Hepatitis C virus: an overview of current approaches and progress

Michael A. Walker

Hepatitis C virus (HCV) was unambiguously identified in the year 1989 as the agent responsible for most cases of non-A, non-B hepatitis, a chronic disease that often leads to cirrhosis and hepatocellular carcinoma. Having developed the means to detect the virus in the general population, it is now apparent that HCV infection is widespread and is likely to remain a health threat unless effective treatments are developed. The inability to propagate the virus in tissue culture and the scarcity of convenient animal models have proved to be major obstacles in drug discovery. Despite these limitations, several opportunities exist for targeted drug development based on the viral enzymes that have been characterized so far. These targets and inhibitors reported to be active against them are discussed in the following review.

At the beginning of this century, hepatitis A (HAV) and hepatitis B (HBV) viruses, so called 'infectious' and 'serum' hepatitis, were viewed as the major agents of viral hepatitis. Although there was evidence for a third type of hepatitis as early as 1950, it was not until the 1970s, when serological tests for HAV and HBV became available, that the new agent referred to as non-A, non-B hepatitis (NANBH) was recognized[1]. After the introduction of the HBV blood screen, it became apparent that NANBH was responsible for a large portion of transfusion-associated hepatitis cases[2]. Not surprisingly, a major effort was mounted to isolate and study the virus to prevent further infection and to develop effective treatments for those already infected.

Hepatitis C virus (HCV), as the major agent of NANBH came to be known, has been described as an insidious disease and a 'silent epidemic', mainly because the infection is often subclinical. Acute infection occurs in only a few patients. In most cases, the virus results in chronic infection taking 10–20 years before the emergence of liver disease, this often being accompanied by only mild or vague symptoms. Despite the seemingly benign onset of the disease, a significant number of patients with chronic hepatitis develop cirrhosis and its complications[3]. In addition, HCV-cirrhosis has been linked to hepatocellular carcinoma, although the contribution of additional factors cannot be ruled out[4,5]. In the US, almost four million individuals might be infected, with up to 170 million people infected worldwide[6]. Transmission of the virus is primarily parenteral, and hence, screening blood donors for the virus has been effective in reducing transfusion-associated HCV. Unfortunately, the virus is also spread amongst other groups such as intravenous drug users, and there appears to be a high number of cases resulting from unidentifiable risk factors.

Current therapies

There is currently no vaccine available for HCV and progress in this area is hampered because there are several different subtypes worldwide and the virus exists as a quasispecies during infection. Interferon-α (IFN) is currently the recommended treatment for HCV and is believed to act indirectly by binding to specific cellular

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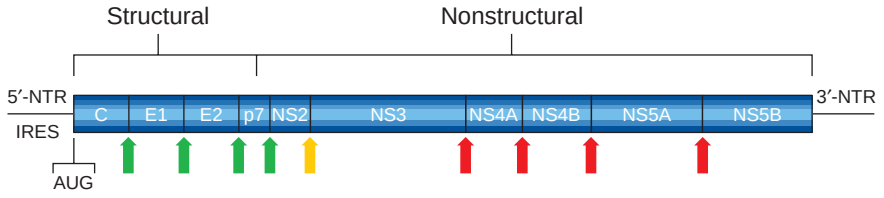

Target	Size (kDa)	Function
C	21	Nucleocapsid
E1–E2	31, 70	Envelope glycoprotein (heterodimer)
p7	7	Unknown function
NS2	23	Forms autoprotease with NS3
NS3	70	Serine protease, NTPase/helicase
NS4A	8	NS3-protease cofactor
NS4B	27	Unknown function
NS5A	56	Interferon sensitivity determining region
NS5B	68	RNA-dependent RNA polymerase

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Figure 1. *The structure of the hepatitis C virus genome is shown. The 3′- and 5′-nontranslated regions are defined as well as the regions coding for the structural and nonstructural components of the virus. Cellular signal peptidase (shown in green), the NS2–NS3-protease (shown in yellow) and the NS3-protease (shown in red) cleave the polyprotein at the sites indicated. The structural and nonstructural proteins are identified in the table.*

receptors that induce an intracellular antiviral response[7] (for reviews, see Refs 8,9). The standard regimen is three million units of IFN given subcutaneously three times-a-week for up to 12 months. A complete response, defined by normalized alanine aminotransferase (ALT) levels and the disappearance of HCV-RNA from the serum, occurs in <50% of patients treated, but almost 50% of these individuals suffer a relapse after the treatment has ended. Improvement in the response rate, but not the relapse rate has been achieved through optimizing dosing and/or chemical modification of IFN.

In the past couple of years, combination therapy of IFN plus ribavirin, has proven to be more effective as an initial treatment and in patients who have relapsed[10,11]. Ribavirin is a guanosine analogue that has activity against several different viruses. The mechanism of action of this agent in HCV-combination therapy has not been determined[12]. Although the IFN–ribavirin combination therapy represents a major advance in the treatment of HCV, it still has several shortcomings:

- IFN must be given by injection
- There are significant side-effects associated with both IFN and ribavirin
- The cost of the treatment is high
- HCV1b, the most common genotype in the US, is less responsive than the others.

Hence, while IFN therapy has proven to be beneficial for some patients, there clearly is a need for drugs that target the virus directly as it is generally believed that such agents would be more effective and less prone to unwanted side-effects.

Problems of HCV research

The study of HCV has been hampered considerably by the lack of a tissue culture system capable of supporting efficient replication of the virus and a convenient animal model other than the chimpanzee. Even so, the cloning of the HCV genome in 1989 (Ref. 13) has proven very useful in characterizing the virus and identifying several potential targets for drug development. Genetic analysis has revealed that HCV is a positive-stranded RNA virus having a genome of approximately 9000–9300 nucleotides in length and is classified as a separate genus of the Flaviviridea family. The genome contains a single open reading frame (ORF) coding for up to ten proteins that are cleaved from the initially translated polyprotein by cellular and viral proteases. Reading from 5′ to 3′, these include a core protein C and two envelope proteins E1 and E2, followed by the nonstructural proteins NS2–NS5 that are essential for viral replication, translation and polyprotein processing (see Fig. 1). Further characterization of the non-structural proteins has yielded that:

- NS2–NS3 is an autocatalytic protease cleaving the polyprotein at NS2/NS3
- NS3 is a multifunctional protein coding for a NTPase-dependent helicase and a serine protease that processes all downstream nonstructural proteins
- NS4A forms an essential complex with NS3 that activates the NS3-protease as well as possibly anchoring the complex in the endoplasmic reticulum membrane
- NS5A is highly phosphorylated post-translationally and appears to regulate interferon sensitivity
- An RNA-dependent RNA polymerase (RdRP) is coded for by NS5B.

In addition, the genome contains two nontranslated regions (NTRs), the first a 59-domain that functions as an

internal ribosomal entry site (IRES) and a second site located at the 39-end that probably plays a role in initiating replication of (–)RNA. Hence, despite the reluctance of the virus to replicate in tissue culture, several promising opportunities for drug development have already been discovered through molecular biology14,15.

Of the potential targets currently known, it is difficult to decide which will offer the best target for drug discovery. Several reviews have recently appeared that delineated the function and properties of the viral proteins and evaluated their utility as potential drug targets based on their putative role in the virus lifecycle[14,15]. However, in addition to considering the biological importance of a potential target, consideration must be given to how tractable the enzyme is to work with and what chemical functionalities are required for inhibition. These factors will directly affect how quickly an inhibitor can be discovered and whether an oral drug is feasible. Previous attempts indicate that, while certain enzymes and receptors represent reasonable targets for controlling a biological event or process *in vitro*, the agents required to inhibit them make poor drug candidates because of unwanted physical or chemical properties. Therefore, this review examines the inhibitors of HCV that have been reported so far, using their potential as drugs as a means to evaluate and compare the various targets. In addition, this review describes how the state of the pharmaceutical industry is affecting its response to this challenge. Absent from this review are anti-HCV agents for which either the target has not been defined or that act indirectly against the virus.

R&D alliances

HCV infects a large number of individuals, a property that has not escaped the attention of the pharmaceutical industry. Thus, most companies with existing programs in the anti-infective area are focusing towards discovering agents that are active against this virus. Human immunodeficiency virus (HIV) provides a useful paradigm for HCV antiviral drug development, especially when considering that both lead to chronic infection and are highly mutatable. However, it is likely that combination therapy involving at least two drugs directed against separate targets will be more effective at reducing viral load and preventing the emergence of resistant strains than monotherapy. Based on the recent successes observed against HIV, it is not surprising to find that HCV protease and polymerase have often been mentioned as probable candidates. In addition, several companies are also focusing on translation inhibitors.

It is tempting to speculate that the discovery of drugs effective against HIV was hastened because the compound collections used for screening were fortunately enriched with polymerase and aspartyl-protease inhibitors from discovery efforts against unrelated therapeutic indications. However, as judged by the lack of disclosures, the discovery of anti-HCV agents in this manner has not been successful despite the functional similarity of several HCV-enzymes with targets from other programs. Admittedly, part of this failure is because of the lack of a tissue culture system, which in turn limits primary screens to isolate viral protein targets. However, even when the enzyme and assays were known, the discovery of potential drug candidates has met with little success. Based on this, it might be concluded that the putative chemotypes for inhibition of HCV-targets are poorly represented in most industrial compound collections.

Because of the apparent failure to discover truly novel anti-HCV agents, many companies are searching for outside technologies and resources. Hence, during the time from when the HCV genome was identified, several companies have expanded their search for anti-HCV agents by forming alliances. An overview of the partnerships that have emerged is given in Box 1. These partnerships are founded on several different strategies including shared technology, compound libraries and proprietary viral targets.

NS3-protease, being the most characterized enzyme of HCV, is the basis for the majority of the partnerships. Over the past few years, both Agouron (see Warner Lambert, Morris Plans, NJ, USA) and Vertex (Cambridge, MA, USA) have enjoyed success in the area of antiviral chemistry as evidenced by the recent introduction of two HIV-protease inhibitors. Building on this track record, both companies have made impressive advances in characterizing the NS3-protease, publishing concurrent papers on the X-ray crystallographic structure of the enzyme. The alliances of Japan Tobacco (Tokyo, Japan) with Agouron and of Eli Lilly (Indianapolis, IN, USA) with Vertex are built upon this expertise. By contrast, companies such as Corvas (San Diego, CA, USA) and Axys (South San Francisco, CA, USA) have pioneered novel methods for drug discovery, in particular, the serine protease inhibitors that form the basis for their respective partnerships with Schering-Plough (Kenilworth, NY, USA) and Bristol-Myers Squibb (New York, NY, USA). Corvas has developed a proprietary combinatorial strategy while Axys is relying on their 'delta technology' to aid in the discovery of protease inhibitors. The number of alliances in this area highlights the unusual structural requirements of the protease as traditional serine protease inhibitor chemotypes perform poorly against the enzyme.

Of the alliances where the target has been disclosed, inhibitors of protein translation are the second most prevalent behind protease inhibitors. Reported inhibitors of viral protein translation usually target viral mRNA and, more

Box 1. Hepatitis C research alliances

Companies	Target[a]
Japan Tobacco/Agouron[b]	NS3
BioChem Therapeutics/XTL Biopharmaceuticals	NA
BioChem Therapeutics/Structural Bioinformatics (SBI)	NA
Structural Bioinformatics/CyberChemics	NS3
Bristol-Myers Squibb/Axys	NS3
Chiron/AMRAD	NA
ViroPharma/Chiroscience	NA
DuPont/Signal	Viral gene regulation
ISIS/Chem-Sero/Mochida	IRES
Lilly/Vertex	NS3
Lilly/Ribozym	Replication
Chiron/Lynx	IRES
Merck/Tularik	Transcription factor
Merck/ISIS	Replication
Oncogene Science (OSI)/BioChem Therapeutics	NA
Pharmacia Upjohn/Chiron	NA
Roche/Hybridon	NA
Schering-Plough/Corvas	NS3
Boehringer Ingelheim/ViroPharma	NA

[a]Information obtained from Internet home pages of listed companies and the Id*db* (Investigational Drug database, Current Drugs Ltd).

[b]A subsidiary of Warner Lambert.

Abbreviations: IRES, internal ribosome entry site; NA, not announced; NS3, NS3-protease.

specifically, the HCV-IRES by using antisense agents. Hence, companies such as ISIS (Carlsbad, CA, USA), Hybridon (Milford, MA, USA) and Lynx (Hayward, CA, USA), which have been developing antisense agents, have a lot of expertise to offer in an area not traditionally pursued by large pharmaceutical companies. This has led Merck (Whitehouse Station, NJ, USA), Roche (Basel, Switzerland) and Chiron (Emeryville, CA, USA) to enter into research agreements with these companies, although the Roche–Hybridon agreement was terminated in 1998. After taking into account those alliances based on NS3-protease and IRES, there are still many alliances for which no target has been specifically disclosed. These are most likely based on at least one of the companies having expertise in virology, HCV or anti-infective agents.

Compared to other programs of drug discovery, HCV is a relative newcomer. The increase in the current understanding of the viral lifecycle and elements responsible for each step is slow because of the difficulties of getting this virus to grow using standard techniques. If the recent past is any indication, it is likely that as advances in HCV-virology are made, the R&D landscape will change, with new targets being found and new alliances formed to meet the challenge of discovering inhibitors. The NS3-protease and the HCV-IRES have gathered much attention recently because these targets are well characterized. Based on the literature, it appears that NS3-helicase and NS5B-polymerase might not be too far behind. Both from a drug discovery and a purely scientific point-of-view, HCV promises to be a very interesting field to keep track of in the next few years.

NS3 protease

The recent successes achieved against HIV using viral-protease inhibitors are encouraging and suggest that other viruses that carry an analogous target might be treated in a similar manner. HCV encodes a serine protease located in the N-terminal domain of NS3 that, along with an NS2–NS3 protease, is responsible for processing the nonstructural portion of the HCV-polyprotein. Analysis of the cleavage products has identified four processing sites, one being cleaved *in cis* (NS3/NS4A) and three being cleaved *in trans* (NS4A/NS4B, NS4B/NS5A and NS5A/NS5B). The consensus sequence, D(E)XXXXC(T)↓S is found at the processing sites of each of these peptides with cysteine located at P_1 (P_n, P_n', S_n and S_n' are defined according to the nomenclature of Schechter and Berger[16]) for the *trans*-cleaved proteins. The protease plays a crucial role in the formation of the viral polymerase (NS5B) and other essential proteins (NS4B and NS5A), and hence makes an attractive target for drug development[17].

Although the NS3-protease belongs to the chymotrypsin family of serine proteases, there are several differences, including a requirement for a peptide cofactor (NS4A), an unusual preference for cysteine in the P_1-position and the inability to cleave small peptide substrates. In addition, whereas most serine proteases have loops (connecting β-strands) lining the substrate-binding groove, NS3 lacks these. As a result, the substrate-binding domain is relatively flat, wide and exposed to the solvent. The major protease–substrate interactions span the entire binding domain and include a highly discriminating S_1-pocket, hydrogen bonds to the substrate backbone and complementary electrostatic or hydrophobic contacts across the entire groove. Based on these observations, it might be expected that an inhibitor of NS3 would need to be large to fully occupy the substrate-binding site and take advantage of these interactions[18].

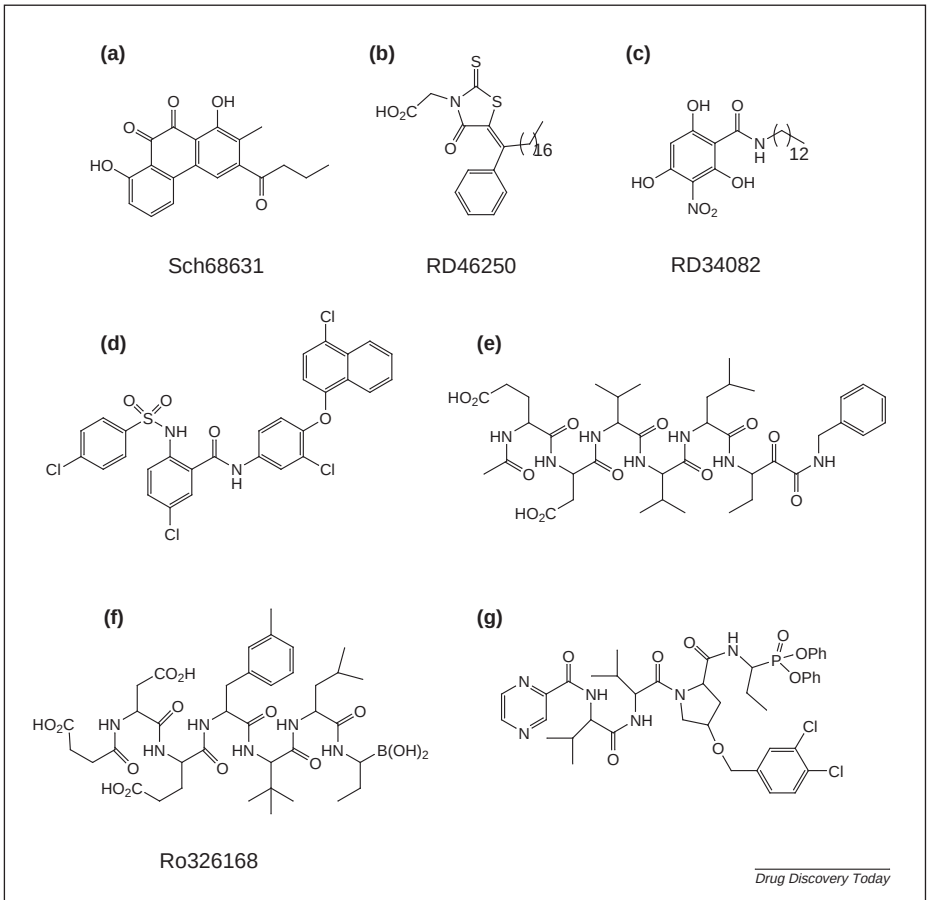


Figure 2. *Inhibitors of hepatitis C protease.*

Small-molecule inhibitors

There have been several small-molecule inhibitors reported to be active against HCV-protease *in vitro* (see Fig. 2). These include murayaquinone (Sch68631, IC_{50} = 7 μM, Fig. 2a), a natural product isolated from *Streptomyces* sp.[19], thiazolidinone-based inhibitors (e.g. RD46250, IC_{50} = 4.4 μM, Fig. 2b)[20], 2,4,6-trihydroxy-3-nitrobenzamides (e.g. RD34082, IC_{50} = 5.8 μM, Fig. 2c)[21] and benzoylamides (e.g. Fig. 2d, IC_{50} = 6.5 μM)[22]. Mechanistic evaluation has revealed that the thiazolidinones and benzoylamides are noncompetitive with the substrate, while the 2,4,6-trihydroxy-3-nitrobenzamides are mixed noncompetitive/uncompetitive inhibitors. Unfortunately, the selectivity of these inhibitors for NS3-protease over other serine proteases appears to be low making them non-optimal leads.

Peptide-based inhibitors

By contrast, three classes of competitive peptide-based inhibitors have been discovered, these being noncleavable substrates, product-based inhibitors and transition-state mimetics. In a study of the mechanism of NS4A activation, Vertex reported that replacement of the P_1'-serine residue of an NS5A/NS5B-decapeptide substrate resulted in poor enzyme turnover. Moreover, for certain P_1'-residues, k_{cat} (turnover number) was reduced while K_M (Michaelis constant, substrate affinity) remained the same or improved, resulting in enzyme inhibition when assayed against efficiently cleaved substrates. This was most noticeable for cyclic amino acids such as proline, pipecolinic acid (Pip) and tetrahydroisoquinoline-3-carboxylic acid (Tic) (see Box 2). The pronounced affinity of the Tic-containing peptide (Box 2d) for NS3 is consistent with previous models indicating that the S_1'-pocket can accommodate residues much larger than serine. Further analysis showed that while the P-side residues contributed more to binding than the P′-side, a favorable interaction could be detected between the P_4'-tyrosine residue of the inhibitor and NS4A (Ref. 23).

During the course of substrate specificity studies it was discovered that HCV NS3-protease was susceptible to product inhibition. Compared to most proteases, the K_Is measured for the N-terminal cleavage products were lower than the K_Ms of the corresponding substrates (NS4A/NS4B, NS4B/NS5A and NS5A/NS5B). An initial screen identified the hexapeptide, Ac-DEMEEC-OH (Box 3a), corresponding to P_6–P_1 of the NS4A/NS4B substrate as the most active product-based inhibitor (K_I = 0.6 μM) and implicated the C-terminal carboxylate as a major contributor to binding.

Box 2. Non-cleavable substrate inhibitors[23]

Compound	Sequence	K_I (μM)
a	EDVVAbuCPAbuSY	80.0
b	EDVVAbuCPipAbuSY	18.0
c	EDVVAbuCTicAbuSY	4.0
d	EDVVLCTicNleSY	0.3

Abbreviations: Abu, α-aminobutyric acid; Nle, norleucine; Pip, pipecolinic acid; Tic, tetrahydroisoquinoline-3-carboxylic acid.

Box 3. Product-based inhibitors of NS3

Compound	Inhibitor sequence	IC_{50} (μM)	Refs
NS4A/NS4B-based inhibitors			
a	Ac-DEMEEC-OH	1.0	25
b	Ac-D*Gla*LIChaC-OH	0.015	25
NS5A/NS5B-based inhibitors			
c	Ac-D*D*IVPC-OH	28.0	26
d	Ac-D*D*IVPC-OH	4.0	26
e	Ac-D*D*IVPNva-OH	17.0	27
f	Ac-D*D*IVPNva-CF_3	22.0	27
g	Ac-D*D*IVPNva-H	1.1	27
h	Ac-D*D*IVPNva-CONHBn	0.64	27

Abbreviations: *D*, D-aspartic acid; *Gla*, D-γ-carboxy glutamic acid; Nva, norvaline-ketoamide.

Mutagenic analysis suggests that this interaction is mediated through K136, located in close proximity to the oxyanion hole[24]. The essential determinants for activity were found to be cysteine at P_1 and an acidic pair of residues at P_6–P_5. Improvements in activity were gained using traditional and combinatorial methods to optimize the P_6–P_2 amino acids. Several inhibitors with submicromolar IC_{50}s were found, the most active of which is shown in Box 3b (Ref. 25). An analogous study was carried out using the P_6–P_1 peptide, DDIVPC-OH, corresponding to the NS5A/NS5B cleavage site. Modest inhibition was observed with the parent peptide, Ac-DDIVPC-OH (Box 3c, IC_{50} = 28 μM), although this was improved by inverting the configuration of the P_5-residue to yield Ac-D*D*IVPC-OH (*D* = D-aspartic acid) with an IC_{50} of 4 μM (Ref. 26).

Attempts to replace the C-terminal carboxylate with functional groups that will covalently bind to the active site serine of the NS3 protease have not produced significant improvements in activity. This is exemplified by peptides f–h in Box 3. Replacement of the P_1-cysteine with norvaline results in a fourfold loss of activity (Box 3e) compared to the parent NS5A/NS5B-product (Box 3d). However, this loss of activity can be recovered by replacement of the carboxylic acid with an aldehyde or ketoamide (Box 3g,h). This is encouraging as it suggests that the activated carbonyls are interacting with the enzyme as they were designed to. Unfortunately, these inhibitors are only slightly more active than the parent peptide and in follow-up studies, they were found to be much less selective for NS3 (Ref. 27). Analogous studies have been carried out by others and the active peptides described in these reports are shown in Fig. 2. Figure 2e is representative of a family of inhibitors containing ketoamide functionality that have modest activity (K_i <1 μM) against NS3 protease[28]. One of the most potent peptide-based inhibitors is the boronic acid, Ro326168 (Fig. 2f, IC_{50} = 38 nM)[29]. Interestingly, phosphonate diester (Fig. 2g) is the only inhibitor currently reported that does not require an acidic pair of residues at the N-terminus.

Box 4. Inhibitors of NS4A binding[31]

Compound	Inhibitor sequence[a]	IC_{50} (μM)
a	KGSLVIRGVIVVCK	0.2
b	KGSLVIRGVIVK	5.0
c	CVVIVGRIVLSGK	0.2
d	SLVIRGVIV	0.6
e	KGSLVIRGVIVVC	2.0
f	Bio-KGSLVIRGVIVVCKK[b]	0.4
g	KGSLVIRGVIVVCKK[c]	0.4

[a]D-Amino acids.

[b]Bio = biotin.

[c]Biotin attached to the ε-amino group of lysine.

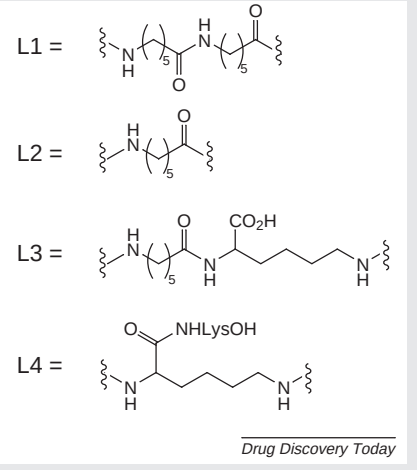
NS4A inhibitors

An interesting strategy has been developed using NS4A-analogues alone or linked to an NS5A/NS5B peptide yielding inhibitors capable of binding to both domains simultaneously. The NS4A-inhibitors were designed to act at the NS4A-binding region of NS3 but were assembled from D-amino acid residues in standard (Box 4c) or reverse order (Box 4a,b,d–g). As shown in Box 4, a reasonable level of inhibition is achieved by these peptides. It is worth highlighting that Shimizu and coworkers have previously described an NS4A-inhibitor of NS3-protease based on NS4A (21–34) (IC_{50} = 20 μM), wherein arginine at position 28 was replaced with glutamine (Ref. 30). The mechanism of inhibition has not been reported and several questions are raised by the inhibitory activity associated with these derivatives. In particular, which determinants lead to NS3-inhibition versus activation, and whether these inhibitors effectively compete with wt-NS4A during an infection if the initial binding of NS4A to NS3 occurs cotranslationally. Several bivalent inhibitors (Box 5) with two enzyme-binding domains have also been described. These inhibitors employ a P_6–P_1 element (EDVVCC or DEVVCC)

Box 5. Dual site inhibitors of NS3 protease[31]

Compound	Inhibitor sequence	IC_{50} (μM)
a	EDVVCC-L1-CVVIVGRIVLSGK	0.6
b	EDVVCC-L2-CVVIVGRIVLSGKK	3.0
c	EDVVCC-L3-(KGSLVIRGVIVVC)[a]	3.0
d	EDVVCC-L4-(KGSLVIRGVIVVC)[a]	3.0–30.0
e	EDVVCC-L1-KGSLVIRGVIVVC[b]	0.2
f	DEVVCC-L3-(CVVIVGRIVLSGK)[a,b]	2.0
g	DEVVCC-L2-KGSLVIRGIVVCK[b]	0.2
h	DEVVCC-L4-(CVVIVGRIVLSGK)[a,b]	0.2

[a]Peptide sequence in parentheses is written from C-terminus to N-terminus.
[b]Last 12 residues are D-amino acids.



attached via a linker (L1–L5) to the NS4A inhibitors already described[31].

Macromolecular inhibitors

Protein- and RNA-based macromolecular inhibitors have also been described in the literature. Protein-based inhibitors include:

- A competitive specialized V_H-antibody construct (cV_HE2, IC_{50} = 300 nM) discovered through phage display[32]
- A noncompetitive minibody inhibitor (Mbip, IC_{50} = 1500 nM)
- An analogue of the human pancreatic secretory trypsin inhibitor (hPSTI-C3, IC_{50} = 540 nM) that was found to be competitive with the substrate[33]
- Mutant eglin c analogues where the native binding-loop of the inhibitor was replaced with NS5A/NS5B and NS4A/NS4B sequence elements (IC_{50} = 0.06 and 2.0 μM respectively)[34].

RNA constructs include:

- 10G-1, which binds selectively to NS3 (apparent K_d = 650 nM) yielding modest inhibition of proteolytic activity (≈20% at 3.5 μM)[35]
- Second-generation aptamers, G6–16 and G6–19 produced by the same group having an IC_{50} of 3 μM (Ref. 36). Although it is not yet known whether these molecules can act as drugs, structure–activity relationship studies using these inhibitors can provide valuable information for the design of small molecules.

As demonstrated above, despite the challenges presented by the NS3 protease, inhibitors of this enzyme are beginning to emerge. Thus far, three classes of inhibitors have been disclosed:

- Small molecules acting by a noncompetitive mechanism
- Product-based analogues competitive with substrate and larger peptide
- RNA macromolecules operating by a variety of mechanisms.

In general, noncompetitive inhibitors target nonessential pockets on the enzyme with high specificity. The principal problem with the noncompetitive inhibitors of NS3 described so far is their low selectivity for the viral protease over other serine proteases. However, noncleavable substrate and product-based inhibitors have proven to be highly active and selective against NS3-protease. This class of inhibitors is disadvantaged because peptide-based molecules with relatively high molecular weights (i.e. >500) are known to be inferior drug candidates because of poor bioavailability. However, the development of HIV-protease inhibitors was burdened with the same challenges but these were eventually overcome, providing some hope for the HCV inhibitors. In addition, new prodrug strategies that improve oral bioavailability and/or target the liver continue to be developed that might also be useful in producing oral drugs[37].

Helicase

An enzymatic domain comprising 456 amino acids coding for a type II helicase is located in the first two-thirds of the C-terminal end of NS3 (Refs 38,39). The importance of this enzyme in the viral life cycle is shown by the fact that it has been found in all known members of the Flaviviridae family. Mechanistic studies have revealed that the NS3-helicase attaches to single-stranded RNA segments and then catalyses the unwinding of duplex RNA (3′ to 5′ direction) in an ATP-dependent manner. Additionally, the structure of the helicase has been determined using X-ray crystallography by two separate groups, as the free

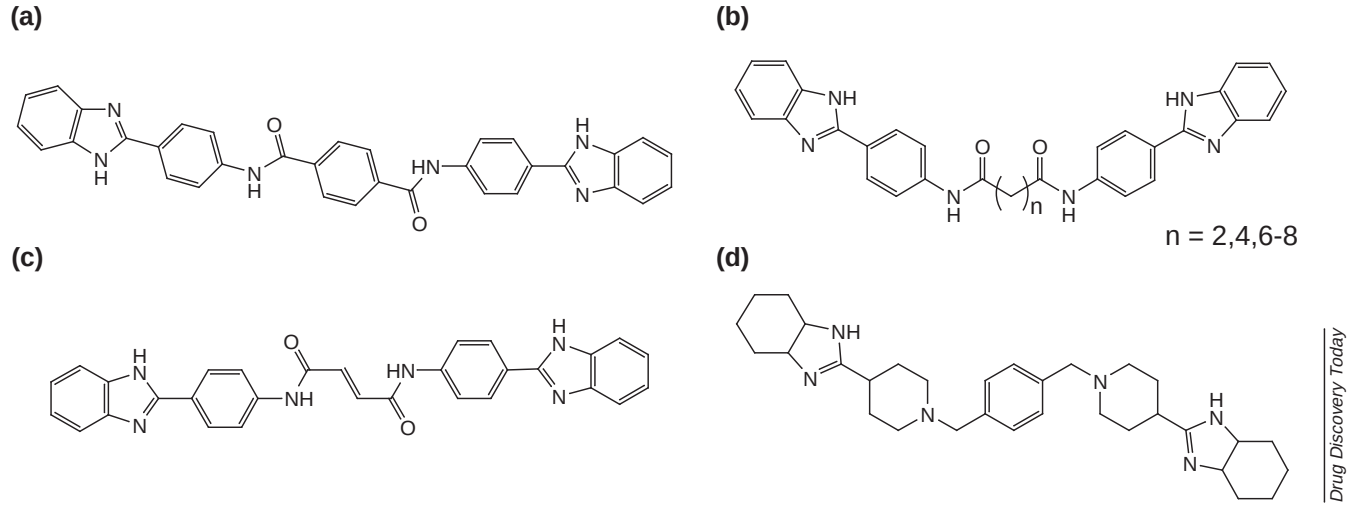


Figure 3. *Inhibitors of helicase.*

enzyme or bound to single-stranded DNA (Refs 40,41). These structures expose several potential sites for drug targeting including the ATP and oligonucleotide binding regions. It could also be suggested that, in addition to blocking replication, any helicase inhibitor might indirectly stimulate a cellular antiviral response because of the expected build-up of double-stranded RNA.

So far, there have been only a few inhibitors reported for the helicase portion of NS3. This is surprising considering the quantity of structural information available for this enzyme. ViroPharma (Malvern, PA, USA) disclosed two related series of compounds having benzimidazole or benzimidazole-like moieties attached to a long symmetrical linker (Fig. 3, IC_{50} = 0.7–10 μM). The mechanism of inhibition has not been disclosed, although their long structure suggests that they might compete with single-stranded RNA for the corresponding enzyme binding site[42,43].

Internal ribosomal entry site (IRES)

Protein translation inhibitors are well known in antibacterial therapy (such as tetracycline, erythromycin and the oxazolidinones). By contrast, there are relatively few antiviral agents that target protein translation despite several viruses possessing unique elements (*cis*-acting RNA or enzymes) that are essential for protein synthesis. The 5′-end of the HCV-genome is a good example. This *cis*-acting RNA element is composed of a highly structured nontranslated domain of 340–342 bases in length [5′-NTR (nontranslated region)] that functions as an IRES. The IRES allows HCV to bypass the normal cellular pathway for initiating protein synthesis by facilitating attachment of the viral RNA to the ribosome, hence enabling cap-independent translation. The HCV-IRES has been classified as a type 3 IRES possessing four hairpins (H1–H4), two of which are considered essential (H2 and H3). The AUG-start codon lies at the extreme 3′-end of the IRES (position 342), although some studies have demonstrated that sequences downstream of this might also be required. In addition to its unique function, the RNA sequence of the 5′-NTR of HCV is highly conserved (>85% sequence identity among the various genotypes), making it an attractive target for antiviral therapy[44,45].

It is believed that viral-protein translation involves cellular enzymes and proteins in addition to those coded for by the virus. Hence, there is a degree of uncertainty in evaluating inhibitors in the absence of an authentic cell culture system as the complete collection of cellular components that form the translation complex or that might contribute to the antiviral effect are absent. *In vitro* assays currently include translation models such as the rabbit reticulocyte lysate and transient expression in HepG2, H8Ad17c or Huh7 cell lines. In general, these systems comprise either a reporter gene (e.g. luciferase) or E1/E2 under the translational control of one of several HCV-IRES constructs [the 5′-NTR and sequences extending into the core (C)]. An *in vivo* model has also been developed in

Table 1. Antisense inhibitors of HCV-IRES-directed translation

Chemistry	Sequence targeted[a]	Assay	Substrate	% Inhibition (conc.)	Refs
ODN	38–65, 134–161,148–175, 312–339, 341–377	Lysate[b]	NTR-C–ΔE1	50–98[c]	48
PS-ODN	326–348	Lysate, HepG2 cells	NTR-ΔC–luc	96 (4.4 μM), 94 (0.3 μM)	49
PS-ODN	342–359, 346–363	MT-2C cells	HCV	100 (10 μM)[d]	50
PS-ODN	328–347, 326–345	Lysate	NTR-C–E1–ΔE2	90 (1.2 μM)	51
PS-ODN, 2′-MeEt, 5-MeC	260–279, 330–349, 340–359	H8Ad17c cells, BALB/c mice	NTR-C–ΔE1, NTR-C–luc	50 (100–200 nM)[e], 52 (20 mg kg^{-1})	46,52
ODN, α-ODN	104–123, 105–124, 106–125, 107–126, 108–127, 338–357, 341–347, 351–377	Lysate, wheat-germ extract	NTR-C–ΔE1	47–95[f] (20-fold excess)	53
PS-ODN	340–353, 348–365, 371–388	Lysate, HepG2 cells	NTR-ΔC–luc, pCMV-NTR–luc	42–94 (4.1 μM), 89 (0.3 μM)	54
PS-ODN, Me-ODN, Bn-ODN	326–348	Cellular	Not specified	96 (5 μM)	55
ODN, PS-ODN, 2′-F–PS-ODN	14–20mer starting at 355	Lysate, H8Ad17c cells	NTR-C–ΔE1	50 (100–200 nM)[g]	56
PS-ODN	258–277, 324–345	Huh7 cells	NTR-ΔC–luc	99 (0.1 μM), 88 (60 μM)	57

[a]Refers to the sequence of HCV-RNA (5′ to 3′ direction) targeted by the antisense agent; [b]RRL, rabbit reticulocyte lysate; [c]Tenfold excess; [d]No (−)RNA detected by RT−PCR five days post-inoculation; [e]IC_{50}; [f]20-fold excess; [g]EC_{50}.

Abbreviations: Bn-ODN, benzylphosphonate-modified oligonucleotide; C, sequence coding for the core protein; CMV, cytomegalovirus; ΔC, sequence coding for the truncated core; E1/E2, sequences coding for envelope proteins 1 and 2; ΔE1/ΔE2, sequences coding for truncated envelope proteins 1 and 2; 2′-F, 2′-fluoro; HCV, hepatitis C virus; luc, luciferase; 5-MeC, 5-methylcytidine; 2′-MeEt, 2′-methoxyethoxy; Me-ODN, methylphosphonate-modified oligodeoxynucleotide; NTR, nontranslated region; ODN, oligodeoxynucleotide; α-ODN, oligodeoxynucleotide having the α-configuration at the anomeric centers; PS, phosphorothioate.

which an adenovirus vector was used to deliver a similar 5′-NTR-reporter gene expression system to BALB/c mice[46]. Alternatively, chimeric viruses have been constructed wherein the HCV-IRES is used in place of the analogous domain on poliovirus or bovine viral diarrhoea virus (BVDV). Interestingly enough, chimeric (HCV-IRES)–BVDV viruses constructed in this fashion were susceptible to pseudo-revertant mutations in the 5′-NTR region (recombination or deletion), yielding a more efficiently replicating virus[47].

Most inhibitors of the HCV-translation reported so far have been antisense agents directed at sequences in the IRES or in the 5′-end of the open reading frame (ORF). Table 1 provides a comprehensive survey of recently discovered agents outlining the chemical composition of the oligodeoxynucleotide (ODN), the targeted sequences in the IRES, the assay system used for screening, and the representative activity[48–57]. Various ODN chemistries are represented in these studies including unmodified DNA (ODN), ribose-modified analogues [2′-methoxyethoxy (2′-MeEt), 2′-fluoro (2′-F) or α-anomeric sugars (α-ODN)], and backbone phosphate ester-modified ODNs [phosphorothioate (PS-ODN), methyl- or benzyl-phosphonates (Me-ODN, Bn-ODN) or peptide nucleic acid (PNA)]. While the sequence targeted by most of these agents is proximal to the initiating AUG-codon, there are some discrepancies as to the exact location of the optimal site for inhibition. These differences probably correspond to the ODN-chemistry used, the assay conditions and the context of the IRES-containing substrate.

To advance these agents, several issues need to be addressed, the principal one being their mechanism of action. Several mechanisms have been observed and/or proposed for antisense agents including inhibition of translation by hybrid arrest, RNAse H-cleavage of the targeted mRNA sequence, stimulation of cellular antiviral pathways, inhibition of viral uptake, and/or non-sequence specific mechanisms[58]. Unfortunately, tissue culture and *in vivo* control experiments using the whole virus are required to unambiguously determine the mode of inhibition. However, the results obtained so far suggest that while RNAse H probably plays a role in whole cell activity, antisense agents directed towards HCV-IRES are capable of inhibiting RNA translation in the absence of RNAse H. This was demonstrated in the rabbit reticulocyte assay where the activity of RNAse H is low and in the whole cell

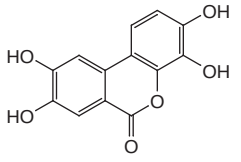


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Figure 4. *Small-molecule inhibitor of hepatitis C internal ribosomal entry site.*

assay of the 2′-MeEt and 2′-F-ODNs, constructs known not to induce RNAse H.

Another concern arises from the unfavorable physicochemical and pharmacokinetic properties of ODN analogues. Because of their large, highly charged and enzymatically labile structure, the primary challenges in developing an ODN drug are drug delivery, cellular uptake, stability and distribution. Despite these difficulties, several antisense agents have recently been evaluated in the clinic as antiviral therapies against papilloma virus, cytomegalovirus (CMV) and HIV. Recently, the anti-CMV PS-ODN drug, formvirsen (Vitravene™), which was discovered by ISIS Pharmaceuticals, has received Food and Drug Administration (FDA) approval for the treatment of CMV-retinitis. This represents a significant milestone in being the first antisense drug approved for marketing in the US.

The IRES folds in a highly ordered way to form a complex structure. Like a protein, it would be expected that this structure possesses pockets suitable for binding small molecules that could inhibit association of the IRES to the ribosome or other translational co-factors. However, only a few reports have appeared that describe small-molecule-based inhibitors of HCV-translation. In one of these reports, analogues of the ellagic acid-derivative shown in Fig. 4 inhibited HCV translation by 30–70% in the rabbit reticulocyte lysate assay. These compounds bound to the HCV-IRES and not to the cellular components of the translation complex and should therefore selectively inhibit IRES-dependent translation over cap-dependent translation[59]. Being highly conserved, the IRES is also an attractive target for ribozyme-based antiviral agents. In theory, ribozymes can be designed that incorporate the catalytic sequences as well as sequences complimentary to selected target sites in the 5′-NTR. Reports describing such agents have appeared in the literature[60–62]. Even so, this is a novel method of antiviral therapy and several challenges, such as the molecular size, delivery and stability of the ODN structure, need to be addressed before these agents can be used in the clinic.

Polymerase

Sequence analysis of the HCV genome reveals that NS5B contains four motifs commonly found among RNA polymerases, the most notable being a GDD sequence at positions 2737–2739, indicating that this domain encodes a RdRP (Ref. 63). Additional evidence comes from the *in vitro* RNA polymerase activity of isolated NS5B expressed in insect cells or in *Escherichia coli*. Although both Mg^{2+} and a primer are generally required for activity, certain heteropolymeric templates can be replicated without added primer by a 'copy back' mechanism where the 3′-terminus folds back on itself[64]. NS5B synthesizes both positive- and negative-stranded RNA, is membrane bound, and forms a replication-complex that includes other HCV-nonstructural proteins such as NS3 (Ref. 65). Because of the lack of a tissue culture system, several questions remain unanswered concerning the timing of the various replication events, the identity of the complete replication complex, and the function of the 5′- and 3′-nontranslated RNA regions.

Viral polymerases are attractive targets for antiviral therapy as demonstrated by the clinical success of nucleoside- and non-nucleoside-inhibitors of HBV and HIV replication. It might therefore be expected that an inhibitor of NS5B will be equally effective in treating HCV. Unfortunately there are some fundamental differences between NS5B and the polymerases encoded for by HBV and HIV. In particular, NS5B is an RdRP, a class of enzyme for which there are few, if any, known specific inhibitors. Furthermore, as with translation-inhibitors, the issue is whether *in vitro* activity obtained with the isolated enzyme is predictive of antiviral activity or merely an artifact of the experimental conditions. To avoid this, some groups have opted to use surrogate viruses (flavi- or pesti-viruses) that are related to HCV. So far, only a few compounds have been identified as HCV-polymerase inhibitors including ribavirin (IC_{50} = 100 μM)[66], cerulinin (IC_{50} ≈500 μM) and cordycepin (3′-dATP, IC_{50} >100 μM)[67].

Other targets

In addition to the viral targets already discussed, information is emerging concerning the function of the remaining structural and nonstructural proteins of HCV such as the NS2–NS3 protease, NS5A and E1–E2. The NS2–NS3 protease is highly unusual in that it does not share significant sequence homology with any known protease, leading to conflicting opinions as to whether it is a metalloprotease or a zinc-dependent cysteine protease. This protease has received little attention as an antiviral target, probably because the protease acts cotranslationally. There is evidence to suggest that resistance to IFN is mediated by NS5A

directly interfering with the IFN-induced intracellular antiviral pathway[68]. If correct, this might represent a possible target for drug development or at least, could be used as a screening tool to aid in treatment decisions. More recently, CD81 has been identified as the putative receptor for viral attachment by E2 (Ref. 69). While there is still more to be learned about HCV tropism and viral attachment and entry, this step in the viral life-cycle has proven to be an effective point to attack other viruses such as influenza and HIV.

Conclusion

As discussed, current successes have been hard won and HCV continues to defy traditional paradigms of medicinal chemistry in that advances have been made in unexpected areas or by exploiting unusual binding properties of the viral enzymes. For example, the NS3-protease has the unique property of binding the N-terminal product more tightly than the substrate to such an extent that inhibitors with nanomolar binding affinities (K_I) have been developed. While work is still required to attenuate the peptidic nature of these inhibitors, it is hoped that a suitable candidate for clinical studies will result. By contrast, the development of HCV-polymerase inhibitors is proceeding at a much slower pace. This is at first surprising when considering that polymerase inhibitors compromise the backbone of HIV, HBV, herpes simplex virus (HSV) and human CMV therapies. Perhaps no HCV-polymerase inhibitors have been reported because the enzyme is an RdRP and, as far as the author is aware, there have been no reports of small-molecule inhibitors active against this class of enzymes. Fortunately, there are other opportunities for inhibiting HCV, such as the HCV-IRES and NS3-helicase, which represent relatively new and unproven targets for antiviral drug development. Although several antisense compounds inhibit IRES function, delivery of these compounds still remains an issue. Reports of helicase inhibitors are only beginning to emerge, and hence, further research needs to be carried out to establish their mechanism of inhibition.

In conclusion, a lot of progress has been made in developing anti-viral agents in the relatively short time that the genome of HCV has been available. Further opportunities are expected to arise as more is learned about the virus and its lifecycle. Despite the number of obstacles that remain, it appears that an effective anti-HCV treatment will emerge within the next ten years.

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REFERENCES

1 Sherlock, S. (1996) *Digest. Dis. Sci.* 41, 3S–5S
2 Houghten, M. (1996) in *Fields Virology* (3rd edn) (Fields, B.N. *et al.*, eds), pp. 1035–1058, Raven Press
3 Sharara, A.I., Hunt, C.M. and Hamilton, J.D. (1996) *Ann. Intern. Med.* 125, 658–668
4 Rice, C.M. (1996) in *Fields Virology* (3rd edn) (Fields, B.N. *et al.*, eds), pp. 931–952, Raven Press
5 Kew, M.C. (1997) *Yale J. Biol. Med.* 70, 119–125
6 Herrine, S.K. and Weinberg, D.S. (1999) *Infect. Med.* 16, 111–117
7 Peters, M. (1996) *Hepatology* 23, 909–916
8 Poynard, T. *et al.* (1998) in *Therapies for Viral Hepatitis* (Schinazi, R.F., Sommadossi, J-P. and Thomas, H.C., eds), pp. 371–378, International Medical Press
9 Hoofnagle, J.H. and Bisceglie, A.M. (1997) *New Engl. J. Med.* 336, 347–356
10 McHutchison, J.G. *et al.* (1998) *New Engl. J. Med.* 339, 1485–1492
11 Davis, G.L. *et al.* (1998) *New Engl. J. Med.* 339, 1493–1499
12 Patterson, J. and Fernandez-Larsson, R. (1990) *Rev. Infect. Dis.* 12, 1139–1146
13 Choo, Q-L. *et al.* (1989) *Science* 244, 359–362
14 Major, M.E. and Feinstone, S.M. (1997) *Hepatology* 25, 1527–1538
15 Blight, K.L. *et al.* (1998) in *Therapies for Viral Hepatitis* (Schinazi, R.F., Sommadossi, J-P. and Thomas, H.C., eds), pp. 207–217, International Medical Press
16 Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162
17 Lohmann, V., Koch, J.O. and Bartenschlager, R. (1996) *J. Hepatol.* 24(Suppl. 2), 11–19
18 Kwong, A.D. *et al.* (1998) *Antiviral Res.* 40, 1–18
19 Chu, M. *et al.* (1996) *Tetrahedron Lett.* 37, 7229–7232
20 Sudo, K. *et al.* (1997) *Biochem. Biophys. Res. Commun.* 238, 643–647
21 Sudo, K. *et al.* (1997) *Antiviral Chem. Chemother.* 8, 541–544
22 Kakiuchi, N. *et al.* (1998) *FEBS Lett.* 421, 217–220
23 Landro, J.A. *et al.* (1997) *Biochemistry* 36, 9340–9348
24 Steinkühler, C. *et al.* (1998) *Biochemistry* 37, 8899–8905
25 Ingallinella, P. *et al.* (1998) *Biochemistry* 37, 8906–8914
26 Llinàs-Brunet, M. *et al.* (1998) *Bioorg. Med. Chem. Lett.* 8, 1713–1718
27 Llinàs-Brunet, M. *et al.* (1998) *Bioorg. Med. Chem. Lett.* 8, 2719–2724
28 Tung, R.D. *et al.* (Vertex Pharmaceuticals Inc.) (1998) WO9817679
29 Attwood, M.R. *et al.* (F. Hoffman-La Roche) (1998) WO9822496
30 Shimizu, Y. *et al.* (1996) *J. Virol.* 70, 127–132
31 Zhang, R., Mui, P.W. and Weber, P.C. (Schering Corp.) (1997) WO9743310
32 Martin, F. *et al.* (1997) *Protein Eng.* 10, 607–614
33 Dimasi, N. *et al.* (1997) *J. Virol.* 71, 7461–7469
34 Martin, F. *et al.* (1998) *Biochemistry* 37, 11459–11468
35 Urvil, P.T. *et al.* (1997) *Eur. J. Biochem.* 248, 130–138
36 Kumar, P.K.R. *et al.* (1997) *Virology* 237, 270–282
37 Gangwar, S. *et al.* (1997) *Drug Discovery Today* 2, 148–155

38 Jin, L. and Peterson, D.L. (1995) *Arch. Biochem. Biophys.* 323, 47–53
39 Kim, D.W. *et al.* (1995) *Biochem. Biophys. Res. Commun.* 215, 160–166
40 Yao, N. *et al.* (1997) *Nat. Struct. Biol.* 4, 463–467
41 Kim, J.L. *et al.* (1998) *Structure* 6, 89–100
42 Diana, G.D. and Bailey, T.R. (ViroPharma Inc.) (1997) US5633388
43 Diana, G.D., Bailey, T.R. and Nitz, T.J. (ViroPharma Inc.) (1997) WO9736554
44 Lemon, S.M. and Honda, M. (1997) *Semin. Virol.* 8, 274–288
45 Wang, C. and Siddiqui, A. (1995) *Curr. Top. Microbiol. Immunol.* 203, 99–115
46 Zhang, H. *et al.* (1999) *Antimicrob. Agents Chemother.* 43, 347–353
47 Frolov, I., McBride, M.S. and Rice, C.M. (1998) *RNA* 4, 1418–1435
48 Wakita, T. and Wands, J.R. (1994) *J. Biol. Chem.* 269, 14205–14210
49 Alt, M. *et al.* (1995) *Hepatology* 22, 707–717
50 Mitzutani, T. *et al.* (1995) *Biochem. Biophys. Res. Commun.* 212, 906–911
51 Seki, M. and Honda, Y. (1995) *J. Biochem.* 118, 1199–1204
52 Hanecak, R. *et al.* (1996) *J. Virol.* 70, 5203–5212
53 Vidalin, O. *et al.* (1996) *Antimicrob. Agents Chemother.* 40, 2337–2344
54 Alt, M. *et al.* (1997) *Arch. Virol.* 142, 589–599
55 Eisenhardt, S. *et al.* (1997) *Nucleosides Nucleotides* 16, 1669–1672
56 Lima, W.F. *et al.* (1997) *J. Biol. Chem.* 272, 626–638
57 Wu, C.H. and Wu, G.Y. (1998) *Gastroenterology* 114, 1304–1312
58 Branch, A.A. (1996) *Hepatology* 24, 1517–1529
59 Ikeda, N. *et al.* (Eisai Co. Ltd) (1996) JP8268890
60 Lieber, A. *et al.* (1996) *J. Virol.* 70, 8782–8791
61 Sakamoto, N., Wu, C.H. and Wu, G.Y (1996) *J. Clin. Invest.* 98, 2720–2728
62 Welch, P.J. *et al.* (1996) *Gene Ther.* 3, 994–1001
63 Poch, O. *et al.* (1989) *EMBO J.* 8, 3867–3874
64 Behrens, S-E., Tomei, L. and De Francesco, R. (1996) *EMBO J.* 15, 12–22
65 Ishido, S., Fujita, T. and Hotta, H. (1998) *Biochem. Biophys. Res. Commun.* 244, 35–40
66 Yuan, Z-H. *et al.* (1997) *Biochem. Biophys. Res. Commun.* 232, 231–235
67 Lohmann, V. *et al.* (1997) *J. Virol.* 71, 8416–8428
68 Enomoto, N. *et al.* (1996) *New Engl. J. Med.* 334, 77–81
69 Pileri, P. *et al.* (1998) *Science* 282, 938–941

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