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Helicase inhibitors as specifically targeted antiviral therapy for hepatitis C

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Abstract

The hepatitis C virus (HCV) leads to chronic liver disease and affects more than 2% of the world's population. Complications of the disease include fibrosis, cirrhosis and hepatocellular carcinoma. Current therapy for chronic HCV infection, a combination of ribavirin and pegylated IFN- α , is expensive, causes profound side effects and is only moderately effective against several common HCV strains. Specifically targeted antiviral therapy for hepatitis C (STAT-C) will probably supplement or replace present therapies. Leading compounds for STAT-C target the HCV nonstructural (NS)5B polymerase and NS3 protease, however, owing to the constant threat of viral resistance, other targets must be continually developed. One such underdeveloped target is the helicase domain of the HCV NS3 protein. The HCV helicase uses energy derived from ATP hydrolysis to separate base-paired RNA or DNA. This article discusses unique features of the HCV helicase, recently discovered compounds that inhibit HCV helicase catalyzed reactions and HCV cellular replication, and new methods to monitor helicase action in a high-throughput format.

Keywords

ATPase; DNA; HCV; helicase; motor protein; protease; RNA; STAT-C

Approximately 2.2% of the population worldwide is infected with the hepatitis C virus (HCV), or one in every 45 persons alive today [1,2]. Although country-specific infection rates vary widely, HCV infection is astonishingly high in certain populations. For example, an estimated 24% of all Egyptians are HCV infected [3]. The clinical course of chronic HCV infection varies widely; 15–45% of patients clear HCV spontaneously, and 55–85% progress to a chronic viral infection. Typically, less than 15% of all those infected by HCV experience acute symptoms, such as fever or jaundice [4], and as a result, most patients infected by HCV are unaware of any abnormality until they develop fibrosis, cirrhosis or hepatocellular carcinoma years later [5]. As a result, chronic HCV patients receive the majority of all liver transplants.

HCV genome, proteins & experimental techniques

One reason that chronic HCV infection is extraordinarily difficult to study and treat is because the virus evolves very rapidly, with new variants continually being discovered [6]. Together, all HCV variants comprise the genus *Hepacivirus*, which is a member of the family *Flaviviridae*. The closest HCV relatives are members of the genera *Flavivirus* (e.g., Dengue virus, West Nile virus and Yellow fever viruses) and *Pestivirus* (e.g., bovine virus diarrhoea). Major HCV variants are classified into numbered genotypes, which are subdivided into dozens of different distinct lettered subtypes [7]. Even in individual patients, HCV evolves rapidly into genetically distinct variants called ‘quasispecies’. The RNA sequences of HCV variants differ by as much as 35%, complicating HCV treatment and hindering development of new vaccines or antiviral therapies.

The single-stranded RNA HCV genome contains one main open-reading frame that encodes an approximately 3000 amino acid-long protein. Host and viral proteases cleave this polyprotein into mature structural (core, E1 and E2) and nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The NS proteins are responsible for replication and packaging of the viral genome into capsids formed of the structural proteins. Several of the NS proteins have enzymatic activities. NS2/NS3 is an autocatalytic protease, the NS3 protein is an ATPase and helicase, the NS3/NS4A complex is a serine protease and NS5B is an RNA-dependent RNA polymerase that synthesizes new viral RNA. Each of these enzymes and proteins could be considered a target for HCV therapies and, consequently, they all have been studied in great detail both on the molecular and atomic levels [8]. However, translating enzyme inhibitors or other lead compounds into antiviral agents was hindered owing to the slow development of systems for studying HCV virology on a cellular level.

Hepatitis C virus grows poorly in the laboratory setting and the only nonhuman animal that can be infected by HCV is the chimpanzee. While reproducing the various clinical, serological and pathological changes observed in HCV, the use of a large primate model is difficult and expensive [9]. The first molecular clones of HCV were isolated from patient blood in 1989 [10], and the first clones infectious to chimpanzees were assembled in 1997 [11,12]; however, it was not until 1999 that it was possible to study HCV replication in cultured cells. These first HCV ‘replicon’ systems utilized recombinant clones that placed sequences encoding the HCV NS proteins downstream from a gene encoding a drug-resistant marker that could be used to select cells possessing autonomously replicating HCV RNA [13]. Adaptive viral mutations that allow HCV replicons to replicate more efficiently in hepatocytes were soon identified [14,15]. More permissive cell lines were later discovered [16] and replicons containing the full-length HCV genome were designed [17,18]. Numerous sequences encoding reporter proteins or fusion peptides have since been added to HCV replicons for more convenient analyses, quantification and direct visualization of HCV proteins in cells [19,20]. Thus, as had been predicted [21], replicons revolutionized HCV drug development and have even been used to identify new drug candidates in high-throughput screens [22,23].

Another important breakthrough in HCV research came after an HCV sequence was cloned from a Japanese patient with fulminant hepatitis (JFH) [24]. This JFH1 sequence can replicate as a subgenomic replicon even in the absence of adaptive mutations and it now forms the basis for recombinant HCV clones that replicate and even produce infectious virions in certain hepatoma cells. Such HCV clones are often called HCVcc to emphasize their unique ability to replicate in cell culture [25–28], yet why HCVcc can replicate while similar strains do not is not absolutely clear. Recent studies comparing JFH1 with a related genotype 2a strain suggest that both NS5B and NS3 are critical in this regard [29], and they may act in concert with NS5A in order to enhance RNA replication in JFH1 systems [30]. It might also be worth noting that JFH1 was isolated in a patient who developed a severe acute disease and, subsequently, cleared

the virus, in contrast to the majority of HCV patients who proceed directly to chronic hepatitis without a fulminant event [24].

HCV therapy

Unlike many other antiviral therapies, many patients treated with current HCV therapy frequently achieve a sustained virologic response (SVR), in which viral loads decrease to undetectable levels and do not rebound after treatment is ended. All currently approved therapies for HCV infection combine the broad-spectrum antiviral ribavirin with pegylated IFN- α 2a (PEGASYS®, Roche, Basel, Switzerland) or 2b (Pegintron™, Schering-Plough, NJ, USA). The mechanisms of actions of pegylated interferon, in which polyethylene glycol is attached to recombinant human interferon, and the nucleoside analog ribavirin have been widely studied and reviewed elsewhere [31,32]. Treatment success varies widely but correlates best with the genotype of the infecting HCV. When the pegylated interferons were first introduced, the frequency of patients achieving a SVR was as high as 76% in patients with HCV genotypes 2 or 3, but as low as 36% in patients with genotype 1, an unfortunately common genotype in North America and Europe [33]. SVR has improved as therapies have been optimized, however, genotype 1 still remains as the one most refractory to current HCV treatments.

Side effects are common with all current HCV therapies, as interferon and ribavirin both induce a systemic antiviral response. The cytokine interferon induces fevers, chills and other ‘flu-like symptoms’ upon injection, and ribavirin causes hemolytic anemia, severe depression leading to suicidal tendencies and may even cause birth defects. As a consequence, approximately 35% of patients withdraw from therapy, even in clinical trials [33]. Many HCV genotypes remain difficult to treat. Most patients with HCV genotype 1 and approximately a third of patients infected with other genotypes who complete the grueling 24- or 48-week therapy regimen relapse or do not respond owing to resistance, which might be linked to a variety of host and viral factors [34]. Current HCV therapies are also expensive, costing US \$30,000–40,000 per person [35], thus putting these regimens out of reach for many patients, even in wealthy nations. When cost, fear of potential side effects and all other contraindications are considered, one study found that 72% of US patients that otherwise might respond to therapy are left untreated [36]. Thus, even though current HCV therapies provide *de facto* ‘cures’, they are making only a small dent in the number of HCV patients who might eventually develop debilitating liver disease. Therefore, new HCV therapies will need to be less expensive, work more quickly, exhibit lower toxicity and eliminate a wider variety of HCV genotypes than current therapies.

Specifically targeted antiviral therapy for HCV

The next generation of HCV drugs has been dubbed specifically targeted antiviral therapy for hepatitis C (STAT-C), an acronym describing compounds designed to directly inhibit HCV or host proteins involved in HCV replication [37,38]. STAT-C attempts to follow the precedent set by highly active antiretroviral therapy (HAART) used to treat HIV, which simultaneously attacks multiple targets using a ‘cocktail’ of drugs. Probably not coincidentally, the three most advanced classes of STAT-C compounds target HCV enzymes that are analogous to those targeted in HAART: protease inhibitors, nucleoside polymerase inhibitors and non-nucleoside polymerase inhibitors. Successful STAT-C will probably require two or more of these compounds to be combined because, as briefly discussed below, mutations conferring viral resistance to most agents in trials have already been discovered using replicons.

The first STAT-C agent that was clinically effective was Boehringer Ingelheim’s HCV protease inhibitor, ciluprevir (BILN2061), which could rapidly reduce HCV RNA in patients [39]. Trials with BILN2061 have since been halted owing to side effects, however, several other NS3

protease inhibitors, such as Vertex Pharmaceutical's (MA, USA) telaprevir (VX-950) [40] and Schering-Plough's boceprevir (SCH503034) [41], appear to be better tolerated. Emergence of viral resistance to protease inhibitors has already been documented with replicons, with HCV replicons resistant to BILN2061 [42], VX-950 [43] and SCH503034 [44] having been isolated. Nucleoside analog inhibitors of the NS5B polymerase include 2'-C-methyl-3'-O-L-valine cytidine (Idenix's [MA, USA] valopicitabine, NM283) [45], 2'-deoxy-2'-fluorocytidine [46], 2'-deoxy-2'-fluoro-2'-C-methyl-cytidine [47] and 4'-azidocytidine [48], and are generally considered less susceptible to resistance mutations, although some have been detected. Non-nucleoside inhibitors of NS5B include diketoacids and benzo-thiadiazines [49], thiophene carboxylic acids [50], benzimidazoles and indoles [51]. Non-nucleoside inhibitors tend to be more susceptible to resistance mutations, and replicons resistant to benzimidazoles [52], thiophenes [53] and benzothiadiazines [53] have all been documented.

With resistance clearly a problem for STAT-C agents, more antiviral targets are needed. Compounds targeting other HCV regions, such as the NS3 helicase, could be combined with STAT-C protease and polymerase inhibitors, with the goal of eliminating any need for either ribavirin or interferon.

HCV NS3 helicase as a possible drug target

Over the past 15 years, many laboratories have studied the helicase portion of NS3 as a possible HCV drug target [54]. Multiple studies have demonstrated that NS3 is necessary for viral replication, both in whole animal and replicon models, validating NS3 helicase as a possible target. HCV RNA harboring mutations inactivating the NS3 helicase is unable to replicate in chimpanzees [55], and similar mutations in subgenomic replicons demonstrate that NS3 helicase activity is absolutely necessary for viral RNA replication [56,57]. Delivery of RNA aptamers [58], small peptides [59], antibodies [60] and small molecules [61] that specifically inhibit the HCV helicase have all been shown to inhibit HCV RNA synthesis in cells bearing HCV replicons.

Role of the NS3 helicase in HCV replication

There are numerous possible biological roles for HCV helicase, such as assisting translation, protein processing or packaging RNA into virions (Figure 1). Based on three lines of evidence, HCV helicase probably assists RNA-dependent RNA replication by tracking along RNA and resolving double-stranded intermediates, either as a result of secondary structures in a single-stranded RNA, or double-stranded RNA intermediates formed during replication. First, NS3 stimulates the ability of NS5B to synthesize long RNAs [62]. Second, the motor action of NS3 on RNA is clearly established [63,64]. Third, HCV replicons without a functional helicase are capable of synthesizing and processing polyproteins from the transfected RNA, but fail to synthesize additional RNA [56]. Two lines of evidence hint at a possible role for the helicase in packaging. First, experiments with related *Flaviviruses* have shown that the analogous NS3 helicase is involved in assembly [65]. Second, point mutations in the NS3 helicase region allow robust virus production of an otherwise defective chimeric (genotype 1a/JFH1) HCV construct [66].

Hepatitis C virus helicase has several peculiar properties that also might hint at a more complex role in viral replication. First, NS3 helicase is able to separate both double-stranded DNA, as well as duplex RNA. Most helicases typically unwind either DNA or RNA, but not both. The mechanism by which HCV helicase unwinds DNA has been studied in great detail, and its interaction with DNA is somewhat different than with RNA [67–69]. Although there is no DNA stage in HCV replication and replication occurs outside the nucleus, HCV clearly affects host gene expression, and there have been reports that NS3 may actually enter the nucleus [70], where HCV helicase could encounter DNA. Thus, the biological importance of the NS3

helicase's ability to unwind DNA remains mysterious but intriguing. Another interesting property that may hold a clue to the biological role of the NS3 helicase is its peculiar pH optimum level at a relatively acidic pH of 6.5 [71]. As HCV replication progresses from the endoplasmic reticulum to the Golgi apparatus, the local cellular pH changes. The pH of the Golgi is typically approximately 6.4, close to the point where HCV helicase unwinds RNA most rapidly, again hinting at a possible role for the helicase in virus maturation or particle assembly [71].

HCV helicase: structure

The helicase portion of NS3 can be separated from the protease portion by truncating at a peptide linker, but the resulting protein (called NS3h) unwinds RNA somewhat less efficiently compared with the full-length NS3 or the NS3–NS4A complex [72,73]. Nevertheless, removing the protease to form NS3h allows one to express much higher levels of recombinant soluble protein in *Escherichia coli*. To date, numerous atomic structures of HCV helicase have been deposited in public databases. These structures can be used to compare the full-length NS3 protein [74] with NS3h [75] or NS3h from genotypes 1a [75] and 1b [76]. Two structures have been deposited with HCV helicase bound to a DNA oligonucleotide: one with genotype 1a helicase bound to a oligonucleotide [77] and another showing multiple genotype 1b NS3h molecules bound to the same oligonucleotide [57]. Although no structures have been deposited with bound duplex DNA, RNA, ATP, nucleotide analogs or helicase inhibitors, clues to where other ligands might bind can be found by aligning HCV helicase structures with those of similar proteins with bound ligands. For example, alignment of the HCV helicase structures with those of a related helicase that was recently crystallized with a DNA duplex [78] yields clues as to how the protein splits complementary nucleic acids (Figure 2A).

The mature NS3 protein has two main functional domains: the N-terminal portion forms a serine protease and the C-terminal portion forms the helicase. The protease and helicase portions can be further subdivided into distinct structural domains: the N-terminal serine protease is composed of two structural domains bound to the NS4A cofactor (i.e., NS3/NS4A protease), and the C-terminal helicase is composed of three structural domains (i.e., HCV NS3 helicase or NS3h) (Figure 2). The NS3/NS4A protease is a serine protease responsible for cleaving the single polyprotein produced during transcription at several sites (Figure 1A) [79]. As such, the NS3–NS4A protease is an intensively investigated drug target in its own right, exemplified by the clinical successes of the protease inhibitors mentioned previously. However, as alluded to above and discussed in more detail below, the protease helicase actions of each may be functionally linked, and there is new evidence that the helicase domains might even assist peptide cleavage [80].

Two HCV helicase structural domains share similar shapes with all other helicases and other motor proteins. Specifically, HCV helicase structural domains 1 and 2 both resemble the *E. coli* RecA protein [81]. In helicases that do not form rings around nucleic acids, such as HCV helicase, two RecA-like domains are repeated in tandem on the same polypeptide and form a molecular motor, allowing the protein to move along nucleic acids. The most striking difference among the various published HCV helicase structures is the relative rotation of the C-terminal RecA-like domain (domain 2) relative to helicase domains 1 and 3. Most molecular models explaining how the HCV helicase functions contend that ATP binding and/or its hydrolysis regulates domain 2 movement and that this change, in turn, leads to movement of the protein along RNA [54]. Conformational changes in domain 2 upon DNA binding have been also confirmed using nuclear magnetic resonance [82].

Key HCV helicase motifs & clefts

The HCV NS3 protein is multifunctional and possesses several distinct molecular motifs and clefts, which could be considered independent targets for pharmacologic modulation. The most obvious targets on HCV helicase are the ATP (Figure 1B) and RNA-binding sites (Figure 1C), however, other unique features of the protein exist and may be exploitable as drug targets. Aligning the HCV NS3 sequence with sequences of related helicases and motor proteins has identified some important sites. Such alignments are used to classify all helicases into various families and superfamilies, whose members all share certain conserved signature sequences, known as helicase motifs. Specifically, HCV helicase is a superfamily (SF)2 helicase and shares six heli-case motifs with related proteins. The roles of the various SF2 helicase motif residues in HCV helicase-catalyzed reactions have been reviewed before in detail [54,83] and are discussed below in the context of the various sites on NS3 that might accommodate ligands or inhibitory compounds. For annotated primary sequences of HCV helicase and its various motifs see [54,84,85].

ATP-binding site

ATP most likely binds to HCV helicase in the cleft between the RecA-like motor domains (Figure 2B). Domain 1 contains one of the most common motifs found in nature, the phosphate-binding loop (P-loop) 'Walker-type' NTPase fold [86]. Proteins with Walker-type NTP-binding sites all bind ATP using a P-loop with a conserved lysine (K210 in NS3) in the Walker A-site (SF2 helicase motif I) and an acidic residue that interacts with a required divalent metal cation (D290 in NS3) in the Walker B-site (SF2 heli-case motif II). A conserved catalytic base near the P-loop (E291 in NS3) activates the water molecule needed for hydrolysis [87]. A basic residue on another domain or peptide, typically called an 'arginine finger', stabilizes the pentavalent transition state that occurs when an activated water molecule attacks the γ -phosphate of ATP.

Several arginines in SF2 helicase motif IV could act as an arginine finger, but a leading candidate is R467, which is also a target of a host arginine methyltransferase that regulates the enzyme [88]. The molecular details of the ATP-binding site of the related Dengue virus NS3 helicase have been recently visualized in exquisite detail [89]. Luo *et al.* reported atomic structures of the Dengue virus NS3 helicase co-crystallized with various ligands representing substrate, intermediates and products along the ATP hydrolysis reaction coordinate both in the presence and absence of a bound RNA molecule. Their work unambiguously demonstrated the relationships between ATP binding, RNA binding and various enzyme conformational changes [89]. Alignments of the new Dengue structures with those of HCV heli-case reveal how ATP might interact with the HCV enzyme (Figure 2B).

Known nucleic acid-binding site

The cleft separating the two RecA-like motor domains from the rest of the NS3 helicase (Figure 2C) is known to bind DNA oligonucleotides and, presumably, also accommodates one strand of RNA [57,77]. The most noteworthy residue in this cleft is Trp501, which stacks like a bookend between the nucleic acid bases and is critical for both the unwinding reaction and HCV RNA replication [56]. Another key residue is Glu493, which must be protonated for the helicase to be optimally active. Glu493 has an abnormally high pK_a values of approximately 7, meaning it becomes protonated when the pH falls to the enzyme's pH optimum [71,90]. Glu493 has an unusual electrostatic profile because it lies in the center of an acidic patch on the surface of the protein, which might provide the driving force for moving the helicase along RNA or DNA. If negatively charged RNA binds in this negatively charged cleft (Figure 2C), then potential energy would build up and its release owing to ATP binding or hydrolysis could propel the enzyme along the RNA fiber [84]. Binding of RNA near Glu493 conversely activates

helicase-catalyzed ATP hydrolysis [90]. DNA is held in this cleft by an arginine clamp centered around Arg393, which rotates upon ATP binding releasing the nucleic acid and permitting the protein to translocate [84]. Interestingly, alignment of various NS3 helicase sequences reveals that five of the six residues in the arginine-clamp motif are invariant amongst HCV and related helicases, however, they are completely absent in other SF2 helicases, including 21 human DEAD-box proteins [84].

Phe-loop

The primary structural difference between the two RecA-like motor domains in HCV helicase is that the C-terminal domain (domain 2) contains an extended β -loop that connects domain 2 with domain 3. This motif has been designated the 'Phe-loop' (Figure 2B) because it contains two phenylalanines (Phe438 and Phe444) at its tip, which are conserved in all HCV strains and are required for helicase activity [84]. Phe438 and Phe444 pack into a cluster of other aromatic residues on domain 3 and provide a pivot point for the rotation of domain 2 [75]. Similarly positioned but notably shorter, β -loops are present in related helicases and play a critical role in strand separation by disrupting base pairs [91]. Alignments of HCV helicase with structures of similar helicases crystallized with duplex substrates [78] reveal how the Phe-loop might separate DNA or RNA (Figure 2D).

Helicase–protease interface: an additional RNA-binding site?

The main feature that distinguishes HCV helicase from cellular SF2 helicases is the presence of an attached serine protease. Until recently, it was believed that the NS3 and protease activities functioned independently because they could be expressed alone as NS3 fragments, and as fragments they still maintained their respective activities. However, since full-length NS3 unwinds RNA better than a truncated NS3 protein lacking the protease [72], the protease region could play a more direct role in modulating the enzyme's unwinding activity. If RNA directly interacts with the protease region, one potential location could be the cleft separating the protease from the helicase, which is positively charged and could accommodate RNA (Figure 2e) [54]. Recent experiments comparing RNA unwinding by full-length and truncated NS3 proteins support such a model [73,92]. If RNA interacts at this helicase–protease interface, the site provides yet another potentially unique drug target where ligands might interfere with normal RNA binding.

Interaction with other proteins & formation of higher order structures

As a component of the HCV replication complex, NS3 interacts intimately with other HCV NS proteins [93], and it may be possible that compounds disrupting these interactions could function as antivirals. The most clearly defined of these protein–protein interactions is between NS3 and NS4A, the cofactor needed for activation of HCV protease. Although clearly required for the protease reaction, any influence of NS4A in NS3-catalyzed ATP hydrolysis or RNA unwinding remains under investigation [80,94,95], however, NS4A clearly serves a structural role, tethering NS3 to membranes in a manner that might help coordinate polyprotein processing and interprotein interactions [96].

Interaction between NS3 and NS5B polymerase has also been studied in some detail, with NS3 stimulating NS5B-catalyzed RNA-dependent RNA synthesis through an interaction requiring helicase-catalyzed ATP hydrolysis [62]. Conversely, the presence of NS5B polymerase in assays increases both NS3-catalyzed DNA [97] and RNA unwinding [68]. As previously noted, a role for the helicase in virus assembly could be explained by an interaction between NS3 and HCV core protein [66]. In addition, an interaction with human arginine methyltransferase 1 that results in regulation of NS3 helicase activity has been uncovered [88].

Another key interaction of NS3 helicase is the formation of dimers and higher order quaternary structures. In the replicase, HCV helicase probably functions as an oligomer, and it is well established that NS3-catalyzed DNA and RNA unwinding is highly dependent on protein concentration, even under conditions in which the amount of protein greatly exceeds the amount necessary to saturate the single-stranded region of the substrate [72,98–101]. There has even been some progress in localizing oligomerization motifs, although studies differ in their conclusions. One study points out key residues in domain 1 [102] while another identifies key protein–protein contacts between domain 2 of one NS3h molecule and domain 3 of a neighboring NS3h [57].

HCV helicase inhibitors: the continued search for small molecules

Few small molecules that inhibit HCV helicase have been reported in the literature and even fewer have been studied enough to reveal structure–activity relationships. This is somewhat perplexing given the need for new drugs to treat HCV and the relative success in targeting other HCV enzymes. Recombinant HCV helicase and high-resolution structures were available at about the same time as when work began on NS5B and the NS3 protease. It is possible that early screens using assays monitoring ATP hydrolysis yielded few specific hits that do not also inhibit key cellular motor proteins. It is also possible that NS3 helicase projects were dropped to pursue other HCV antivirals. Nevertheless, recent screens of small libraries have found compounds that inhibit HCV-catalyzed DNA unwinding, some of which also prevent HCV replication in cells. These compounds are discussed below, and they could be used as positive controls for new screens or as scaffolds to discover more potent compounds.

Compounds targeting the ATP-binding site

To date, only one small molecule that has been directly verified to inhibit HCV helicase by occupying the ATP-binding site has been reported. Using virtual screening and x-ray crystallography, Hu *et al.* identified a series of compounds that can bind the cleft separating the RecA-like motor domains of HCV helicase [103]. The compound with the highest affinity for NS3h was soluble blue HT (Figure 3), which directly interacts with the lysine (K210) in SF2 motif I that is required for ATP hydrolysis, contacting the aspartate that coordinates metal ions (D290) through a water molecule [103].

Nucleotides and their derivatives are also assumed to exert their action at the putative ATP-binding site (Figure 3). Common nonhydrolyzable nucleoside triphosphates are, however, surprisingly poor inhibitors of NS3-catalyzed ATP hydrolysis [104] and DNA unwinding [105]. An intact triphosphate moiety appears to be needed for the interaction between HCV helicase and either canonical or modified NTPs. For example, when a 5' triphosphate is added to ribavirin, the compound binds tighter to HCV helicase, although neither compound inhibits HCV helicase-catalyzed strand separation; ribavirin triphosphate actually fuels the unwinding reaction [106]. The products of ATP hydrolysis, (ADP and inorganic phosphate) and tripolyphosphate are weak HCV helicase inhibitors, yet, surprisingly, pyrophosphate and its nonhydrolyzable analog, imidodiphosphate, are relatively potent inhibitors of NS3-catalyzed ATP hydrolysis with K_i 's of 20 and 12 μM , respectively [104].

Other nucleoside analogs that inhibit HCV helicase-catalyzed reactions (Figure 3) include compounds, such as dichloro(ribofuranosyl) benzotriazole (DRBT), and base-analog inhibitors include compounds, such as tetrabromo-benzotriazole (TBBT) [107]. TBBT and DRBT inhibit *in vitro* helicase assays with IC_{50} s of 20 and 1.5 μM , respectively [107], and they also inhibit HCV RNA replication in cells. Depending on the cell line, TBBT inhibits HCV replicons with an IC_{50} ranging from 60 to 65 μM and DRBT inhibits with an IC_{50} ranging from 10 to 53 μM [61].

Ring-expanded 'fat' nucleosides inhibit HCV and related *Flavivirus* helicase-catalyzed reactions *in vitro*, with IC₅₀s in the 5–10 μ M range. One fat nucleoside analog compound 24 (Figure 3), inhibits HCV helicase-catalyzed RNA unwinding with an IC₅₀ of 5.5 μ M [108, 109]. Another recently disclosed nucleoside analog with a diaminodihydro-triazine substituent replacing the 5-amino group of the nucleoside metabolite, nucleoside 4 (4-carbamoyl-5-[4,6-diamino-2,5-dihydro-1,3,5-triazin-2-yl]imidazole-1- β -D-ribofuranoside) inhibits HCV helicase-catalyzed DNA unwinding with an IC₅₀ of 37 μ M but does not effect HCV helicase-catalyzed RNA unwinding [110].

Compounds targeting the RNA-binding site

Few compounds are known to inhibit HCV helicase by binding to the known RNA-binding cleft, although there are a couple important recent leads in this area (Figure 4). By screening small libraries of compounds that are known to bind HIV reverse transcriptase at the non-nucleoside binding site, Maga *et al.* identified a series of compounds that inhibit HCV helicase-catalyzed DNA unwinding but not its associated ATPase activity. After optimizing these compounds using docking and molecular modeling, they discovered QU663 (Figure 4A), which acts as a competitive inhibitor of HCV helicase catalyzed DNA unwinding with a K_i of 750 nM. Docking studies suggest that QU663 binds in place of the RNA substrate, interacting with both W501 and R393 [111].

Clues as to how small molecules might interact with the known RNA-binding cleft of HCV helicase might also be derived from a recently reported small peptide inhibitor (Figure 4). This 14 amino acid-long peptide (p14), which has the same sequence as that surrounding the putative HCV helicase arginine finger (SF2 helicase motif IV), inhibits HCV helicase-catalyzed DNA unwinding with an IC₅₀ of 200 nM [112], and p14 inhibits the replication of HCV replicons in cells with an IC₅₀ of 83 μ M [59]. The p14 amino acid sequence (RRGRTGRGRRGIYR) renders the peptide quite basic, suggesting that the peptide might function by sequestering the nucleic acid substrate. However, several key controls argue for a more specific mechanism of action. For example, slightly longer or shorter peptides derived from HCV helicase domain 2, as well as the analogous peptides from related viruses, interact with HCV helicase with much lower apparent affinities [112], and crosslinking studies support the notion of a direct p14–HCV helicase interaction [59]. Nuclear magnetic resonance data and molecular modeling suggest that p14 binds near to the SF1 helicase motif 1 (the P-loop), in order to prevent any interaction with the amino acid sequence on domain 2, which is the same as p14. The p14 peptide then appears to twist along domain 1 to also occupy the RNA-binding cleft in the region of Trp501 [59].

Compounds binding to unknown sites

Libraries of tropolone derivatives, which are known to act as antiviral, anticancer and anti-fungal agents, have been screened as inhibitors of HCV helicase-catalyzed DNA unwinding. The derivative of the seven-member tropolone ring system that most potently inhibits HCV helicase, called 3,7-dibromo-5-morpholinomethyltropolone (DBMTr) (Figure 5), acts with an IC₅₀ of 17.6 μ M. DBMTr has no effect on HCV helicase-catalyzed ATP hydrolysis [113] nor, curiously, HCV helicase-catalyzed RNA unwinding [114]. Unlike related compounds, DBMTr is not particularly toxic to yeast cells, suggesting that it might be an appropriate agent to use in eukaryotic cells.

Acridone derivatives, some of which are potent anticancer and antiviral agents, have also been screened as inhibitors of HCV helicase-catalyzed DNA unwinding. Stankiewicz-Dragon *et al.* found several acridone-4-carboxylates that inhibit both HCV helicase-catalyzed DNA unwinding and HCV RNA replication [115]. Two of the most potent derivatives, compound 20 (9-oxo-9,10-dihydro-acridine-4-carboxylic acid pyridin-4-ylamide) and its close relative,

compound 27 (9-oxo-9,10-dihydro-acridine-4-carboxylic acid pyridin-2-ylamide), inhibit HCV helicase-catalyzed DNA unwinding with IC_{50} s of 9 and 4 μ M, respectively. Although acridone-carboxylates might function by simply interacting with the DNA substrate, the best derivatives in this series inhibit T7 RNA polymerase catalyzed RNA synthesis with a two- to ten-fold lower potency, indicating they are selective for HCV helicase. Most importantly, these compounds also inhibit replication of HCV replicons (with IC_{50} s of approximately 10 μ M) and are not particularly toxic to cells [115].

High-throughput HCV helicase assays

Now that it is clear that HCV helicase inhibitors can function in cells as HCV antiviral agents, the time may be right to try to screen larger or combinatorial chemical libraries for more potent compounds. The simplest method to screen for HCV helicase inhibitors is to monitor helicase-catalyzed ATP hydrolysis using any of the common high-throughput ATP assays [116]. Simply screening for inhibitors of helicase-catalyzed ATP hydrolysis, however, is problematic because many other ATPases are present in cells, and a number of counter-screens are necessary to eliminate nonspecific agents. As a result, the direct measurement of helicase activity is probably a better route for finding HCV helicase inhibitors.

Measuring helicase-catalyzed DNA or RNA unwinding is difficult because the reaction products (single-stranded DNA or RNA) will rapidly re-anneal and, therefore, cannot be detected. To solve this problem, one can perform helicase assays either at very low DNA concentrations, where the annealing reaction is slow, at high helicase concentrations or with added additional complementary DNA (or ssDNA-binding proteins) to trap the single-stranded products. There are a variety of assays available for monitoring HCV-catalyzed DNA or RNA unwinding in high-throughput format. Typical helicase assays are performed with radiolabeled oligonucleotides and, in the case of HCV helicase, one of these oligonucleotides must be longer than its complement such that, when combined, they form a partial duplex with a single-stranded 3' tail. Released radiolabeled oligonucleotides can be captured for scintillation proximity assays using a bead with a covalently attached oligonucleotide complementary to the DNA substrate [117]. Biotinylated oligonucleotides can also be used to capture radiolabeled substrates for measurement [118], or to measure nonradio active substrates with ELISAs [119]. The main problem with the above assays is that they only yield a single time point per reaction because the reactions must be terminated before observation. More data can be collected in real time if helicase substrates are labeled with fluorescent moieties. For example, if one strand labeled on the 5' end with a fluorescent probe is annealed to another strand with a quenching molecule at the 3' end and incubated with HCV helicase, fluorescence will increase in an amount proportional to the amount of DNA unwound [120,121]. Such fluorescent assays were used to identify many of the compounds listed previously, however, they are difficult to use to screen large libraries containing compounds that absorb in the visible range. Such compounds can appear to inhibit the helicase reaction if they simply absorb light emitted by the fluorescent probe, and differentiating these compounds from true inhibitors can be cumbersome. Another problem with these fluorescent assays is that high concentrations of 'capture' strands must be added to reactions to prevent the products from reannealing. These capture strands limit assay reproducibility and sensitivity because they can interact both with the enzyme and the compounds being tested.

To help facilitate screens for HCV helicase inhibitors, we developed a molecular beacon-based helicase assay (MBHA), which uses dual-labeled DNA oligonucleotides that form hairpins (i.e., molecular beacons [122]) annealed to a complementary DNA or RNA oligonucleotide as helicase substrates [105]. Unlike the assays discussed before, the MBHA is irreversible as, upon strand separation, the products form hairpins. This means that no capture strands or ssDNA-binding proteins are needed to trap the products, simplifying the protocol and

enhancing reproducibility and sensitivity. Another advantage of MBHA is that hairpin formation brings a fluorescent molecule closer to a quencher molecule, resulting in a decrease in fluorescence proportional to the amount of substrate unwound. This allows a screener to quickly identify compounds that simply quench substrate fluorescence, as quenchers would decrease fluorescence at the start of the reaction. This feature decreases the overall number of hits, possible false leads and the need for a counter screen to identify quenching compounds.

Conclusion & future perspective

The prospect that the first generation of STAT-C agents will include helicase inhibitors is unlikely but not unimaginable at this time. In all likelihood, HCV helicase inhibitors will not be the first STAT-C agents approved to treat HCV infection. HCV helicase inhibitors lag years behind protease and polymerase inhibitors; protease and polymerase inhibitors have already advanced to stage 3 clinical trials, while no helicase inhibitors have been reportedly tested in animals or even the JFH1 system.

The lack of reported progress with helicase inhibitors is surprising for several reasons. First, HCV with a defective helicase will not infect cells. Second, when helicase inhibitors are added to cells, HCV does not replicate. Third, a few potent helicase inhibitors with submicromolar IC₅₀s have been developed and many of these compounds are active in HCV replicon systems. Three leading scaffolds for development are soluble blue HT (Figure 3), whose binding site is clearly defined, benzotriazoles (Figure 4) and acridone-4-carboxylates (Figure 5), the latter two having been shown to inhibit HCV replicons in cells.

These compounds, or other helicase inhibitors, will need to complete a number of steps before they can ever be considered as drug candidates. First, the compounds will need to be tested against replicons and/or purified helicases derived from a variety of HCV genotypes. Such experiments could help define compound specificity and possible binding sites. Second, HCV replicons resistant to each lead compound will need to be isolated to understand how rapidly resistance develops and whether amino acid combinations conferring resistance already exist in nature. Third, these compounds could serve as parent molecules for the synthesis of analogs, structure-activity studies and possible lead identification for drug design efforts. Fourth, compounds could be co-crystallized with HCV helicase to provide a definitive picture of ligand-binding sites and facilitate structure-based drug design. Finally, before any clinical trials are started, absorption, distribution, metabolism, and excretion must be analyzed in animal models. Owing to the inherent difficulties in working with chimpanzees, other surrogate animal models have been developed, such as murine systems involving transplanting human hepatocytes into immunodeficient mice [123].

Helicase inhibitors reported thus far could also serve another purpose in the near future if they are used in JFH1 infectious clone models. The benefits of using helicase inhibitors as molecular probes would be twofold: simple determination of efficacy in cell-based systems and investigation of the role of HCV helicase in the viral lifecycle.

Now may also be the time to initiate broader screens for small molecule HCV helicase inhibitors. A new, more sensitive, high-throughput HCV helicase assay has been developed, which could be used to screen diverse libraries of natural or synthetic compounds or combinatorial libraries derived from some the inhibitors described above. Recent advances in understanding the molecular basis for helicase action might also spur interest in rationally designing compounds that might target key motif or clefts. New well-defined helicase inhibitors will also be useful for chemical genetics. Knocking out HCV helicase in certain cellular environments or at various stages of infection could finally elucidate the role of the helicase in virus replication. HCV helicase is clearly an undeveloped drug target, although all

the recent advances might encourage new attempts to find better helicase inhibitors. Such continued work and recent advances in the field will hopefully soon result in the discovery of more compounds for use in the laboratory and clinic. If this happens, the probability of using helicase inhibitors as STAT-C could change in the near future.

Executive summary

Hepatitis C & hepatitis C virus

- Hepatitis C is a liver disease caused by chronic infection by the hepatitis C virus (HCV).
- HCV infects approximately 2% of the population worldwide and can cause fibrosis, cirrhosis and hepatocellular carcinoma.
- Current HCV treatments combine ribavirin and pegylated interferon, but they are expensive, cause serious side effects and are not fully effective in eliminating many common HCV genotypes.
- Specifically targeted antiviral therapy for HCV (STAT-C) includes all new antiviral agents that act upon proteins involved in HCV replication.
- Leading STAT-C agents are protease and polymerase inhibitors, however, additional agents will be needed to combat HCV resistant to these compounds.
- Surrogate systems to study HCV include the chimpanzee, chimeric mice, HCV replicons and a recently developed cell-culture system based on the JFH1 strain.

HCV NS3 helicase as a potential drug target

- The nonstructural (NS) protein 3 encoded by HCV possesses protease, ATPase and helicase activities.
- The HCV helicase unwinds both duplex RNA and DNA.
- The HCV helicase could be involved in translation, replication, virus assembly or host gene regulation; its precise biological role is still unclear.
- The NS3 helicase is absolutely required for infectivity *in vivo* and in cell culture.
- The NS3 helicase possesses unique motifs and ligand-binding clefts that could accommodate small molecules.

HCV helicase inhibitors

- The commercially available dye, soluble blue HT binds the NS3 ATP-binding cleft to inhibit the enzyme.
- Nucleoside and base analogs known as benzotriazoles inhibit HCV helicase *in vitro*, with IC_{50} s ranging from 2 to 20 μ M, and cellular HCV RNA replication with IC_{50} s of approximately 10–60 μ M.
- A compound called QU663 (K_i = 750 nM) and a 14 amino acid-long peptide (IC_{50} = 200 nM) are suspected to bind to the NS3 RNA-binding cleft.
- The best tropolone derivative inhibits HCV helicase-catalyzed DNA unwinding, with an IC_{50} of 18 μ M.
- Acridone-4-carboxylic acid derivatives inhibit HCV helicase *in vitro* (IC_{50} = 4 μ M) and HCV RNA replication in cell culture (IC_{50} = 10 μ M).

- A new molecular beacon-based helicase assay has been developed to facilitate inhibitor analysis and new high-throughput screens.

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Figure 1. Possible roles of HCV NS3 in viral replication

(A) Polyprotein processing. The protease domain of the NS3 protein is depicted cleaving the viral polyprotein to release mature HCV nonstructural proteins. The helicase could assist this process by interacting with the RNA being translated. (B) RNA replication. The helicase is shown aiding the action of the NS5B polymerase by separating double-stranded RNA duplexes resulting from genome replication. (C) Packaging. The helicase is depicted packaging HCV RNA into virions with the help of the HCV core protein. HCV: Hepatitis C virus; IRES: Internal ribosome entry site; NS: Nonstructural.

Figure 2. Ligand-binding clefts and key amino acids in the HCV NS3 helicase

(A) Model of HCV helicase unwinding DNA. The full-length NS3 structure (Protein Data Bank [PDB] accession number 1CU1) was aligned with the costructure of a related helicase–DNA complex (archaeal Hel308, PDB file 2P6R) to reveal how NS3 might split a duplex. Shown are the NS3 protease (green), NS4A (blue), helicase domain 1 (yellow), domain 2 (purple) and domain 3 (tan), along with the DNA (sticks) bound to Hel308, which is not shown. (B) Putative ATP-binding site. An alignment of the crystal structure of HCV NS3h (PDB file 1A1V) with that of a related helicase cocrystallized with a nonhydrolyzable ATP analog (Dengue virus NS3, PDB 2JLV) to show the coordination of ATP. K210 is in SF2 helicase motif I and coordinates the γ -phosphate of ATP. D290 is in SF2 motif II and coordinates the bridging divalent metal cation (Mg^{2+}). R467 is a likely ‘arginine finger’ that might coordinate the γ -phosphate in the transition state and E291 is the catalytic base that activates the water that attacks the γ -phosphate. The ATP analog is depicted as sticks. (C) Known RNA-binding site. The nucleic acid-binding cleft of PDB file 1A1V is shown as an electrostatic surface (red, negatively charged; blue, positively charged). The surface was calculated without DNA bound, and the DNA was added back to mark the binding site. The oligonucleotide is held in a positively charged pocket centered on E493, held in place by the Arg-clamp motif (R393). (D) Phe-loop motif. A β -loop (ribbon) containing the conserved Phe-loop motif acts to separate double-stranded nucleic acids. (E) Possible second RNA-binding site in the cleft between the protease and helicase domain 2. The surface is colored by domains as in (A). Molecular models were aligned using UCSF Chimera [201], ligands were docked using UCSF DOCK [202], electrostatic surface was calculated with APBS [203] and models were rendered using pyMol [204]. APBS: Adaptive Poisson–Boltzmann Solver; HCV: Hepatitis C virus; NS: Nonstructural; SF: Superfamily; UCSF: University of California, San Francisco.

Figure 3. Compounds that likely bind to the ATP site
DRBT: 5,6-dichloro-1-(β-D-ribofuranosyl)benzotriazole; TBBT: 4,5,6,7-tetrabromobenzotriazole. Data taken from [103–110].

Figure 4. Compounds that likely to bind the RNA site
(A) QU663 [111]. (B) The p14 peptide RRGRTGRGRRGIYR [59].

Figure 5. Compounds binding to unknown sites

(A) DBMTr (3,7-dibromo-5-morpholinomethyltropolone) [113]. (B) Acridone-4-carboxylic acid derivative: 27,9-oxo-9,10-dihydro-acridine-4-carboxylic acid pyridin-2-ylamine. (C) Acridone-4-carboxylic acid derivative: 20, 9-oxo-9,10-dihydro-acridine-4-carboxylic acid pyridin-4-ylamine [115].