Challenges and successes in developing new therapies for hepatitis C

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Hepatitis C virus (HCV) will continue to be a serious global health threat for many years to come because of the chronic nature of the infection, its high prevalence and the significant morbidity of the resulting disease. Recently, a small number of molecules have produced encouraging results in proof-of-concept clinical trials. At the same time, preclinical evidence is accumulating that development of resistance will eventually limit the efficacy of new drugs. Thus, combinations of multiple agents will be required to treat chronic HCV infection.

With an estimated 170 million infected individuals worldwide, hepatitis C virus (HCV) exacts a heavy toll on public health[1]. Despite considerable reduction of the incidence of new infections, the prevalence of HCV infection is predicted to remain constant in the near future[2]. Our current interferon (IFN)-based therapies are effective in only a fraction of the patients and are plagued with adverse effects (see the review by Hoofnagle in this issue, page 967). New treatment regimens are needed that are more efficacious and better tolerated by all patients. Investigators have taken several different approaches to address this pressing medical need. Major research efforts have focused on the identification of agents that inhibit specific steps in the life cycle of the virus. These 'HCV-targeted drugs' include small-molecule, orally bioavailable inhibitors of the HCV enzymes as well as nucleic-acid-based agents that attack the viral RNA. In addition, agents that can modulate the host immune response are being investigated for their ability to control and possibly eradicate HCV infection. In spite of the difficulties posed by the lack of readily available laboratory models of viral infection, a handful of investigational compounds have just started to show promising results in early-phase clinical trials (Table 1).

Drug-resistant viruses emerge rapidly under the selective pressures exerted by antiviral drugs, however. This is a major concern for successful anti-HCV therapy. The fast turnover rate and the intrinsic low fidelity of the HCV replication machinery endows the virus with the ability to fully explore its genome space and quickly come up with mutations that render it resistant to antiviral drugs. Each newly generated HCV genome is expected to exhibit, on average, one nucleotide change per replication cycle. As a consequence, even in untreated individuals, HCV exists as a genetically heterogeneous viral population, termed 'quasispecies'. Thus, the clinical success of HCV-targeted drugs will depend on their ability to suppress all viral variants as well as prevent the emergence of resistant viruses. In view of these considerations, it is crucial that the optimization of anti-HCV drug candidates is guided by the study of their spectrum of action on the different genotypes as well as by their resistance profile.

In the absence of an efficient *in vitro* infection system, preclinical studies of HCV antiviral resistance were made possible by the availability of HCV replicons (see Box 1 and the review in this issue by Lindenbach and Rice, page 933). So far, it has been possible to select replicons that are resistant to several HCV enzyme inhibitors as well as replicons that escape the action of small interfering RNAs (siRNAs) directed against the HCV genome[3]. For all agents analysed so far, a single mutation in the target gene seemed sufficient for conferring drug resistance *in vitro*. Depending on the genetic make-up of the replicon, some of these resistance mutations affected the replication fitness in tissue culture. Given the many unknowns and the different requirements for efficient HCV replication *in vitro* and *in vivo* (see the review in this issue by Lindenbach and Rice, page 933), these findings cannot be directly extrapolated to viruses in infected individuals. Nonetheless, these *in vitro* data demonstrate the magnitude of the problem and allow an estimate of the genetic barrier that the virus has to overcome to acquire resistance to a given antiviral agent. Ultimately, it is likely that the combination of multiple drugs, possibly directed at viral as well as at host targets, will be required to contain the emergence of drug-resistant HCV variants and efficaciously treat chronic HCV infection.

This review provides an overview of recent progress in the identification of new targets and approaches. We will discuss, in this order, progress towards developing drugs targeting the viral enzymes or the viral genome, and then novel immunomodulatory molecules, with particular emphasis on the agents with demonstrated antiviral activity in the clinics.

Small-molecule inhibitors of viral enzymes

The development of direct antivirals that block essential viral enzymes represents a straightforward approach to developing new anti-HCV agents. Although all HCV enzymes are, in theory, equally appropriate for therapeutic intervention, the NS3-4A serine protease and the NS5B RNA polymerase have emerged as the most popular targets. A number of competitive inhibitors of the NS3 protease as well as nucleoside and non-nucleoside inhibitors of the NS5B polymerase are being developed. The efficacy shown by NS3 serine protease and the NS5B RNA-dependent RNA polymerase inhibitors in recent proof-of-concept clinical trials have validated the efforts spent in search of clinical candidates and triggered a renewed interest in this arena.

Inhibitors of the NS3-4A protease

The NS3-4A protease is a heterodimeric protease, comprising the amino-terminal domain of the NS3 protein and the small NS4A cofactor. Its activity is essential for the generation of components of the viral RNA replication complex (see the review in this issue by Lindenbach and Rice, page 933). Structurally, the HCV enzyme is a member of the chymotrypsin serine protease family, but its het-

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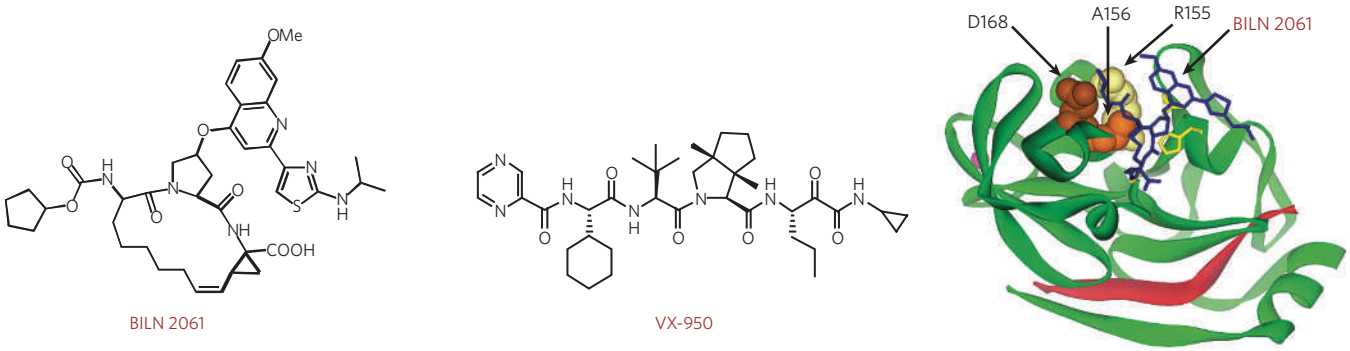

Figure 1 | The structures of inhibitors BILN 2061 and VX-950 and of the NS3 serine protease domain (green) complexed with the central domain of NS4A (red). The residues that constitute the enzyme catalytic triad (histidine 57, aspartate 81 and serine 139) are shown as yellow (stick representation). The structural zinc atom is indicated in purple. The chemical structures of the protease inhibitors BILN 2061 and VX-950 are shown on the left. BILN 2061 is also modelled in the active site. Residues responsible for resistance to peptidomimetic protease inhibitors are shown in space-filling mode and colour-coded. See text for details.

erodimeric nature and the presence of a structural zinc atom differentiate the NS3-4A protease from the other members of the family (Fig. 1). The enzyme cleaves the viral polyprotein at four junctions with a temporal sequence that is presumably crucial for replication. In addition, the NS3-4A protease activity has been implicated in blocking the host cell's ability to mount an innate antiviral response (see the review in this issue by Gale and Foy, page 939). This observation has fostered the hope that inhibitors of this enzyme will block virus replication with a double hit, leading to an increased antiviral efficacy.

The substrate specificity of the NS3-4A protease is very different from that of related host enzymes[4]. Although this made the identification of inhibitors for the HCV enzyme conceivable, early biochemical and structural studies provided the first glimpse that developing such agents as drugs was no trivial task. The enzyme requires a long peptide substrate, with which it establishes multiple weak interactions distributed along an extended surface. The requirement for such long substrates instilled the fear that the enzyme could only be inhibited by molecules sufficiently large to mimic the natural substrate, and large molecules cannot be easily converted into drugs. The resolution of the three-dimensional structure of the enzyme provided another blow to the enthusiasm of drug developers. The substrate-binding cleft of NS3-4A protease seemed flat and featureless, lacking the cavities, holes and flaps that had been exploited as anchor points to design potent and selective inhibitors of other proteases[5–7]. In spite of these difficulties, a few groups continued their search for inhibitors capitalizing on the observation that the enzyme is susceptible to marked inhibition by the N-terminal peptide products released from the substrates upon enzymatic cleavage[8,9].

BILN 2061 (Ciluprevir; Boehringer Ingelheim), a macrocyclic mimic of peptide product inhibitors (Fig. 1), was the first HCV protease inhibitor to enter clinical trials[10]. The development of BILN 2061 was built upon the finding that the carboxy-terminal carboxylic acid of the hexapeptide products provided an active-site affinity anchor and a moiety that warranted the desired selectivity for the HCV enzyme over the cellular proteases[11]. Structural information guided optimization of the side chains and the conversion of the linear peptidic leads into macrocyclic inhibitors with enhanced potencies and improved biopharmaceuticals properties[12,13], culminating in the selection of BILN 2061 as a clinical candidate. When administered to patients infected by genotype-1 HCV twice a day for only two days, BILN 2061 induced a rapid, dose-dependent decline of the viral load, exceeding a 2 $\log_{10}$ reduction in all individuals receiving the higher doses of the drug[10,14]. Treatment with BILN 2061 was somewhat less effective on genotype-2 and -3 viruses, although it resulted in a clinically meaningful reduction of the viral titre in several patients[15]. Although the effect of BILN 2061 was transient, the hope is that longer treatments with HCV protease inhibitors will achieve high rates of sustained viral response and particularly so in patients that do not respond well to IFN therapy.

Unfortunately, the clinical development of BILN 2061 was halted because of the observation of cardiac toxicity in laboratory animals[15]. Toxicity may not be the only concern, however. As this agent progressed to clinical trials, development of resistance to BILN 2061 was observed in the HCV replicon system[16,17]. A single mutation is sufficient to confer resistance to BILN 2061 *in vitro*, predicting that escape mutants might emerge with high frequency in HCV-infected patients. Substitution for either arginine 155, alanine 156 or aspartate 168 in the NS3 protease domain (Fig. 1) induced a high degree of resistance to BILN 2061 and to related analogues[16–18]. Intriguingly, replacement of aspartate 168 with glutamine is naturally found in the NS3 protein of genotype 3 viral isolates, possibly explaining the reduced activity of BILN 2061 strain patients infected with this viral strain[19].

VX-950 (Vertex/Mitsubishi) is a product-derived peptidomimetic inhibitor of the NS3-4A protease that is stabilized into the enzyme's

Box 1 | Laboratory tools for evaluating HCV antiviral resistance

Historically, the lack of readily available animal models for HCV infection and the inability to infect cultured cells have been major obstacles in establishing reliable antiviral assays. A major breakthrough for evaluating candidate antiviral agent in cell culture is represented by the so-called HCV replicons. HCV replicons are engineered subgenomic HCV RNAs capable of autonomous replication once introduced in cultivated cells.

The lack of a robust laboratory infection system makes preclinical studies of HCV antiviral resistance also quite challenging. However, it has been recently shown that the HCV replicon system, combined with biochemical assays and reverse genetics, can be exploited to study the emergence of resistance to anti-HCV agents *in vitro*.

This type of *in vitro* evolution and selection is made possible by two replicon features. On one hand, the low fidelity of RNA replication in tissue culture generates a genetic diversity in the replicon population that is likely to recapitulate the heterogeneity of the natural HCV quasispecies. On the other hand, the inclusion of a dominant selectable marker in the replicon sequence, such as neomycin phosphotransferase, allows the selection of replicon variants that become insensitive to the antiviral agent used during the selection. In brief, the ability of the replicon cells to survive in the presence of the antibiotic neomycin relies on replicon-driven expression of neomycin phosphotransferase. Thus, inhibitors of viral RNA replication abolish expression of resistance to neomycin in cells that harbour wild-type replicons. Under these conditions, cells containing replicons sensitive to the inhibitor are eliminated because of the antibiotic effect, while cells containing replicon variants with decreased sensitivity to the antiviral agent under study will survive and give rise to clones that can be isolated and characterized. Taking advantage of these features, it is possible to isolate cell clones containing resistant replicons by culturing a relatively small number of cells in the presence of neomycin and of a given inhibitor.

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active site through the inclusion of an α-ketoamide[20] (Fig. 1). The α-ketoamide moiety is an enzyme active-site anchor capable of forming a reversible covalent bond with the catalytic serine. The incorporation of such a 'warhead' is a customary approach to making potent serine-protease inhibitors but is often disregarded because covalent protein binding is potentially responsible for an unfavourable safety profile. Not only did VX-950 efficiently inhibit HCV replication in cell culture, it was also sufficiently tolerated in laboratory animals to prompt its advancement in clinical trials. Interim results of phase Ia/Ib clinical trials indicate that VX-950 was well tolerated and demonstrated outstanding antiviral activity[21]. Treatment with VX-950 induced a fast decline of the viral load in patients infected by genotype-1 HCV, with a median reduction in HCV RNA ranging between 2 and 4.4 $\log_{10}$ at the end of 2 weeks of therapy. Although this relatively short treatment was not sufficient to eradicate the virus, and viraemia returned to baseline after stopping therapy, these encouraging results have stimulated the planning of additional clinical studies.

VX-950 interacts covalently with the protease but this does not render it immune to the development of viral resistance[17]. There is only partial cross-resistance between BILN 2061 and VX-950, possibly reflecting the differences in the interaction of different inhibitor types with the enzyme. In fact, substitutions for aspartate 168 of NS3 confer resistance to BILN 2061 but not to VX-950. Conversely, replacement of alanine 156 yields different outcomes depending on the nature of the mutation. Thus, replacement of alanine 156 with serine confers selective resistance to VX-950 (ref. 17), whereas replacement of the same residue with threonine or valine confers significant cross-resistance to VX-950 and BILN 2061 as well as to structurally different protease inhibitors[22,23].

Several other peptidomimetic inhibitors of the NS3-4A protease are at various stages of development. However, since cross-resistance to diverse protease inhibitors can be achieved with the mutation of a single amino acid, the combination of multiple protease inhibitors could turn out to have limited value in the clinical practice.

NS5B polymerase inhibitors

The RNA-dependent RNA polymerase (RdRp) contained within the NS5B protein is the catalytic component of the HCV RNA replication machinery[24]. This enzyme synthesizes RNA using an RNA template. This biochemical activity is not present in mammalian cells, offering the opportunity to identify very selective inhibitors of the viral enzyme. In common with other polymerases, the structure of the NS5B enzyme is imaginatively assimilated to that of a right hand[25–27] (Fig. 3). The palm domain contains the active site of the enzyme whereas the fingers and the thumb modulate the interaction with the RNA chain. In the conformation more frequently seen in the crystal, two loops extending from the fingers domain — the fingertips — are in contact with the thumb domain, leading to the formation of a 'closed' active site tunnel in which the RNA template, the nascent RNA strand and the nucleotide substrates are accommodated during the polymerization reactions. The NS5B polymerase can also adopt a more 'open' conformation, where the contact between the fingertips and the thumb is disrupted, resulting in a wider access to the catalytic site[28]. As noted below, the resolution of the structure in complex with allosteric inhibitors suggests that some compounds work by freezing the enzyme in the 'open', inactive conformation.

Unlike the NS3-4A protease, the N5B RdRp is a very tractable drug discovery target. The rational search of substrate analogues led to the identification of several nucleoside analogues, whereas high-throughput screening efforts have uncovered a variety of non-nucleoside inhibitors (NNIs) (Fig. 2). Nucleoside analogues need to be converted by the host-cell machinery to the corresponding nucleotides, which in turn inhibit synthesis of viral RNA as 'chain terminators', that is, substrate analogues that are incorporated by the viral polymerase in the nascent RNA molecule and induce premature termination of the RNA synthesis. Conversely, NNIs are almost invariably allosteric inhibitors believed to block the enzyme, preventing a conformational transition needed for initiation of RNA synthesis[29]. Interestingly, several different binding sites for NNIs exist on the HCV polymerase. Furthermore, the different classes of nucleoside analogues and NNIs elicit diverse patterns of resistance. Thus, although these agents target the same viral enzyme, they offer the potential to be developed for combination therapy.

Nucleoside analogues

NM283 (Valopicitabine; Idenix/Novartis) is so far the only inhibitor of the NS5B polymerase with demonstrated antiviral activity in the clinics[30]. NM283 is an oral prodrug of 2′-C-methyl-cytidine (Fig. 2). The

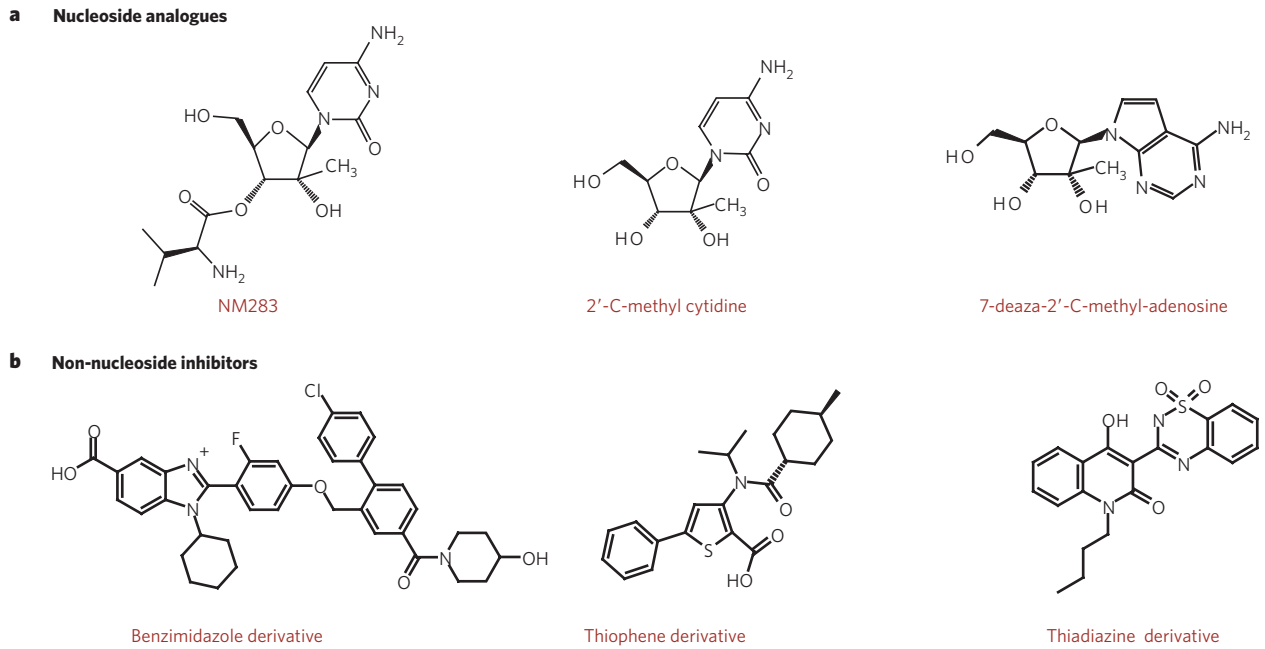

Figure 2 | Examples of inhibitors of the HCV RNA-dependent RNA polymerase. a, Structures of 2′-C-methyl-nucleosides. NM283 is a valine ester of NM107. **b**, Structures of representative examples of different classes of non-nucleoside inhibitors of the NS5B polymerase, each presumably binding at a different allosteric site on the enzyme: benzimidazoles, benzothiadiazines and thiophenes.

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latter compound was initially identified as an inhibitor of the HCV-related bovine viral diarrhoea virus (BVDV) and later shown to inhibit HCV RNA replication in the replicon assay[31,32]. Interestingly, 2′-C-methyl purine nucleosides (Fig. 2) also inhibit replication of HCV, BVDV and other Flaviviruses, indicating that the addition of a methyl group at the 2′ position of the ribose is sufficient to transform the nucleotide substrates into specific chain-terminators of the *Flaviviridae* RdRps[33,34]. When administered to genotype-1 HCV patients for at least 2 weeks, NM283 induced a dose-dependent decline of the viral load to less then 10% of the initial levels in patients receiving an 800 mg daily dose[30]. As expected, in all patients viraemia returned to pre-treatment levels after stopping therapy. Although NM283 was not as effective or rapid as BILN 2061 and VX-950, it demonstrated antiviral activity at tolerated doses. This encouraged the progression to longer term clinical trials in combination with pegylated IFN-α. Interim results after 24 weeks of therapy show that this combination caused a mean reduction in viral load of more than 10,000-fold, and HCV RNA was undetectable in a significant fraction of the patients[35]. More extensive evaluation in combination trials is needed to assess whether it will achieve a sustained viral response rates superior to the current standard of care.

2′-C-methyl-nucleosides inhibit NS5B enzymes and replicons derived from different HCV genotypes and may have potential for treatment for all viral strains[32,36]. But these agents are also readily susceptible to resistance development[33,34]. Selection of replicons resistant to 2′-C-methyl-nucleosides has shown that HCV quickly learns to discriminate between these agents and the natural nucleotides. The virus acquires resistance to 2′-C-methyl-nucleosides by replacing serine 282 of NS5B with threonine. Ironically, from a chemical viewpoint, this mutation corresponds to the addition of a methyl group, the same modification used to convert the enzyme substrates into inhibitors. Replicons carrying the replacements of serine 282 with threonine show a decreased replication fitness[33]. It remains to be defined whether this mutation will have similar debilitating effects on HCV replication also in a more physiological setting.

Non-nucleoside inhibitors

Among the NNIs of the NS5B polymerase, at least three different classes of compound are being evaluated in the clinic, but limited data have been disclosed on these drug candidates. The first NNIs of the NS5B polymerase to enter clinical trials were two oral agents JTK-109 and JTK-003 (Japan Tobacco). Very little has been so far disclosed on the clinical development of these compounds except that JTK-003 has been advanced to phase II. From the patent literature[37], JTK-109 and JTK-003 are assumed to be part of a heterogeneous series of 6,5-fused heterocyclic compounds based on a benzimidazole or indole core (Fig. 3). These compounds act as allosteric inhibitors and block polymerase activity before elongation, presumably impeding the conformational transition needed for the formation of a productive polymerase–RNA complex[38,39].

The very recent determination of the structure of the polymerase in complex with related analogues showed that these inhibitors bind on the surface of the thumb domain in a cavity that in the free enzyme is normally occupied by one of the fingertips (Fig. 3)[40]. Inhibitor binding disrupts the fingertips–thumb interactions and forces the enzyme into an 'open', inactive conformation. Resistance to this class of inhibitor arises through a single mutation within the inhibitor binding site — replacement of proline 495 with alanine or leucine — which strongly reduces affinity for the inhibitors[39]. Replicons carrying substitutions for proline 495 replicate inefficiently, but their fitness can be restored by mutations elsewhere in the NS5B coding region. In spite of the observation that proline 495 is absolutely conserved across all HCV isolates, compounds with this mechanism of action are significantly less active on enzymes and replicons derived from genotype-2 clinical isolates[32], potentially limiting their clinical use.

A number of other polymerase NNIs progressed to clinical trials: these include R803 (Rigel) and HCV-371, HCV-086 and HCV-796

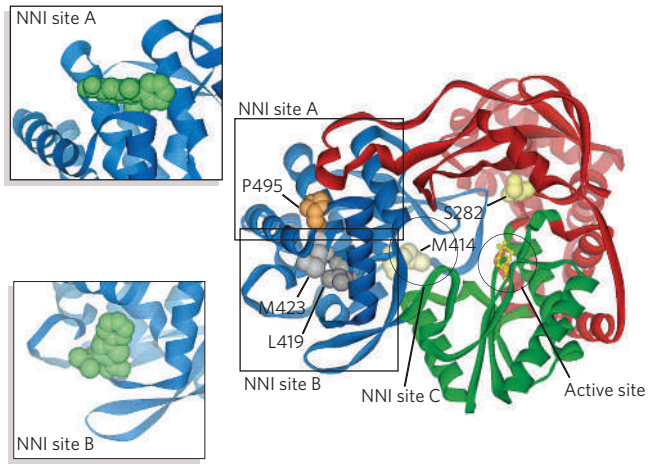

Figure 3 | The crystal structure of the NS5B RNA-dependent RNA polymerase. The thumb, palm and fingers domains are coloured in blue, green and red, respectively. The side chains of the two catalytic aspartates are in yellow in the active site (stick representation). The residues responsible for resistance to 2′-C-methyl nucleoside analogues (serine 282), benzimidazole (proline 495), thiophene (methionine 419 or threonine 423) or thiadiazine inhibitors (methionine 414) are shown in space-filling style and indicated in the figure. The insets show the details of the polymerase in complex with a 5,6-fused heterocyclic inhibitor (NNI site A) or a thiophene inhibitor (NNI site B). For clarity, space-filling models of the inhibitors are shown in the insets in bright green. In the apoprotein structure, the thiophene binding pocket is occupied by the solvent while the benzimidazole binding site is occupied by the short α-helix at the extremity of one of the fingertip loops. NNI site C indicates a tentative location of the binding site for the inhibitors of the thiadiazine class based on resistance and indirect biochemical data.

(ViroPharma/Wyeth). R803 was identified and optimized using the replicon assay[41]. Subsequently, biochemical and resistance studies demonstrated that R803 and related inhibitors targeted the NS5B polymerase. In fact, substitutions for tyrosine 452 or arginine 465 of the NS5B polymerase conferred resistance to a close analogue of R803. The unique resistance pattern of this class of compound suggests that they are mechanistically distinct from other known NNIs. R803 was selected for clinical development, but the clinical investigation was soon terminated because of poor efficacy of the compound, presumably because of inadequate pharmacokinetics[42]. Related compounds are being developed, including a prodrug of R803 and derivatives of R803 with improved pharmacokinetic properties.

No data have been disclosed yet on the preclinical characterization of HCV-371, HCV-086 and HCV-796, but they are allegedly allosteric inhibitors of the NS5B polymerase. Development of HCV-371 and HCV-086 was also halted because of insufficient antiviral activity. Nonetheless, the data obtained with HCV-086 expedited clinical studies of the related compound HCV-796, a more potent analogue of HCV-086 with demonstrated antiviral activity in an animal model of hepatitis C infection[43].

Aside from the compounds that have made it to the clinic, several other NS5B NNIs have been reported to inhibit HCV replication in tissue culture and are being considered for development[29]. Among these, a class of thiophene derivatives has been characterized extensively[44,45]. These compounds are reversible allosteric inhibitors of the NS5B polymerase and bind to the enzyme at the base the thumb domain, in a long cleft that is close to, but distinct from, the site occupied by benzimidazole-based inhibitors[28] (Fig. 3). Intriguingly, binding of thiophene inhibitors also induces a conformational change of the enzyme toward a more 'open' form, suggesting that the two classes of compound inhibit RNA synthesis with a similar mechanism. *In vitro* studies have shown that resistance to thiophene inhibitors arises through substitution for either of two residues — methionine 419 or threonine 423 — located in the hydrophobic region of the inhibitors binding site[3]. Interestingly, although the thiophene-binding region is

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well conserved among HCV genotypes, compounds of this class are significantly less potent on polymerases of non-1 genotypes[32]. The crystallographic evidence that the same binding pocket is also targeted by structurally different inhibitors based on a phenylalanine or dihydropyranone scaffold provides important information for further optimization of inhibitors binding at this site[46,47].

Another class of allosteric inhibitor of the HCV polymerase is based on a benzothiadiazine scaffold[48] (Fig. 2). Similarly to other NNIs of the NS5B polymerase, compounds from this class inhibit RNA synthesis acting before the formation of an elongation complex[49,50]. The available biochemical data, however, suggest that benzothiadiazines bind the enzyme at a different site and possibly act through different mechanisms from the allosteric inhibitors described above[50]. This hypothesis was strengthened by the unique pattern of resistance mutations obtained in the replicon system with these compounds[50,51]. Indeed, several different single NS5B mutations, mapping to different regions of the polymerase, have been reported to induce resistance to benzothiadiazines in the replicon system. Of these, only the mutants of methionine 414 demonstrated a clear resistance to inhibition in biochemical assays using the purified enzyme. The observed resistance could be ascribed to reduced affinity for the inhibitor, thus implicating methionine 414 as part of the inhibitor-binding site. On the basis of the location of this residue in the inner surface of the thumb domain, close to the active site, it is tempting to speculate that the binding site of benzothiadiazines is distinct from those of other allosteric inhibitors (Fig. 3). Sequence analysis of natural HCV isolates revealed that the NS5B residues involved in resistance to benzothiadiazines are not entirely conserved among HCV genotypes, implying that some naturally occurring HCV isolates might be resistant to this class of inhibitor. In line with this consideration, representative benzothiadiazines have been found to inhibit only a limited subset of enzymes and replicons derived from clinical isolates of different genotypes[32,36].

Nucleic-acid-based antiviral agents

The concept of using synthetic nucleic acids as drugs has received increasing attention over the past few years. In particular, antisense oligonucleotides, ribozymes and, more recently, siRNAs are being explored as therapeutic agents in a number of areas. One of the major issues that will determine the success of nucleic-acid-based drugs is efficient delivery of the synthetic polymers to the appropriate cells *in vivo*. Because of their size and chemical nature, nucleic-acid polymers are not orally bioavailable and can be administered only parenterally. Perhaps reflecting anatomical and physiological features, systemic administration seems to work best for the liver and less efficiently for other organs[52]. In this respect, HCV could be viewed as the ideal target for successful development of effective nucleic-acid-based drugs.

The HCV internal ribosome entry site (IRES) found at the 5′ end of the viral genome has been considered the most attractive target for the development of RNA-based drugs, both because of the wealth of data available on IRES structure and function and because of the conservation among HCV genotypes. Ribozymes and antisense oligonucleotides designed to target the HCV IRES reduced the HCV RNA translation and replication in cell culture. The ribozyme RPI.13919 (Heptazyme; RPI) and the antisense oligonucleotide ISIS-14803 (ISIS Pharmaceuticals) have both progressed to early-phase clinical studies in HCV-infected patients[53], but their development was halted because of adverse effects or because of the limited efficacy.

RNA interference approach to HCV antivirals

RNA interference (RNAi) is the latest addition to the list of nucleic-acid based approaches being explored for HCV therapy[54–58] (Box 2). In tissue culture, siRNA, as well as vector-encoded short hairpin RNA (shRNA) directed against the viral genome, effectively blocks the replication of HCV replicons. Not all siRNAs showed the same efficiency, but all regions of the HCV genome were susceptible to RNAi, including conserved sequences. The extent and the duration of silencing varied greatly between the different studies, but the most effective siRNA seemed capable of completely eradicating HCV from more than 98% of the replicon-bearing cells[58]. The positive outcome of these *in vitro* studies stimulated scientists to take up the challenge of identifying appropriate means for efficient *in vivo* delivery of siRNA. Given the notorious lack of readily accessible laboratory models for HCV infection, initial animal studies simply addressed the ability to knockdown messenger RNA expression in mouse liver. High-pressure injection in the tail vein of siRNA or shRNA targeting the surface receptor FAS, caspase 8 or a reporter gene containing HCV sequences silenced the cognate mRNAs[59–61]. Despite studies proving that naked, unmodified siRNA or shRNA can effectively induce RNAi *in vivo*, it is doubtful that the hydrodynamic injection method employed has the potential for clinical application. Possibly more promising is the use of chemically modified siRNA. Several avenues are being explored and the most promising seem capable of enhancing siRNA stability and cell penetration without reducing the silencing efficiency. Interestingly, chemically modified siRNA seem able to inhibit HCV replication not only in tissue culture but also in a mouse model of HCV infection[62].

Resistance development is a potential obstacle also for RNAi-based therapy. HCV can develop resistance to prolonged treatment with siRNA through the accumulation of nucleotide point mutations within the siRNA target sequence[63]. As expected, replicons resistant to a given siRNA remain susceptible to siRNAs targeting different HCV RNA sequences, and the emergences of resistant replicons is diminished by the combination of two or more siRNAs. Thus, the use of two or more siRNAs targeting different sequences of the viral genome may provide a way to control the development of resistance. Indeed, BLT-HCV (Benitec), the first clinical candidate to treat HCV infection through RNAi, consists of three components targeting different HCV sequences, underlining the importance of a multi-targeting approach to prevent resistance development[64].

Novel immunomodulatory agents

The experience with IFN-based therapies has demonstrated that HCV infection can be eradicated by agents that stimulate the host innate and adaptive immunity. Fuelled by this observation, synthetic agonists of Toll-like receptors (TLRs) 7 and 9 have progressed through early-phase clinical trials and have now begun to show their potential in controlling HCV infection.

Toll-like receptors are molecular sentinels that sense the presence of invading microorganisms through the recognition of molecular patterns characteristic of pathogens such as bacteria, viruses and parasites[65]. They are expressed by immune cells, which include macrophages, monocytes, dendritic cells and B cells[66]. Signalling by stimulated TLRs initiates acute inflammatory responses by induction of antimicrobial genes and pro-inflammatory cytokines and chemokines.

So far, ten different TLRs have been identified in humans, each recognizing molecular patterns associated with a specific class of microbial agents[66]. TLRs 3, 7, 8 and 9 are intracellular receptors specialized in the recognition of viral nucleic acids: they detect, respectively, double-stranded RNA (TLR-3), single-stranded RNA (TLR-7 and -8), and unmethylated deoxycytosine-deoxyguanosine (CpG) DNA sequences (TLR-9) in late endosomes and lysosomes[67]. Recognition of ligands by cognate TLRs expressed on cells that are effectors of the innate immune response leads to the rapid activation of inflammation and microbicidal pathways[66]. In addition, owing to their ability to induce the activation of antigen-presenting dendritic cells, a subset of TLRs can initiate T-cell priming and contribute to the establishment of an adaptive immune response[68]. Signalling through TLRs also induces the production of T-helper (T_H) cells type 1, promoting cytokines and chemokines, steering the course towards the T_H1 phenotype, as needed to combat chronic viral infections and favour viral clearance. Stimulation of the appropriate TLRs may help restore the innate and adaptive immune functions that are dysfunctional in hepatitis-C-infected hosts[69]. As discussed below, the immune response observed

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Box 2 | Therapeutic RNA interference

RNA interference (RNAi) is a natural process used by eukaryotic cells to recognize and destroy abnormal or exogenous RNA[76]. RNAi is triggered by the presence of RNA molecules containing double-stranded regions (dsRNA) which are recognized as exogenous and chopped into 21–23-nucleotide long duplexes, designated short interfering RNA (siRNA). siRNA associate with a number of cellular proteins to form an RNA-induced silencing complex (RISC) which recognizes RNA complementary to either of the siRNA strands and catalyses its cleavage.

The existence of RNAi in mammalian cells was initially masked by the fact that long dsRNA activate the interferon pathway resulting in a non-specific shutdown of translation. Only in 2001 did scientists discover that it was possible to avoid the interferon response and activate gene-specific RNAi by directly feeding siRNA to mammalian cells[77], opening the way to the therapeutic application of RNAi. The initial hype over the therapeutic use of RNAi was based on its potential to overcome some of the shortcomings of the previous antisense technology, the most important advantage being the catalytic nature of RNAi. Unfortunately, soon it became evident that RNAi was not exempt from the quandaries observed with antisense, and in particular from the problems connected with *in vivo* delivery and stability. Chemical modification of the siRNA is emerging as a valid approach to improve stability and permeability characteristics[78]. Alterations of the phosphodiester-ribose backbone, such as the addition of 2′-O-methyl groups, have been shown to increase the siRNA resistance to nucleases and prolong the silencing effect. Likewise, various tricks have been used to enhance cell penetration both in cell culture and *in vivo*, one of the most popular being the conjugation with lipophilic molecules such as cholesterol. A different solution to the delivery problems is the use of vectors expressing siRNAs in the form of short hairpin RNA (shRNA) that are converted into siRNA by the cellular processing machinery.

during the course of acute viral infections can be mimicked by the use of synthetic agonists of TLRs.

Antiviral activity of TLR-9 and TLR-7 agonists

TLR-9 and TLR-7, in humans, are both expressed in B cells and in plasmacytoid dendritic cell (PDCs). In addition, TLR-7 is expressed in myeloid dendritic cells[66]. PDCs are the key effectors in the innate immune response to invading viruses because of their extraordinary capacity to produce very high levels of type 1 (α and β) IFNs in response to TLR-9 and TLR-7 stimulation by the viral nucleic acids[70]. As a consequence of the secretion of type 1 IFNs, a number of secondary effects are induced that link the innate to the adaptive immune response, such as stimulation of natural killer (NK) cells as well as maturation of PDCs to potent antigen-presenting cells.

Short synthetic oligonucleotides containing one or more unmethylated CpG motifs flanked by specific sequences are potent agonists of TLR-9 (ref. 71). Stimulation of PDCs with such oligonucleotides results in the production of tumour necrosis factor-α (TNF-α), interleukin (IL)-12 and high levels of IFN-α. Additionally, TLR-9 ligands are potent stimulators of B-cell proliferation and antibody secretion. A CpG-containing oligonucleotide, CPG-10101 (Actilon; Coley Pharmaceutical Group), was evaluated in HCV-infected patients, and yielded promising results[72]. The drug was administered subcutaneously twice weekly over a period of four weeks to individuals with chronic HCV infection who had failed previous IFN-based therapy. One-third of the patients demonstrated early viral level reduction of at least an order of magnitude during the treatment. The viral level reduction observed was consistent with the elevation of IFN-α and other markers associated with an antiviral immune response, thus validating the rationale for exploiting TLR-9 agonists for the treatment of chronic HCV infection.

TLR-7 recognizes several synthetic compounds that are structurally related to nucleic acids. These include imidazoquinolines, loxoribine (7-allyl-7,8-dihydro-8-oxoguanosine), and bropirimine (2-amin-5-bromo-6-phenyl-4(3)-pyrimidinone)[66]. The antiviral properties of synthetic TLR-7 ligands have been long known: these molecules induce a potent, broad-spectrum antiviral response owing to their ability to induce the release of inflammatory cytokines, especially IFN-α.

ANA245 (7-thia-8-oxoguanosine or Isatoribine; Anadys Pharmaceuticals) is a guanine nucleoside analogue whose immunostimulatory activity depends on its agonistic activity on TLR-7 (ref. 73). To provide the evidence that an agonist of TLR-7 could show anti-HCV activity, ANA245 was administered subcutaneously to a small group of HCV patients infected with different HCV genotypes[74].

Patients administered the highest daily dose of ANA245 for a week showed a statistically significant reduction in viral load, with some of them achieving reduction of more than 90% at the end of the treatment. Although these results are preliminary, they provide proof that a compound interacting with TLR-7 can reduce viral load in HCV-infected patients. In addition, the patient responses did not appear to be dependent on the viral genotype. Following the encouraging results in clinical study, ANA975, an oral prodrug of ANA245, is currently being developed[75]. If successful, ANA975 could combine the broad-spectrum efficacy of an immune-based therapy with the convenience of administration of an oral drug.

Outlook and future challenges

Several novel drugs have entered or will soon enter clinical evaluation to establish their clinical usefulness for HCV patients. Aside from the safety and efficacy requirements common to all new drugs, the success of HCV-targeted agents will be heavily influenced by their ability to inhibit all viral variants and prevent the emergence of escape mutants. Although agents targeting host rather than viral factors are less likely to fail owing to this problem, no drug can be considered totally exempt from the risk of resistance development. As is the case for HIV, combinations of several antiviral agents attacking different viral and possibly host targets will almost certainly be required to control infection and prevent the emergence of drug-resistant viral variants.

Although the first wave of experimental HCV-targeted drugs is yielding promising results in early clinical trials, a wider repertoire of

Table 1 | A sample of the drug pipeline for hepatitis C

a HCV-targeted drugs

Compound name(s)	Company	Clinical phase	Target	Mechanism of action
BILN 2061 (Ciluprevir)	Boehringer-Ingelheim	Phase II*	NS3-4A protease	Product-derived serine protease inhibitor
VX-950	Vertex/Mitsubishi	Phase Ib	NS3-4A protease	Serine protease reversible covalent inhibitor
NM283 (Valopicitabine)	Idenix/Novartis	Phase II	NS5B polymerase	Nucleoside analogue (chain terminator)
JTK-103	Japan Tobacco	Phase II	NS5B polymerase	Non-nucleoside allosteric inhibitor
HCV-796	ViroPharma/Wyeth	Phase Ia	NS5B polymerase	Non-nucleoside allosteric inhibitor

*Development halted due to cardiotoxicity in monkeys

b Host targets/immunomodulators

Actilon (CpG-10101)	Coley Pharmaceutical Group	Phase Ib	Toll-like receptor-9	Immunomodulator
ANA245 (Isatoribine)	Anadys Pharmaceuticals	Phase Ib	Toll-like receptor-7	Immunomodulator
ANA975	Anadys Pharmaceuticals	Phase Ia	Toll-like receptor-7	Immunomodulator (prodrug of ANA245)

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novel antiviral agents is needed. For this, drug discoverers must consider and pursue all other known viral targets for which there are not yet clinically useful inhibitors. These include the p7 ion channel, the NS2-3 cysteine protease and the NS3 helicase. Moreover, the advancement in the field of therapeutic RNAi makes it conceivable to target a variety of host factors that are essential for viral replication or persistence and for which development of small-molecule inhibitors may turn out to be prohibitively difficult.

The past clinical experience has demonstrated that, when IFN-based therapies are successful, the hepatitis C virus can be permanently cleared from the host tissues. Thus, the goal for future therapeutic regimens will be that of achieving a complete and sustained viral clearance in as many patients as possible.

What combination will turn out to be successful for HCV remains an open question. After initial proof-of-concept demonstration of antiviral activity, most new drugs are being tested in combination with IFN-α or IFN-α and ribavirin. In the future, as more agents become available, it will become possible to conceive combination schemes based entirely on novel drugs. Considering the limitations imposed by the severity of the side effects associated with the agents currently approved for hepatitis C, a shift away from IFN-based treatments would constitute a major breakthrough. A crucial question that future clinical studies need to address is whether combination therapy with solely HCV-targeted drugs will be sufficient to cure patients or whether the stimulation of the host immune system by immunomodulators or therapeutic vaccines will be necessary to completely eliminate the virus. ■

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