechanism treatment options may provide alleviation for chronic nonresponders, HIV-1/HCV co-infected persons and subjects experiencing relapses, while providing a foundation for elimination of virus across genotypes for all infected individuals.

CONCLUSIONS

New data defining sentinel factors in the HCV replication cycle together with an expanded knowledge of immunological and host factors that impact SVR continue to provide the foundation for design of novel therapies designed to eradicate all genotypes of HCV. Significant progress has been made in the field of STAT-C drug development. Nevertheless, high antiviral potency is not enough for novel anti-HCV targets. The ultimate drugs must present with low, if any, toxicity, excellent pharmacokinetic profile, including high oral bio-availability and extended elimination half-life and most importantly, act synergistically, when co-administrated with other approved drugs to minimize the emergence of drug resistance. To eradicate HCV, the ultimate goal is to exploit a collection of novel drugs targeting different components of the HCV replication cycle and host factors for evaluation in clinical trials without interferon and ribavirin. Achieving these goals represents a significant challenge; however, success is not beyond the reach of the scientific community. The words of the dean of science fiction writer Robert Anson Heinlein resonates with astounding clarity: 'Everything is theoretically impossible, until done'.

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Abbreviations

BVDV	bovine viral diarrhoea virus
CsA	cyclosporine A
eIF2-α	eukaryotic initiation factor α
HCV	Hepatitis C virus
IRES	internal ribosome entry site
JAK-STAT	Janus activated kinase and signal transducer and activators of transcription
NI	nucleosides inhibitors
NNI	non-nucleosides inhibitors
peg-IFN-α	pegylated interferon- α
PI	protease inhibitors
PK	protein kinase
RdRp	RNA-dependent RNA polymerase
RVR	rapid virological response
SAR	structure–activity relationship
SCARB1	scavenger receptor class B Type 1
STAT	specifically targeted antiviral therapy
SVR	sustained virological response
TP	triphosphate

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	Preclinical	Phase 1	Phase 2	Phase 3
3' UTR				
NS5B	<i>Polymerase inhibitors</i>			
	INX-189 PSI-879 PSI-938	ABT-072 ABT-333 ANA-598 IDX-184	PF-00868554 PSI-7851 R-7128 VCH-916	Filibuvir (FLV) GS-9190 VCH-759
NS5A			<i>NS5A inhibitors</i> A-832 BMS-790052	
NS4B				
NS4A	<i>Helicase inhibitors</i>		<i>Protease inhibitors</i>	
NS3	BTN10 BTN11 Triksalen	ABT-450 ACH-1625 BI-201335 ITMN-191 (R7227) PHX-1766 VX-500 VX-813	MK-7009 SCH-900518 TMC-435350	Bocepravir Telaprevir
NS2				
P7		<i>P7 inhibitors</i> BIT225		
E2	<i>Entry inhibitors</i>		<i>Entry inhibitors</i>	
E1	PRO-206 REP-9C SP-30		ITX-5061	
Core				
5' UTR	<i>IRES inhibitors</i> DNAzymes HH-363-50			

Fig. 1. Anti-HCV therapies under clinical investigation arranged by phase of clinical investigation.

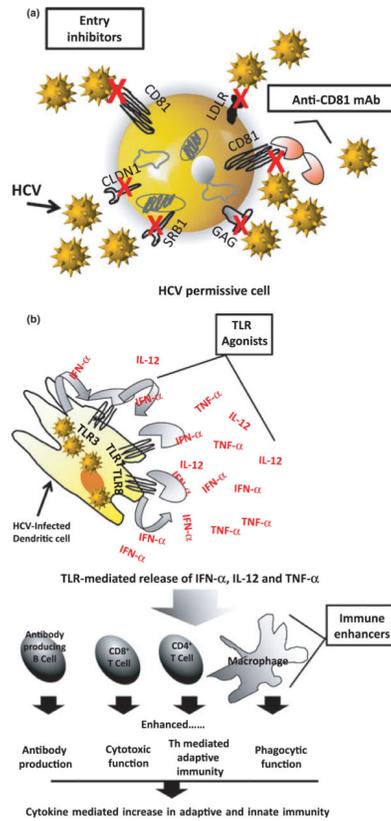


Fig. 2.

(a) Mechanism of action for HCV entry inhibitors. Potential sites for targeted inhibition include receptors implicated in HCV entry including CD81, Claudin 1 [CLDN1], Glycosaminoglycan [GAG], Scavenger receptor type B1 [SRB1], and Low density lipoprotein receptor [LDLR]. (b) Mechanism of immunomodulators for treatment of HCV infection. Toll-like receptor [TLR] mediated production of proinflammatory cytokines TNF- α , IL-12 and IFN- α results in enhanced adaptive and innate immunity hallmarked by increased antibody production, cytotoxic function and increase phagocytic activity (1, 2, 11–12, 1522, 77, 79, 107).