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Review

HCV antiviral resistance: the impact of *in vitro* studies on the development of antiviral agents targeting the viral NS5B polymerase

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The high prevalence of the disease caused by hepatitis C virus (HCV) and the limited efficacy of interferon-based therapies have stimulated the search for safer and more effective drugs. The development of inhibitors of the HCV NS5B RNA polymerase represents a promising strategy for identifying novel anti-HCV therapeutics. However, the high genetic diversity, mutation rate and turnover of HCV are expected to favour the emergence of drug resistance, limiting the clinical usefulness of polymerase inhibitors. Thus, the characterization of the drug-resistance profile of these antiviral agents is considered crucial for identifying the inhibitors with a higher probability of clinical success. In the absence of an efficient *in vitro* infection system, HCV sub-genomic replicons have been used to study viral resistance to both nucleoside and non-nucleoside NS5B inhibitors. While these studies suggest that drug-resistant viruses are likely to evolve *in vivo*, they provide a wealth of information that should help in the identification of inhibitors with improved and distinct resistance profiles that might be used for combination therapy.

Keywords: hepatitis C, HCV, resistance, NS5B, RNA-dependent RNA polymerase, RdRp, NNI, nucleoside analogue, pyrophosphate mimic, allosteric inhibitors

Introduction

Due to their compact genome size, fast replication and high mutation rate, viruses evolve swiftly, enabling rapid adaptation to selective pressures, including antiviral drugs. RNA viruses evolve at particularly high rates due to the inherent low fidelity of their replication machinery. The hepatitis C virus (HCV) is no exception, as demonstrated by its high genetic diversity. HCV infects about 3% of the world population and causes a chronic liver disease in the majority of the infected individuals (McHutchison, 2004). There is no vaccine available for HCV and the current therapy, based on interferon-α (IFN) and ribavirin (RBV), is inconvenient, poorly tolerated and of limited efficacy, in particular for genotype 1 patients. Therefore, the development of new therapeutic agents to treat HCV infection is considered of paramount importance.

Similar to the human immunodeficiency virus (HIV) and the hepatitis B virus (HBV) antiviral research, efforts to develop new therapeutic agents for HCV have mostly focused on the discovery of direct antiviral agents which inhibit HCV replication by targeting the viral enzymes. The HCV polymerase has been regarded as a promising target for discovering novel antiviral agents, based on the clinical success of anti-HIV and anti-HBV drugs directed at the cognate enzymes. Development of HCV polymerase inhibitors has been significantly delayed by the lack of animal models and appropriate cell culture systems. Nonetheless, a decade of efforts from several biopharmaceutical laboratories has resulted in the identification of numerous classes of potent and selective inhibitors, the most advanced of which are progressing in clinical trials. As these new agents are approaching clinical use, scientists are beginning to anticipate the next big challenge in HCV-drug discovery, namely the emergence of resistant viruses. Indeed, the ultimate clinical success of HCV polymerase inhibitors depends on their ability to suppress all viral variants and prevent the emergence of resistant viruses.

As exemplified by HIV and HBV, resistance to antiviral therapy has become a major determinant in the management of chronic viral infections. Therefore efforts to assess resistance development and define the effect of inhibitors on different viral genotypes are now viewed as integral parts of antiviral drug discovery, even in the early stages of drug development. As described below, the advent of a robust *in vitro* replication system has both changed the scenario of HCV research and accelerated drug discovery efforts.

In this review, we summarize the findings in the emerging field of HCV antiviral resistance, focusing specifically on characterizing the molecular determinants of resistance to inhibitors of the NS5B polymerase.

HCV epidemiology and natural history

HCV was identified as the agent responsible for the majority of blood-borne hepatitis approximately 15 years ago and is still one of the leading causes of chronic liver disease throughout the world (Choo *et al.*, 1989; Houghton, 1996). It has been estimated that HCV infects more than 170 million persons worldwide, with population prevalence ranging from 1–5% in most countries (Wasley & Alter, 2000). The incidence of new infections has declined significantly after the introduction of tests to identify contaminated blood in the early 1990s, and nowadays well over half of new cases are acquired as the result of injection-drug use (McHutchison, 2004). HCV displays a high degree of genetic heterogeneity and, based on sequence analysis, can be classified into six major genotypes (designated 1–6) with >100 subtypes (designated a, b, c and so on) (Simmonds *et al.*, 1993). In addition, even viral sequences derived from the same infected individual exhibit such a considerable diversity to be defined as quasispecies. The various genotypes differ by more than 30% in their nucleotide sequence and have a different geographical distribution: genotypes 1, 2 and 3 account for more than 90% of the infections in western countries, with genotype 1 being predominant in the US, Europe and Japan. Genotypes 4, 5 and 6 are very rare in these countries but are predominant in other areas such as Egypt (genotype 4), South Africa (genotype 5) and southeast Asia (genotype 6).

Acute HCV infection is usually asymptomatic and therefore it is only seldom diagnosed. Only a minority of patients infected with HCV resolve acute infection spontaneously and more than 60% of infected individuals develop a slowly progressive chronic disease characterized by liver fibrosis and non-specific symptoms (McHutchison, 2004). The late sequelae of HCV infection include chronic hepatitis, cirrhosis, liver failure and occasionally hepatocellular carcinoma. Although average life-expectancy of chronic HCV patients is not dissimilar from that of the uninfected population, chronic HCV infection accounts for significant morbidity and calls for better treatment for many patients.

Current treatments

Treatment of chronic HCV infection is aimed at cure. Indeed, the main goal of therapy is to produce a sustained virological response (SVR) (no detectable viraemia 6 months following cessation of therapy) which is believed to correlate with complete resolution of the disease (Bacon, 2004). Limited epidemiological studies indicate that also a partial virological response accompanied by improvement in hepatic histology is associated with decreased risk of progression to cirrhosis and hepatocellular carcinoma (Poynard *et al.*, 2002). Current HCV therapies are based on different forms of interferon-α (IFNs) and have undergone impressive advances in the last decade, in particular with the introduction of pegylated forms of IFN (pIFNs) with improved pharmacokinetic (PK) properties (Chander *et al.*, 2002; Di Bisceglie & Hoofnagle, 2002). IFNs and pIFNs are moderately effective as monotherapy and are administered via subcutaneous injection. In the standard clinical practice, pIFNs are replacing unmodified IFNs mostly because of slightly better SVR rates and the convenience of a less frequent administration (once a week vs three times a week). RBV is a nucleoside analogue and is administered orally once a day. RBV does not have a clear antiviral effect as a monotherapy but doubles the response rates of IFN therapy. The best clinical results are obtained with pIFNs and RBV combination regimens. Duration of therapy and response rates are genotype-dependent, therefore patients are routinely genotyped in order to select the appropriate therapy protocol. SVR is obtained in ~45% of HCV genotype 1-infected patients and in ~76–80% of genotype 2 and 3-infected patients. Therapies based on IFNs are poorly tolerated because of significant adverse effects, ranging from mild flu-like symptoms to severe haematological, neurological and psychiatric disorders. Because of the limited efficacy and side effects, less than 20% of diagnosed patients undergo treatment.

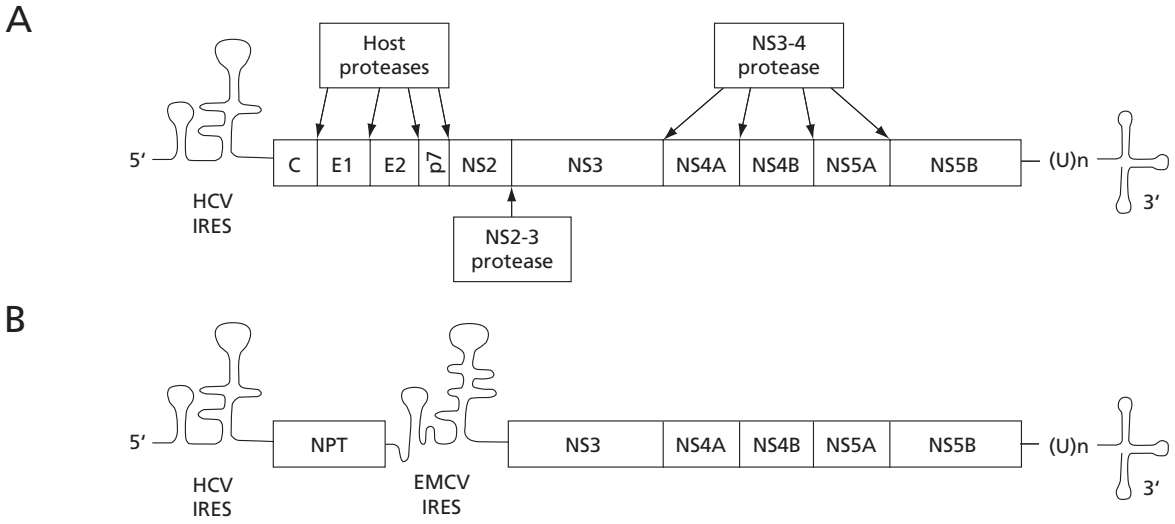
HCV genomic organization and functions of viral proteins

The molecular characterization of HCV became possible with the cloning of the viral genome in 1989, which led to its classification in a separate genus (Hepacivirus) of the *Flaviviridae*, a family of positive strand RNA viruses that include human and animal pathogens (Rice, 1996; Houghton, 1996; Choo *et al.*, 1989). The viral genome is a 9.6 kb-long messenger RNA molecule and has the canonical structure of the *Flaviviridae* genomes, with a single

open reading frame (ORF) flanked by two untranslated regions (UTR) (Figure 1) (Choo *et al*., 1989). The 5′-UTR is highly conserved between different virus genotypes and contains an internal ribosome entry site (IRES) responsible for cap-independent translation of the ORF, as well as essential cis-acting replication elements (Tsukiyama-Kohara *et al*., 1992, Friebe *et al*., 2001). The 3′-UTR encompasses a variable domain of approximately 50 nucleotides, a poly-U sequence of variable length and a highly conserved 98-base element essential for replication (Tanaka *et al*., 1995; Friebe *et al*., 2001; Kolykhalov *et al*., 1997). The ORF encodes a ~3000 amino acid polyprotein that is cleaved co- and post-translationally by the concerted action of cellular and viral proteases, resulting in the generation of at least 10 different mature viral proteins named as follows: NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Figure 1A). The signal peptidase and the signal peptide peptidase of the endoplasmic reticulum (ER) release from the polyprotein the structural proteins: Core (C), E1, E2, and the small transmembrane peptide p7 (Santolini *et al*., 1994; Hijikata *et al*., 1993b; McLauchlan *et al*., 2002). The structural proteins become associated with (C) or integrated into (E1, E2 and p7) the ER membrane and form functional oligomers that will eventually promote virus budding. The non-structural (NS) proteins are released from the nascent precursor by two viral proteases (Figure 1). The NS proteins also become associated with (NS3) or integrated into (NS2, NS4A, NS4B, NS5A and NS5B) the ER membrane where they interact with each other and with host proteins to form the viral replication machinery.

The main role of the C protein is believed to be the formation of the viral capsid, though expression of this protein in mammalian cells has been reported to have pleiotropic effects, including transcriptional modulation, alteration of the lipid metabolism and effects on apoptosis (McLauchlan, 2000). Interestingly, the Core coding sequence also encodes, in an alternative ORF, a second protein, termed ARFP or F (Walewski *et al*., 2001; Xu *et al*., 2001) not strictly required for infectivity in cell culture or *in vivo* (McMullan LK, Grakoui A, Puig M, Mihalik K, Branch AD, Feinstone SM & Rice CM (2004) Multiple stop codons in the HCV ARF suggest that expression of F/ARF is not essential for virus replication but reveal a functional RNA element. *11th International Symposium on Hepatitis C Virus & Related Viruses*, Heidelberg, Germany). The E1 and E2 glycoproteins assemble as a noncovalent heterodimer and are believed to be the viral envelope components responsible for cell attachment and entry. In fact, pseudo-typed retro- or lenti-viral particles harbouring E1 and E2 in their envelope have been shown to infect tissue culture cells of hepatic origin (Hsu *et al*., 2003; Bartosch *et al*., 2003). E2 has been shown to recognize several putative HCV receptors, including the tetraspannin

Figure 1. Schematic diagram of the genetic organization of HCV genome **(A)** and of a prototypical bicistronic replicon **(B)**



C, capsid; E, envelope; NS, nonstructural. The position of the HCV and EMCV IRES and the cleavage sites of host and HCV encoded proteinases are indicated.

CD81, the low-density lipoprotein receptor and the B-I scavenger receptor (Pileri *et al.*, 1998; Scarselli *et al.*, 2002; Flint *et al.*, 2001). p7 is a small transmembrane protein and has been recently shown to act as a cation-selective ion channel (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003; Sakai *et al.*, 2003).

Among the NS proteins, NS2 is an integral membrane protein whose functions are not completely understood. The C-terminal half of NS2 and the N-terminus of NS3 constitute the NS2-3 protease, a zinc-dependent auto-protease responsible for a single cut between NS2 and NS3 (Pieroni *et al.*, 1997; Hijikata *et al.*, 1993a; Grakoui *et al.*, 1993b). NS3 is a multifunctional protein and probably the most characterized HCV polypeptide. In addition to forming the NS2-3 protease, the N-terminal domain of NS3 together with the small NS4A peptide constitutes the NS3-4A protease, a distinct serine protease that cleaves the polyprotein at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (Grakoui *et al.*, 1993a; Bartenschlager *et al.*, 1993; Tomei *et al.*, 1993) (Figure 1). The C-terminal domain of NS3 contains an NTPase/helicase necessary for translation and replication of the HCV genome (Kim *et al.*, 1995). NS4B is an integral membrane protein and contains a nucleotide binding domain in its cytosolic region (Einav *et al.*, 2004; Hugle *et al.*, 2001). NS4B expression in mammalian cells induces characteristic membranous vesicles - the membranous web - which are believed to be the site of viral replication (Egger *et al.*, 2002). NS5A is a highly phosphorylated protein capable of binding one zinc ion (Tanji *et al.*, 1995; Tellinghuisen *et al.*, 2004). Although mutational analysis has revealed that zinc binding is essential for replication in tissue culture, the role of NS5A in HCV replication remains controversial. The presence of tissue culture adaptive mutations in NS5A has reinforced the belief that NS5A might have a direct role in replication of the viral RNA. In addition, experimental evidence suggests that NS5A might also be involved in the resistance to the antiviral effect of interferon (Tan & Katze, 2001). As discussed below, NS5B is the RNA-dependent RNA polymerase (RdRp) and is the catalytic core of the viral replicase (Behrens *et al.*, 1996).

HCV replicons

The lack of adequate laboratory animal models and the inability to efficiently culture the virus *in vitro* have been major obstacles in dissecting the HCV life cycle and in establishing reliable antiviral assays. Only in recent years have cell-based assays and pre-clinical animal systems such as a tissue culture replication system, cDNA clones infectious in the chimpanzee, and mouse models for HCV infection been developed.

Undoubtedly, the major contribution to basic virology studies and drug discovery has come from the development of a reliable tissue culture replication system based on subgenomic 'replicons'. This system provided the long-sought after cell-based model for evaluating antiviral compounds as well as an *in vitro* genetic system for virology and resistance studies (Bartenschlager, 2002). Replicons are genetically modified HCV genomes capable of self-replication in tissue culture cells. Based on experience with other positive-strand RNA viruses, HCV replicons were initially assembled with a bicistronic arrangement by replacing the structural region of the genome of the Con1 isolate with the gene encoding the enzyme neomycin phosphotransferase (NPT) and the IRES of the encephalomyocarditis virus (EMCV, Figure 1B) (Lohmann *et al.*, 1999). In this arrangement, translation of NPT is mediated by the HCV IRES present in the 5′ UTR, whereas the EMCV IRES promotes translation of the HCV replication proteins. Transfection of these replicons in the human hepatoma cell line Huh-7 followed by selection with neomycin resulted in the isolation of cell clones that supported very efficient replication of the replicons. Subsequent studies showed that efficient replication required tissue-culture adaptive mutations in the replicons and also led to the isolation of Huh-7 subclones that supported HCV replication more efficiently (Blight *et al.*, 2000; Blight *et al.*, 2002). These findings opened the way to the development of new types of mono- and bicistronic replicons expressing different selectable markers and/or reporter genes. These novel replicons facilitated HCV genetic studies and paved the way to the development of high-throughput screening assays for the identification of novel inhibitors. The repertoire of available replicons has subsequently been extended by the establishment of replicons derived from other viral isolates and the identification of other cell lines that support HCV replication (Ali *et al.*, 2004; Date *et al.*, 2004; Kato *et al.*, 2003; Zhu *et al.*, 2003).

These improvements also led to the discovery that Huh-7 cells supported replication of full-length HCV genomic RNAs. Very recently, it has been shown that these features can be exploited to produce recombinant viral particles that can infect cultured cells (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). Strikingly, not all viral sequences can be converted to replicons. The limited data currently available suggest that there is no clear concordance between replication in tissue culture and infectivity in the chimpanzee and these properties may even be inversely correlated. Indeed, the HCV isolates that replicate to high titres *in vivo* replicate less efficiently or not at all in tissue culture (for example HCV-con1, HCV-BK, HCV-H77) unless one or more adaptive mutations emerge. Conversely, it has been shown that at least some

cell culture adaptive mutations abolish or significantly reduce infectivity *in vivo* (Bukh *et al*., 2002).

Tissue culture adaptive mutations have been described in all non-structural HCV proteins, except for NS4A and are thought to influence protein-protein interactions critical to modulating the activity of the HCV replication complex or, alternatively, to affect virus ability to counteract the innate cellular antiviral response.

NS5B RNA-dependent RNA polymerase

The NS5B RdRp is the catalytic core of the HCV replication machinery and is essential for viral replication. The properties of this enzyme are different enough from those of host RNA and DNA polymerases to make it likely that specific inhibitors of the HCV enzyme will not inhibit the cellular enzymes, and will therefore be devoid of toxic effects.

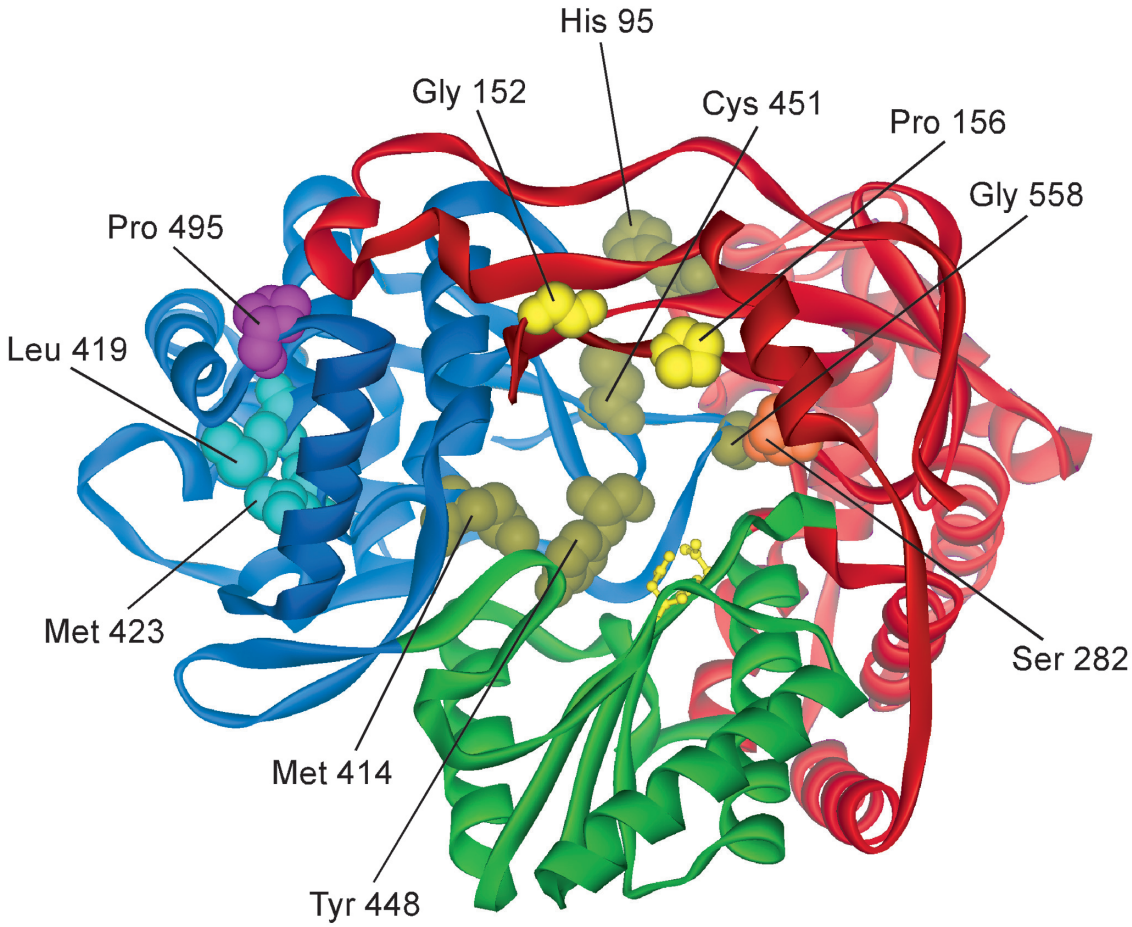
The polymerase activity of the NS5B protein was predicted from the presence of signature motifs in its sequence (Choo *et al*., 1989) and was confirmed when the recombinant full-length protein was purified and shown to be capable of RNA synthesis *in vitro* (Behrens *et al*., 1996; Lohmann *et al*., 1997). Subsequent biochemical and structural studies of the polymerase were facilitated by the expression of more soluble and active forms of the enzyme lacking the hydrophobic transmembrane anchor contained within the last 21 amino acids (Tomei *et al*., 2000; Lohmann *et al*., 1997; Ferrari *et al*., 1999). Interestingly, though it is dispensable for the *in vitro* activity of the recombinant NS5B enzyme, this C-terminal membrane anchor is necessary for viral replication (Lee *et al*., 2004).

In vitro, the NS5B polymerase catalyses the synthesis of RNA using homo- or hetero-polymeric RNA templates, both with and without a primer. The enzyme does not have strict requirements for the length and the sequence of the template RNA, although preference for specific sequences, including the HCV RNA, has been reported (Johnson *et al*., 2000; Oh *et al*., 2000; Shim *et al*., 2002; Kim *et al*., 2002; Kao *et al*., 2000). As expected for an RdRp, NS5B does not accept DNA as a template or deoxynucleotides (dNTPs) as substrates. However, both oligo-ribonucleotides and oligo-deoxyribonucleotides can be utilized as primers by the HCV enzyme. In the absence of a primer, NS5B initiates RNA synthesis by either using the 3′-terminal-OH group of the template as primer, or by means of an authentic *de novo* initiation mechanism using as primer a nucleotide complementary to the base at the 3′ end of the template (Zhong *et al*., 2000; Luo *et al*., 2000; Kim *et al*., 2002). Experimental evidence indicates that *de novo* initiation has specific sequence requirements and that the NS5B polymerase acts by the so-called prime and realign mechanism, whereby the enzyme initially synthesizes short leader RNAs during abortive polymerization and subsequently uses these oligoribonucleotides as primers for elongation (Zhong *et al*., 2000; Shim *et al*., 2002).

A major advance in the understanding of the NS5B polymerase was provided by the resolution of the three-dimensional structures of several truncated forms of the apoenzyme and of complexes with nucleotides or RNA templates (Adachi *et al*., 2002; Ago *et al*., 1999; Bressanelli *et al*., 2002; Bressanelli *et al*., 1999; Lesburg *et al*., 1999; O'Farrell *et al*., 2003). The NS5B has the canonical 'right hand' shape, with the characteristic fingers, palm and thumb sub-domains (Figure 2). Similarly to other RdRps, the HCV polymerase has a compact shape due to the presence of two extended loops – the fingertips – that connect the fingers and thumb domains and completely encircle the active site cavity, to which the RNA template and the nucleoside triphosphate (NTP) substrates have access through two positively charged tunnels (Bressanelli *et al*., 2002). The palm sub-domain constitutes the active site of the molecule and contains the residues responsible for the nucleotidyl transfer reaction within the D-$(X)_4$-D and the GDD motif. The thumb and the fingers sub-domains bind the template and the incoming nucleotide and position them for initiation and elongation. Two structural elements peculiar to the NS5B structure are a β-hairpin (β-loop), protruding from the thumb into the active site, and a C-terminal region, located immediately before the transmembrane domain, that folds from the surface of the thumb towards the active site and establishes a series of hydrophobic interactions with a shallow pocket comprised between the palm and thumb sub-domains (Adachi *et al*., 2002). These elements are probably involved in positioning the 3′ terminus of template RNA and are considered crucial for template selection. Biochemical characterization of deleted enzymes lacking either one of these elements has confirmed that they play an important role in modulating NS5B activity (Hong *et al*., 2001; Adachi *et al*., 2002). Deletion of these elements increased primer-dependent polymerase activity, suggesting that their function is to favour *de novo* initiation of RNA synthesis from the 3′ end of single-stranded template. The structure of the NS5B polymerase in complex with nucleotides (Bressanelli *et al*., 2002) lent further support to the role of these elements in *de novo*-initiation. Three distinct nucleotide-binding sites were observed in the catalytic centre of the HCV RdRp whose geometry was remarkably similar to that observed in the initiation complex of the RNA phage ϕ6 RdRp (Butcher *et al*., 2001), strengthening the proposal that the two enzymes initiate *de novo* replication by similar mechanisms.

The surface of the enzyme shows a number of sites that are potentially involved in the interaction with other components of the viral replication apparatus. A prominent

Figure 2. X-ray crystallographic structure of the NS5B RNA-dependent RNA polymerase



The thumb, palm, and fingers domains are coloured in blue, green, and red, respectively. The two catalytic aspartates are shown in stick and ball style in the active site. Residues responsible for resistance to the various inhibitors are shown in the CPK style and coloured according to the resistance pattern: magenta (benzimidazoles/indoles), light blue (thiophenes), brown (benzothiadiazines), yellow (dihydroxypyrimidines), and orange (2′-C-Me nucleosides).

feature revealed by crystallographic studies is the presence of a non-catalytic GTP-binding site on the enzyme surface, near the interface between fingers and thumb domains (Bressanelli *et al.*, 2002). This GTP pocket was proposed to be an allosteric regulatory site whose occupancy modulates the interactions between the two domains, thus controlling the conformational changes during the polymerization cycle. The presence of a unique nucleotide-binding site distal from the active site potentially provides an attractive target for allosteric inhibitors of the HCV polymerase reaction. As discussed below, crystallographic studies in the presence of inhibitors highlighted the existence of two distinct allosteric binding sites, both located in the thumb domain.

Methodological approaches to resistance studies

Although experimental systems for HCV antiviral resistance studies are less advanced than for HIV, recent progress in biochemical and replicon assays have laid the foundations for resistance testing during drug development and eventually in therapy management.

The first and possibly most important requirement for resistance profiling is the availability of cell-free and cell-based assays for evaluating the activity of antiviral compounds on a panel of viral isolates representative of HCV genetic variability. Recombinant NS5B polymerases from several different viral isolates have recently become

available to test the efficacy of antiviral agents in biochemical assays and more will probably be at hand in the near future (Carroll *et al.*, 2004). The repertoire of reagents for cell-based assays is also growing at a steady pace. To date, replication-proficient HCV replicons have been derived from a handful of genotype 1a, 1b and 2a viral strains. While these replicons already provide an initial hint of the variability of HCV responses to antiviral agents, the availability of more replicons will certainly increase the capacity to predict drug efficacy in patients. Optimistically, efforts to obtain new replicons from all clinically relevant genotypes will be facilitated by the latest expansion of the repertoire of cell lines and clones permissive for replicon replication as well as by the understanding of the role of adaptive mutations. Another successful avenue towards increasing the number of replicons available for testing NS5B inhibitors is the assembly of chimeric replicons derived from genotype 1 replication-competent constructs, in which the NS5B gene was replaced by the corresponding sequences derived from clinical isolates of various genotypes (Ludmerer *et al.*, 2005; Middleton T, Lim HB, Pilot-Mathias T, Tripathi R, Roth A, Koev G, Ng T, Lu L, Pithawalla R, Chen C-M, Kati W, Mo H, Di Bisceglie AM & Molla A (2004) Development of a replicon-based shuttle vector system for phenotypic characterization of HCV patients NS5B genes towards polymerase inhibitors. *11th International Symposium on Hepatitis C Virus & Related Viruses*). As discussed below, these reagents are beginning to be used in order to define the spectrum of activity of various NS5B inhibitors.

Equally important has been the implementation of experimental procedures for selecting and characterizing mutants resistant to specific inhibitors of the HCV enzymes (Trozzi *et al.*, 2003). This advance was made possible by the use of HCV replicons expressing NPT and was based on two replicon features. First, the low fidelity of replicon replication in tissue culture causes the accumulation of mutations resulting in a genetic diversity sufficient to mimic the complexity of the HCV quasispecies in patients. Second, the ability of the replicon cells to survive in the presence of neomycin relies on replicon-driven expression of NPT. Therefore, inhibitors of viral replication abolish resistance to neomycin. Taking advantage of these features, it is possible to select cell clones containing resistant replicons by culturing a relatively small number of cells in the presence of neomycin and of a given inhibitor. Under these conditions, cells containing replicons sensitive to the inhibitor succumb to the antibiotic's toxic effect, while cells containing mutant replicons with decreased sensitivity to the inhibitor survive and give rise to colonies that can be isolated, expanded and characterized. To date, it has been possible to select replicons resistant to several inhibitors of the NS5B polymerase, making it likely that this selection procedure will be broadly applicable to all HCV replication inhibitors. For all NS5B inhibitors described so far, resistance mutations mapped in the NS5B coding sequence and their role was easily defined by reverse genetic analysis. Indeed, the availability of adapted replicon variants and of highly permissive cell lines greatly facilitated the genetic dissection of resistance mutations and also allowed the study of the effect of these mutations on replication fitness. In addition, dissection of the role played by resistance mutations in the context of the recombinant NS5B polymerase has adequately complemented the genetic data obtained with the mutant replicons. In fact, mutant enzymes and replicons turned out to be complementary tools for mechanistic studies and cross-resistance analysis. Even more importantly, the biochemical characterization of resistant enzymes and the possibility of modelling resistance mutations in the 3D structure of the enzymes have greatly contributed to the understanding of resistance mechanisms.

Although resistance studies with authentic HCV viruses cannot yet be performed, the breadth of data that can be obtained with the current *in vitro* systems makes it likely that the resistance patterns observed *in vitro* will also be observed *in vivo* and should contribute to the identification and clinical development of resistance-repellent inhibitors.

Inhibitors of the NS5B polymerase: mechanisms of action and resistance studies

Inhibitors of viral polymerases have been extremely successful in controlling chronic viral infection (De Clercq, 2004). It is therefore not surprising that efforts to develop novel anti-HCV therapeutics have focused on the identification of low molecular weight inhibitors of the HCV NS5B polymerase. These efforts have resulted in the identification of several classes of NS5B polymerase inhibitors, the most advanced of which are being evaluated in clinical trials. In the following section, we will discuss those inhibitors for which resistance studies have been reported. For the purposes of this review, NS5B inhibitors are divided into two categories according to their binding site on the enzyme: active site inhibitors and allosteric inhibitors.

Active site inhibitors

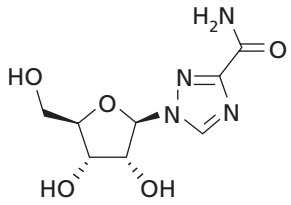
Based on their chemical structure, active site inhibitors can in turn be divided in two subcategories: nucleoside inhibitors (NIs), that inhibit the enzyme as substrate analogues, and active site non-nucleoside inhibitors (NNIs) that inhibit the enzyme as mimics of the pyrophosphate product. With the notable exception of RBV, most, if not all, NIs of the NS5B polymerase function as chain

Figure 3. Active site inhibitors of the NS5B polymerase. Shown are structures of representative examples of nucleosides **(A)** and pyrophosphate mimetics **(B)**

A

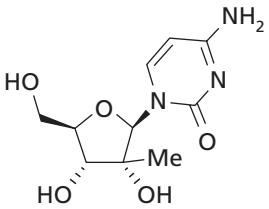
[1]

(ribavirin)



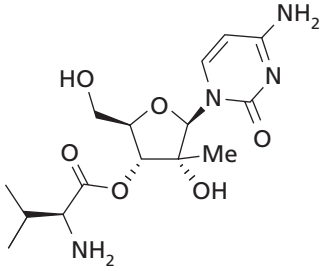
[2]

(NM107)



[3]

(NM283)



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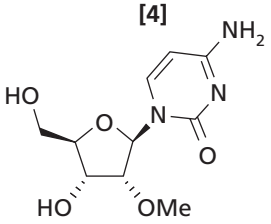

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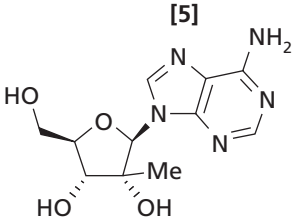

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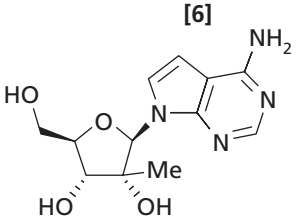


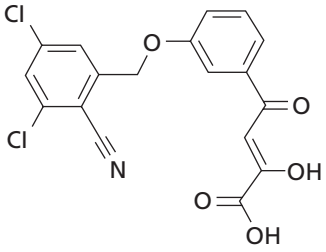
Enzyme IC_{50} (TP) 3.8 μM
Replicon IC_{50} 14 μM
(Carroll *et al.* 2003)

Enzyme IC_{50} (TP) 1.9 μM
Replicon IC_{50} 310 nM
(Carroll *et al.* 2003)

Enzyme IC_{50} (TP) 110 nM
Replicon IC_{50} 300 nM
(Olsen *et al.* 2004)

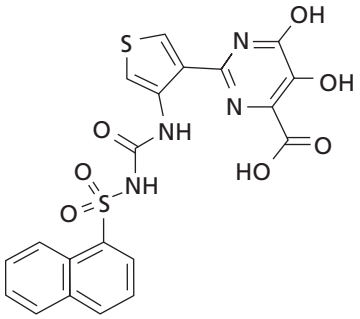
B

[7]



Enzyme IC_{50} 45 nM
(Summa *et al.* 2004b)

[8]



Replicon IC_{50} 1.6 μM
(Migliaccio *et al.* unpublished)

Enzyme and replicon IC_{50} values for these compounds or for the corresponding triphosphates (TP) are derived from the indicated references.

terminators, that is they are incorporated in the nascent RNA molecule and prevent incorporation of the following nucleotide.

Nucleoside inhibitors: ribavirin

RBV (Figure 3A, **[1]**) is a guanosine analogue with a broad-spectrum antiviral activity (McHutchison & Patel, 2002). Though RBV is a mainstay of the approved combination therapies for HCV infection, its mechanisms of action are currently not fully understood (Lau *et al*., 2002). RBV has been proposed to exert its activity through one or more of the following mechanisms: i) enhancement of the immune response; ii) inhibition of the enzyme inosine monophosphate dehydrogenase, resulting in a decreased intracellular GTP concentration with the consequent reduction of viral RNA synthesis; iii) inhibition of the viral polymerase; iv) incorporation of the RBV monophosphate in the viral RNA, resulting in an increased mutation rate. The latter mechanism has received some support from a series of *in vitro* experiments. Biochemical studies have shown that RBV triphosphate is accepted as a substrate by the NS5B polymerase and is incorporated as monophosphate in the nascent RNA, although affinity for the enzyme and incorporation rate are lower than those measured for natural nucleotides (Maag *et al*., 2001; Bougie & Bisaillon, 2003). Furthermore, RBV has been shown to increase HCV RNA mutation frequencies in the replicon system as well as in a DNA-based HCV replication system (Contreras *et al*., 2002; Zhou *et al*., 2003).

In line with these *in vitro* findings, Young *et al*. reported that RBV had a modest mutagenic effect on HCV *in vivo* (2003). The same authors also observed that viruses derived from patients infected by genotype 1a HCV and treated with RBV consistently accumulated mutation of residue Phe415 of the NS5B polymerase into Tyr (F415Y). The role of this substitution in resistance to RBV therapy was investigated using the replicon system. Replicons derived from the Con1 isolate already have a Tyr at position 415 and were poorly sensitive to RBV. Conversely, replicons containing the reverse substitution Y415F in NS5B were more sensitive to RBV, implying that this substitution affected HCV response to RBV and indirectly suggesting that *in vivo* RBV exerts its action by directly interfering with the NS5B polymerase activity. Residue 415 of NS5B is located on the inner surface of the thumb domain of the polymerase. Molecular modelling and structure analysis suggest that the F415Y substitution could narrow the putative RNA binding pocket. However, the mechanisms by which the F415Y substitution induces resistance to RBV are at the moment entirely speculative and await further investigation. Interestingly, while genotype 1a HCV isolates have a Phe at position 415, isolates of other genotypes have a Tyr at this position, suggesting that genotype 1a viruses might be more sensitive to RBV therapy than other genotypes.

Nucleoside inhibitors: 2′-modified nucleosides

Development of NIs of the NS5B enzyme has been complicated by the fact that they are pro-drugs that require conversion to the active triphosphate species by the cellular machinery. For this reason, development of NIs for HCV has required parallel structure-activity relationship (SAR) studies aimed at determining the activity of the NIs in replicon or surrogate cell-based assays and that of the corresponding triphosphates in NS5B enzymatic assays. These studies led to the identification of a number of 2′-modified NIs of the NS5B polymerase that efficiently inhibit HCV replication in tissue culture.

Two pyrimidine derivatives, 2′-C-methyl-cytidine (NM107 from Idenix pharmaceuticals; Figure 3A, **[2]**) and its 3′-valine ester (NM283 from Idenix pharmaceuticals, Figure 3A, **[3]**) are the most advanced NIs in clinical development. Interestingly, NM107 was originally identified as an inhibitor of the HCV-related bovine viral diarrhoea virus (BVDV) in a cell-based infection assay and was subsequently shown to have modest activity in the HCV replicon assay, despite being efficiently converted to the triphosphate in replicon cells (Carroll, 2004; Standring *et al*., 2003). The 3′-valine ester modification yields a pro-drug with improved oral bioavailability. Indeed, pre-clinical PK studies showed that NM283 was efficiently absorbed, rapidly converted to NM107 in plasma and had a sustained half life, making it suitable for once daily oral administration (Godofsky *et al*., 2004). More importantly, NM283 was shown to be efficacious in HCV-infected chimpanzees, inducing a 10-fold reduction of HCV viraemia after 7 days of daily treatment (Standring, 2003).

These data prompted the initiation of human Phase I/II clinical trials to evaluate safety, PK and antiviral activity (Afdhal *et al*., 2004). The trial included five cohorts of patients infected by genotype 1 viruses that were administered a single daily dose of 50, 100, 200, 400 and 800 mg of NM283 for 15 days. Up to 400 mg/day, NM283 showed dose-proportional systemic exposure and no significant accumulation after the 15 days of dosing. NM283 was well tolerated, with some transient gastrointestinal toxicity. More importantly, treatment with NM283 resulted in a dose-proportional antiviral activity, with an end-of-treatment viral load reduction of approximately 10-fold in the cohort receiving the highest dose. Although in all patients viraemia returned to pre-treatment levels after stopping therapy, the positive outcome of this initial trial encouraged the initiation of Phase II trials in combination with pIFNs. No resistance data have yet been reported for NM283. However, mutants resistant to the 2′-C-methyl-purine nucleosides described below were also resistant to NM107,

making it likely that NM283 will share the same mechanism of resistance (Migliaccio *et al.*, 2003; Olsen *et al.*, 2004).

Other 2′-modified NIs, such as 2′-O-methyl-cytidine (2′-O-Me-C, Figure 3A, **[4]**), 2′-C-methyl-adenosine (2′-C-Me-A, Figure 3A, **[5]**) and 2′-C-methyl-guanosine (2′-C-Me-G) are clinically less advanced, but have been more extensively characterized *in vitro* (Carroll *et al.*, 2003; Eldrup *et al.*, 2004). In line with structural resemblance to the natural nucleotide substrates, the triphosphates of these NIs are recognised by the NS5B polymerase as substrate analogues and are incorporated as monophosphate in the nascent RNA chain (Carroll *et al.*, 2003). Incorporation of 2′-modified NIs prevents the addition of other nucleotides resulting in premature termination of RNA synthesis. The mechanism of chain termination is not fully understood, but molecular modelling suggests that 2′-modifications affect the ribose conformation, resulting in incorrect positioning of the 3′-OH group thus blocking subsequent nucleotide addition. As predicted, the ability of these compounds to inhibit replication of HCV replicons correlates with the levels of triphosphate formed inside the cell. In replicon cells, as well as in primary human hepatocytes, 2′-C-Me-G and 2′-O-Me-C are converted to the corresponding triphosphates much less efficiently that 2′-C-Me-A, and therefore are less potent replicon inhibitors, despite the superior or equivalent inhibition of the purified NS5B polymerase.

Notwithstanding its potent replicon activity, 2′-C-Me-A had poor oral bioavailability in rats, indicating that it was not absorbed or metabolized. This observation raised the question of whether this compound could have clinical utility for HCV therapy. SAR of the base led to the identification of 7-deaza-2′-C-Me-A (Figure 3A, **[6]**), a nucleoside with comparable replicon activity, reduced cellular toxicity and very promising PK profiles in pre-clinical animal species, making it an attractive candidate for clinical development (Olsen *et al.*, 2004).

Interestingly, 2′-C-Me-purine nucleosides displayed antiviral activity against HCV-related viruses and did not inhibit human DNA and RNA polymerases, indicating that the 2′-C-Me modification was sufficient to convert the NTP substrates into specific inhibitors of RdRps of the positive strand RNA viruses (Migliaccio *et al.*, 2003; Olsen *et al.*, 2004). Consistent with these findings, 2′-C-Me-nucleosides have been shown to inhibit a panel of NS5B enzymes and replicons derived from different HCV genotypes, indicating their potential to be developed as treatment for all viral strains (Carroll *et al.*, 2004; Ludmerer *et al.*, 2005). Nonetheless, *in vitro* selection of replicons resistant to 2′-C-Me-A and 7-deaza-2′-C-Me-A has shown

Table 1. Effect of NS5B mutations on replicon resistance and cross-resistance to NS5B polymerase inhibitors

			Resistance to compound				
Mutation selected with	NS5B mutation	Conservation across genotypes	**[5]/[6]**	**[8]**	**[9]/[10]**	**[11]**	**[13]**
2′-C-Me-nucleoside **[5]**, **[6]***	S282T	99%	Yes	No	No	No	No
Dihydroxypyrimidine **[8]**†	P156L	89%	No	Yes	No	No	No
	G152E	100%	No	Yes	No	No	No
Benzimidazole **[9]**‡	P495L	99%	No	No	Yes	No	No
	P495A	99%	No	No	Yes	No	No
Benzothiadiazine **[11]**§	H95R	99%	No	No	No	Yes	No
	M414T	86%	No	No	No	Yes	No
	Y448H	100%	No	No	No	Yes	No
	C451R	52%	No	No	No	Yes	No
	G558R	97%	No	No	No	Yes	?
Thiophene **[13]**¶	L419M	78%	No	No	No	?	Yes
	M423T	99%	No	No	No	No	Yes

*(Migliaccio *et al*., 2003; Olsen *et al*., 2004); †(Migliaccio *et al*., unpublished); ‡(Tomei *et al*., 2003); §(Tomei *et al*., 2004; Nguyen *et al*., 2003; Mo H, Lu L, Pilot-Mathias T, Dekhtyar T, Masse S, Pithawalla R, Ng T, Pratt J, Donner P, Maring C, Randolph J, Klein L & Molla A (2004) Identification and characterization of mutations conferring resistance to either a HCV RNA-dependent RNA polymerase or HCV serine protease inhibitor or both in vitro. *11th International Symposium On Hepatitis C Virus and Related Viruses*); ¶(Migliaccio *et al*., unpublished; Le Pogam S, Leveque VJP, Hang J, Rajyaguru S, Oshiro C, Klumpp K, Symons J, Cammack & Najera I (2004) *In vitro* selection and characterization of Con1 replicons resistant to a thiophene-2-carboxylic acid, a new anti-HCV polymerase non-nucleoside inhibitor. *11th International Symposium on Hepatitis C Virus and Related Viruses*)

that HCV can become resistant to these NIs (Migliaccio *et al.*, 2003; Olsen *et al.*, 2004). So far, resistance to 2′-C-Me-nucleosides has been reported to be due to a single mutation in the NS5B polymerase, namely substitution of Ser282 with Thr (S282T). Replicons and NS5B enzymes carrying this mutation were shown to be selectively resistant to 2′-C-Me-nucleosides and to their triphosphates, respectively, but not to 2′-O-Me-nucleosides or to NNIs of NS5B (Table 1).

In the context of the recombinant polymerase, the S282T mutation was shown to confer resistance to 2′-C-Me-nucleosides by a combination of two mechanisms. First, the S282T mutant enzyme has a reduced affinity for 2′-C-Me-nucleosides and only a modest decrease in affinity for the natural substrates, thus reducing the frequency of 2′-C-Me-nucleosides incorporation. Second, although inefficiently, the S282T mutant enzyme can extend the nascent RNA chain after incorporation of 2′-C-Me, thus overcoming chain termination. Modelling of the 2′-C-Me-nucleoside in the NS5B initiation complex provides initial hints on the molecular mechanism of S282T-induced resistance. When the 2′-C-Me-nucleoside is modelled in the NTP site, the 2′-C-Me group is in close proximity of Ser282. Based on this proximity, it is conceivable that the extra methyl group of Thr might account for the reduced affinity for 2′-C-Me-substituted nucleosides of the enzyme carrying the S282T substitution. The ability of S282T NS5B to elongate 2′-C-Me-primed nucleosides is not explained by the model, and may depend on the influence of the S282T mutation on the NS5B structure.

In line with the activity against several HCV isolates, Ser282 is highly conserved across HCV genotypes, possibly explaining the finding that replicons into which the S282T mutation is introduced are attenuated in transient replication assays. The fact that some of the originally selected replicons replicated at a level comparable to wild-type suggests the existence of as yet unidentified compensatory mechanisms (Migliaccio *et al.*, 2003). It remains to be defined whether the S282T mutation will have similar debilitating effects on HCV replication also in a more physiological setting, as well as in the context of other viral sequences.

Active site non-nucleoside inhibitors: diketo acids and pyrimidines

α-γ-diketo acids were recently identified as potent, specific and reversible inhibitors of HCV NS5B polymerase (Summa *et al.*, 2004b). A representative member of this inhibitor class is **[7]** (Figure 3B). The structure of compounds from this class can be viewed as bipartite. The diketo acid moiety has the potential to bind the HCV RdRp by chelating the two Mg^{++} ions found in the enzyme active site, thus constituting an active site 'warhead' or anchor. Conversely, the aromatic substituent(s) in the left-hand side of the molecule can be substituted to improve the inhibitor's specificity and potency (Summa *et al.*, 2004b).

Inhibition of the polymerase enzymatic activity via the chelation of the active site Mg^{++} ions has been reported as the mechanism of inhibition of pyrophosphate analogues, such as Foscarnet and phosphonoacetic acid (Sundquist & Oberg, 1979). These compounds are believed to bind to the pyrophosphate binding site of the enzyme, thus acting as product-like inhibitors of the polymerase reaction. Experiments aimed at measuring the combined effect of diketo acids and classical pyrophosphate analogue(s) on the HCV polymerase have indicated that the two classes of inhibitors interact with the enzyme in a mutually exclusive fashion, suggesting interaction with a common binding site. For this reason, diketo acids are also referred to as 'pyrophosphate mimics' (De Francesco *et al.*, 2003).

Compounds containing the diketo acid chelating moiety have also been found to inhibit a variety of viral enzymes that catalyse the Mg^{++}-dependent transfer of a phosphoryl group via the so-called two-metal-ion mechanism, including the HIV and HBV polymerases, the influenza virus endonuclease, the HIV integrase and HIV RNase H (Grobler *et al.*, 2002; Altamura *et al.*, 2000; Hazuda *et al.*, 2000; Shaw-Reid *et al.*, 2003; Tomassini *et al.*, 1994). These observations suggest that diketo acids have the potential to inhibit a variety of metal-dependent phospho-transferase enzymes that are of therapeutic interest. The potential to be utilized as antiviral agents has been demonstrated for classical pyrophosphate analogues as well as for diketo acid compounds. Foscarnet, a classical pyrophosphate analogue, is used clinically as an anti-herpesvirus agent (De Clercq, 2004). In addition, diketo acid inhibitors of HIV integrase and influenza transcriptase have been reported that inhibit viral replication in cell culture and in animal models (Hazuda *et al.*, 2000; Tomassini *et al.*, 1994). However, no diketo acids have thus far been identified that inhibit HCV replication in cell culture.

Very recently, a new class of HCV polymerase inhibitors was designed from a diketo acid and a meconic acid derivative discovered by screening. In this series, the diketo acid warhead is replaced by dihydroxypyrimidine carboxylate (Summa *et al.*, 2004a) (Figure 3B, **[8]**). These are novel, reversible, and selective inhibitors of the HCV NS5B polymerase that still utilize a Mg^{++}-chelating moiety in order to bind to the enzyme active site, but have improved drug-like characteristics compared to the diketo acids. In fact, dihydroxypyrimidine carboxylates are inhibitors of RNA elongation and are competitive with the classical pyrophosphate analogues. As for the diketo acid series, the substitution of the aryl group modulates the selectivity and potency also for this new class of inhibitors (Summa *et al.*, 2004a). Compounds like **[8]** (Figure 3B) have been found to

efficiently inhibit replication of the HCV replicon in cultured cells.

HCV replicons resistant to the inhibitory action of **[8]** have been selected (Migliaccio *et al.*, unpublished). The characterization of these clones has revealed that either of two specific mutations in the NS5B region of the HCV genome is sufficient to confer resistance to this compound. Specifically, substitution of Pro156 with either Ser or Leu, or substitution of Gly152 with Glu were found to selectively confer resistance to **[8]** (Table 1). Pro156 and Gly152 are located in the Λ2 fingertips loop, within the NS5B RdRp active site channel, in line with the notion that dihydroxypyrimidine carboxylates directly target the enzyme active site (Figure 2). Molecular modelling data suggest that the Λ2 loop is in close contact with the specificity portion of **[8]** but not with its metal chelating portion, thus explaining how mutations in this region may confer resistance without affecting the enzyme catalytic center. Interestingly, this mechanism of acquired resistance seems different from that observed for diketo acid inhibitors of HIV integrase. In this latter case, mutations that conferred resistance to diketo acid inhibitors mapped in amino acids adjacent to the metal coordinating residues of HIV-1 integrase (Hazuda *et al.*, 2000).

Although not part of the enzyme catalytic core, Gly152 is conserved across all six major HCV genotypes. In contrast, in HCV genotype 2a, Pro156 is replaced by Ala. As a crucial step toward the identification of a pan-genotypic anti-HCV agent, it will be very important to determine whether or not this HCV strain is susceptible to inhibition by dihydroxypyrimidine carboxylates.

Non-nucleoside allosteric inhibitors

Screening efforts by many different laboratories have resulted in the identification and characterization of several structural classes of allosteric NNIs. The majority of these compounds have been reported to inhibit the HCV polymerase but not cellular polymerases or polymerases of other viruses. In addition, compounds in each structural class have been shown to block HCV replication in the replicon system with no obvious cytotoxicity, highlighting the potential for further development of several different chemical scaffolds. The detailed mechanism of action of the various classes of allosteric inhibitors has been reviewed recently (De Francesco *et al.*, 2003; Wu & Hong, 2003). Here, we will discuss only those classes of inhibitors for which resistance studies have been reported. Notably, in contrast to the HIV reverse transcriptase for which all known NNIs bind to the same site, genetic, biochemical and structural data indicate that at least three different binding sites exist for NNIs of the HCV polymerase. Consequently, allosteric inhibitors of the HCV polymerase are discussed below, based on their binding site on the enzyme.

Benzimidazoles/indoles

The first group of allosteric NNIs of the HCV RdRp comprises a rather heterogeneous series of compounds based on a benzimidazole or indole core. The initial trisubstituted benzimidazole leads were discovered by high-throughput screening of proprietary compound collections at Boehringer-Ingelheim and Japan Tobacco (Hashimoto *et al.*, 2001; Beaulieu *et al.*, 2004a). Significant increase in potency was achieved by varying the substituents of the benzimidazole scaffold. Compounds like **[9]** (Figure 4A) are both potent inhibitors of the isolated NS5B enzyme and of HCV replicons (Beaulieu *et al.*, 2004a; Tomei *et al.*, 2003). In fact, Japan Tobacco has initiated Phase I/II clinical trials with an oral compound (JTK-003) presumably based on a benzimidazole scaffold (McHutchison & Patel, 2002). Subsequent SAR studies, together with structural information obtained by NMR and X-ray crystallography, further defined the role of the different pharmacophore elements and led to the development of a family of tetrasubstituted indoles, with sub-micromolar blockade of HCV replicons (Figure 4A, **[10]**) (Beaulieu *et al.*, 2004b; Avolio *et al.*, 2004; Harper *et al.*, 2005; LaPlante *et al.*, 2004). These compounds are apparently devoid of the off-target activities associated with the initial benzimidazole leads and show encouraging PK profiles in pre-clinical species, confirming the potential of this series for the development of new anti-HCV agents.

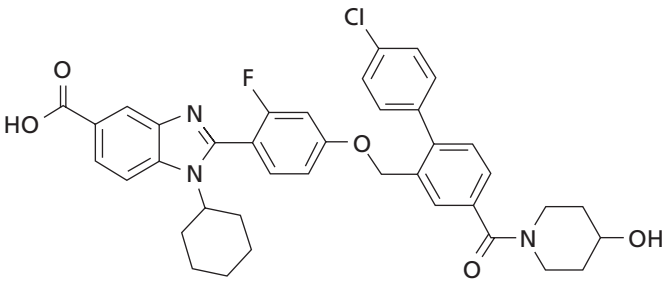
Detailed analysis of their mechanism of action indicated that compounds of this class act as allosteric inhibitors. Through direct interaction with the enzyme at a single binding site (LaPlante *et al.*, 2004; McKercher *et al.*, 2004; Tomei *et al.*, 2003), they inhibit RNA synthesis non-competitively with respect to NTP substrates and interfere with productive RNA binding. As suggested by single-turnover studies, these compounds block RdRp activity during a pre-elongation step, presumably impeding a conformational transition needed for the formation of a productive polymerase/RNA complex.

Replicons resistant to inhibition by benzimidazole-based compounds have been selected by using potent analogues, such as **[9]**, that efficiently inhibit replication of sub-genomic RNA in the cell-based assay (Tomei *et al.*, 2003). So far, a single NS5B substitution, Pro495 to Leu or Ala (P495L/A), has been shown to selectively confer resistance to this class of inhibitors (Table 1). Interestingly, while the enzymes carrying either substitution of Pro495 retained full catalytic activity, replicons where these substitutions were segregated did not replicate efficiently. In fact, supplementary mutations, such as replacement of Ile585 with Thr (I585T), have been shown to compensate for the

Figure 4. Allosteric inhibitors of the NS5B polymerase. Shown are the structures of representative examples of benzimidazoles/indoles **(A)**, benzothiadiazines **(B)** and phenylalanines, thiophenes and dihydropyranones **(C)**

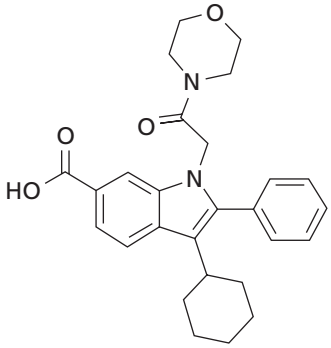
A

[9]



Enzyme IC_{50} 280 nM
Replicon IC_{50} 350 nM
(Tomei *et al.* 2003)

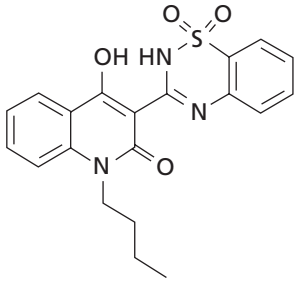
[10]



Enzyme IC_{50} 26 nM
Replicon IC_{50} 800 nM
(Harper *et al.* 2005)

B

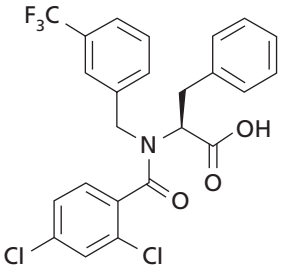
[11]



Enzyme IC_{50} 100 nM
Replicon IC_{50} 550 nM
(Dhanek *et al.* 2002)

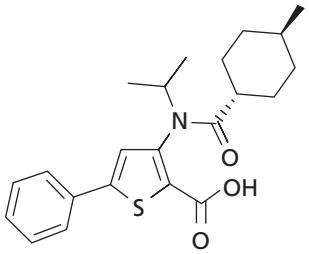
C

[12]



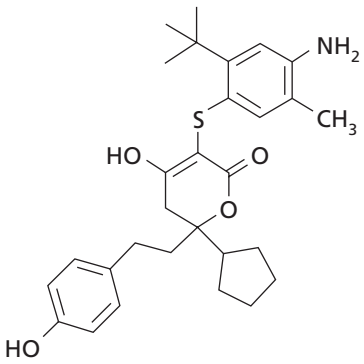
Enzyme Ki 2.2 μM
(Wang *et al.* 2003)

[13]



Enzyme IC_{50} 1.5 μM
Replicon IC_{50} 0.8 μM
(Chan *et al.* 2004a)

[14]



Enzyme IC_{50} 0.9 μM
(Love *et al.* 2003)

Enzyme and replicon IC_{50} or Ki values are derived from the indicated references.

replication defect imposed by Pro495 substitutions. Pro495 is located in the thumb domain of NS5B (Figure 2) and is conserved across natural HCV strains, suggesting a functional role for this residue. X-ray crystallographic data indicated that Pro495 is a key residue involved in the interaction with a non-catalytic GTP molecule on NS5B (Bressanelli *et al.*, 2002). This finding, together with the observation that high GTP concentrations antagonized inhibition by benzimidazoles, led to the speculation that the binding site for this class of inhibitors coincided at least in part with the non-catalytic GTP-binding site (Tomei *et al.*, 2003). Indeed, the very recent elucidation of the structure of the polymerase in complex with representative benzimidazoles or indoles showed that these inhibitors bound the enzyme in a cavity of the thumb domain, on the opposite side of Pro 495 with respect to GTP (Di Marco *et al.*, 2005; Coulombe *et al.*, 2004). Intriguingly, in the apoprotein the same cavity is occupied by the short α-helix A at the extremity of the Λ1 fingertips loop and mediates the interaction between the thumb and the finger domains. In the inhibitor-bound structure, the α-helix A is displaced, suggesting that the inhibitor can disrupt the fingertip-thumb interactions, thus forcing the enzyme into an 'unlocked' conformation. Whether a 'locked/unlocked' conversion is required for productive template binding and whether this conversion is affected by these inhibitors is a fascinating hypothesis that remains to be proved.

Interestingly, despite the conservation of Pro495 across all viral genotypes, representative benzimidazoles have been found to be significantly less active on enzymes and replicons derived from genotype 2 clinical isolates (Carroll *et al.*, 2004; Ludmerer *et al.*, 2005). While the lack of inhibition of genotype 2 isolates is probably due to other, yet unidentified, differences between the NS5B polymerases, it remains to be defined to what extent the lack of activity on these isolates is specific for the tested compounds or whether it is common to other compounds of the same series.

Benzothiadiazines

A second class of allosteric inhibitors of the HCV polymerase is based on a benzothiadiazine scaffold and was initially identified by high throughput screening of the GlaxoSmithKline proprietary compound collection (Figure 4B, **[11]**) (Dhanak *et al.*, 2002; Dhanak *et al.*, 2001). Representative compounds from this class have been thoroughly characterized in biochemical assays and in the replicon system (Dhanak *et al.*, 2002; Gu *et al.*, 2003; Johnston *et al.*, 2003; Tomei *et al.*, 2004). In common with the benzimidazole/indole derivatives, compounds from the benzothiadiazine class have been shown to interact directly with the NS5B polymerase, to be non-competitive with respect to the NTP substrates and to inhibit RNA synthesis, acting prior to the formation of an elongation complex (Dhanak *et al.*, 2002; Gu *et al.*, 2003; Tomei *et al.*, 2004). In contrast to benzimidazoles, however, benzothiadiazines were less potent on deleted forms of the polymerase lacking the β–hairpin or the C-terminal domain (Tomei *et al.*, 2004). Furthermore, synergistic inhibition of the polymerase by representative compounds of the two classes and other subtle differences in *in vitro* assays suggested that benzimidazoles and benzothiadiazines bind the enzyme at different sites and possibly act via different mechanisms (Tomei *et al.*, 2004). This hypothesis was strengthened by the different pattern of resistance mutations and by the lack of reciprocal cross-resistance between benzothiadiazine and benzimidazoles (Nguyen *et al.*, 2003; Tomei *et al.*, 2004; Mo H, Lu L, Pilot-Mathias T, Dekhtyar T, Masse S, Pithawalla R, Ng T, Pratt J, Donner P, Maring C, Randolph J, Klein L & Molla A (2004) Identification and characterization of mutations conferring resistance to either a HCV RNA-dependent RNA polymerase or HCV serine protease inhibitor or both *in vitro*. *11th International Symposium on Hepatitis C Virus & Related Viruses*, Heidelberg, Germany). Five different single NS5B mutations have been reported to induce resistance to benzothiadiazines, namely substitution of His95 with Arg (H95R), Met414 with Thr (M414T), Tyr448 with His (Y448H), Cys451 with Arg (C451R) and Gly558 with Arg (G558R) (Table 1). Notably, comparison of replicons and enzymes carrying these resistance mutations contributed towards clarifying the mechanism of inhibition of the benzothiadiazines. In fact, analysis of the mutant replicons confirmed that all mutations were associated with a resistant phenotype. Unexpectedly, replicons containing C451R or G558R became 'inhibitor-dependent', showing significant levels of replication only in the presence of benzothiadiazines. In biochemical assays, only the M414T mutant enzyme demonstrated clear resistance to inhibition, while the enzymes containing the H95R, C451R and G558R mutations had no or marginal changes in their sensitivity to inhibition. This discrepancy, together with the observation that the mutated amino acids lie in different regions of the polymerase (Figure 2), suggested the existence of multiple mechanisms of resistance and provided a first indirect clue towards identification of the benzothiadiazines binding site on the enzyme. Indeed, only the mutant M414T enzyme lost the ability to interact with benzothiadiazines in a direct binding assay, indicating that only for this mutant was resistance due to reduced affinity for the inhibitor and suggesting that Met414 might be part of the inhibitor binding site. The location of Met414 on the inner surface of the thumb domain, close to the active site, provided additional evidence that the benzothiadiazine binding site was distinct from those of other allosteric inhibitors (see respective sections). Conversely, the other

mutations were proposed to induce resistance with different mechanisms. The C451R/Y448H and G558R mutations are located in the β-hairpin and C-terminal region, respectively, confirming the results obtained with deletion mutants and suggesting that these mutations might affect the interaction of these structural elements. The altered interaction of the β-hairpin/C-terminal region might lead to decreased inhibition by benzothiadiazines and also explain why replicons containing C451R or G558R do not replicate efficiently in the absence of compound. Finally, the mechanism of inhibition arising from the H95R mutation remains speculative. Since His95 has been implicated in binding the RNA template (O'Farrell *et al.*, 2003), it has been proposed that replacement of this residue *in vivo* might influence the ability of the polymerase to recognize the viral genome in a way not reproduced in the available biochemical assays. Taken together, the characterization of resistant mutants confirmed and extended the model for the mechanism of inhibition by benzothiadiazines that emerged from biochemical dissection and replicon studies. In this model, benzothiadiazines bind to the enzyme near Met414 and inhibit polymerase activity through a true allosteric mechanism, possibly by altering the distribution of enzyme between an active and an inactive conformation.

Sequence analysis of natural HCV isolates revealed that two NS5B residues involved in resistance to benzothiadiazines, M414 and C451, are not entirely conserved among HCV genotypes, implying that some naturally occurring HCV isolates might be resistant to this class of inhibitors. 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 (Ludmerer *et al.*, 2005; Carroll *et al.*, 2004). However, it is not yet clear whether the lack of activity on these isolates is specific for the tested compounds or if it is a class effect and whether it is due to the resistance mutations identified for the 1b replicon/enzyme or to other differences in the NS5B polymerase.

Thiophenes, phenylalanines and dihydropyranones

The third group of allosteric inhibitors of the HCV polymerase is more heterogeneous and comprises compounds with different chemical structures, namely thiophene, phenylalanine and dihydropyranone derivatives (Figure 4C, **[12]**, **[13]** and **[14]**). Despite the dissimilarities of their chemical structures, these three classes of allosteric inhibitors can be grouped together based on the crystallographic evidence that they bind the enzyme at the same site (Biswal *et al.*, 2005; Love *et al.*, 2003; Wang *et al.*, 2003). Thiophene and phenylalanine derivatives have been identified by high throughput screening of the Shire BioChem proprietary compound collection while dihydropyranones were identified by a similar approach at Agouron/Pfizer (Chan *et al.*, 2004a; Chan *et al.*, 2004b; Love *et al.*, 2003; Wang *et al.*, 2003). For these structural series, subsequent SAR studies have led to the identification of compounds with potent activity in polymerase assays. Similarly to the other allosteric inhibitors discussed above, these compounds act as reversible inhibitors of the NS5B polymerase and are not competitive with NTP substrates. What is not known is whether they act only at a step preceding elongation or if they also inhibit polymerization. The structure of the polymerase in complex with representative compounds of each of these three chemical series was determined by co-crystallization of enzyme and inhibitors (dihydropyranone) or by soaking apoprotein crystals in inhibitor solutions (phenylalanines and thiophenes). Crystallographic data established that these three types of inhibitors bind in a long cleft at the base of the thumb domain, ~35 Å away from the active site (Biswal *et al.*, 2005; Love *et al.*, 2003; Wang *et al.*, 2003). Comparison of the polymerase structures with and without inhibitors indicates that, in contrast to benzimidazoles/indoles, the inhibitor binding pocket is occupied by the solvent in the native polymerase structure. Interestingly, while dihydropyranone or phenylalanine binding does not induce major rearrangements of the polymerase structure, binding of thiophene inhibitors causes a conformational change of the enzyme toward an open form (Biswal *et al.*, 2005). Comparison of the binding mode highlights similarities and differences in the interactions established by the three classes of compounds with the enzyme. The cyclopentyl moiety of the dihydropyranone, the dichlorophenyl group of the phenylalanines and the benzoyl substituent of thiophenes occupy the hydrophobic pocket formed by Leu419, Arg422, Met423, Leu474, His475, Tyr477 and Try528, while the charged groups of compounds form direct or water-mediated hydrogen bonds with Ser476 and Tyr477. The remaining moieties of the compounds contact different areas of the protein surface, underlining the promiscuous features of this binding site.

Interestingly, the hydrophobic pocket targeted by these compounds is relatively highly conserved among HCV genotypes, displaying only a few conservative substitutions that are not expected to interfere with inhibitor binding (Wang *et al.*, 2003). Surprisingly, initial profiling of thiophene derivatives on replicons and enzymes derived from different viral isolates is only partially in agreement with this hypothesis. In fact, compounds of this class have been reported to retain activity on several replicons expressing genotype 1 polymerases but to be significantly less potent on polymerases of genotypes other than 1 (Ludmerer *et al.*, 2005; Carroll *et al.*, 2004). Thus far, limited resistance

studies have been reported on representative thiophene derivatives (Migliaccio *et al.*, unpublished).

Two different single NS5B mutations have been reported to induce resistance to thiophene inhibitors – substitution of Leu419 with Met (L419M) or substitution of Met423 with Thr (M423T). Based on the location of Met423 and Leu419 in the hydrophobic region of the inhibitors' binding site, it is speculated that substitutions of these residues significantly alter the binding site and thus induce resistance by reducing the enzyme affinity for the inhibitors. Although the full resistance and cross-resistance pattern of these mutants has yet to be determined, preliminary data indicate no or limited cross-resistance with nucleoside, benzimidazole and benzothiadiazine inhibitors (Table 1).

Conclusions and future outlook

The identification of inhibitors of the HCV polymerase that efficiently block viral replication in tissue culture has fostered the hope that these compounds will become effective anti-HCV drugs in the near future. Indeed, an NS5B NI has shown promising results in clinical trials, and efficacy studies in humans have been announced for other compounds. The enthusiasm has been partially curtailed by recent *in vitro* evidence that HCV genetic variability represents a serious hurdle for the clinical success of HCV polymerase inhibitors. Though the relevance of these initial results requires confirmation from more physiological *in vivo* studies, it is likely that they will impact the direction of drug discovery efforts. Indeed, these *in vitro* studies have fully confirmed the suspicion that the HCV genetic variability is a major determinant of the ability of various classes of polymerase inhibitors to block viral replication. Preliminary profiling of prototype compounds on different viral isolates revealed remarkable differences in activity, especially in the case of allosteric inhibitors. Along the same lines, selection experiments have demonstrated that, at least in tissue culture, HCV can become resistant to every class of polymerase inhibitor identified thus far.

Comparison of the available data highlights similarities and differences between the diverse classes of NS5B polymerase inhibitors. Notably, for all inhibitors analysed so far, a single mutation in the polymerase is apparently sufficient for *in vitro* resistance. Yet, while resistance to 2′-C-Me nucleosides and benzimidazoles is apparently due to mutations of one unique residue, mutations of numerous residues have been reported to confer resistance to other NNIs (Table 1). In addition, quantitative data have not always been reported and it is likely that the degree of resistance will not be the same for all mutations. Further, while the majority of mutations had minimal effects on replication, the substitutions of Ser282 or Pro495 have been shown to have a negative effect on replicon fitness and presumably require additional 'compensatory' mutations. Finally, each class of inhibitors elicits a different set of resistance mutations that show limited, if any, cross-resistance, while cross-resistance is in general the rule within the same structural class (Table 1). This finding indicates that inhibitors with different mechanisms of action can be considered as acting on different targets and therefore have the potential to be developed for combination therapy.

Based on all available data, it could be predicted that allosteric inhibitors will likely be effective only on some viral isolates or genotypes. Similarly, these data indicate that the possibility for resistance arising in the clinic exists for all classes of polymerase inhibitors, although some classes are likely to show a higher genetic barrier to resistance development. However, it is obviously too early to draw firm conclusions, since the majority of studies reported so far have been performed with prototype compounds that may not reflect the results that will be obtained with more potent and optimized inhibitors. Furthermore, resistance mutants have been selected with only a couple of viral isolates and therefore it is difficult to anticipate whether comparable resistance patterns will be observed in different genetic contexts. Given the different requirements for replication *in vitro* and *in vivo*, it is not immediately obvious if the debilitating effect of some resistance mutations on replicon fitness will translate into a similar effect on HCV replication in humans. Lastly, inhibitors have been profiled only on a relatively small number of isolates, making it difficult to generalize the results of these studies. For the same reason, it is not yet possible to identify with certainty the molecular determinants of the difference in activity between viral isolates. Only in some cases is it likely that the difference in potency reflects a variation of residues affected by resistance mutations.

What will be the impact of these results on drug development and what should we expect for the future? In our opinion, these initial results strongly argue for the necessity to perform resistance studies and profile inhibitors against a variety of viral isolates even at early stages of inhibitor development.

Based on the current knowledge and methodologies, it is already possible to use the information obtained from *in vitro* resistance studies to assist inhibitor development and to broaden their spectrum of activity on different viral isolates. Indeed, by simply profiling inhibitors against a limited number of natural variants and resistant mutants it is possible to predict the breadth of activity of a given inhibitor and obtain information on the mechanism of action and the site of binding on the enzyme. In addition, comparison of related compounds on sensitive and resistant

viral variants can provide crucial information on the mechanisms and the determinants of resistance that, together with biochemical and structural data, contribute to the design of improved inhibitors.

In the case of HIV, enough data have been accumulated to predict the chance of therapeutic response to most classes of drugs based on the viral sequence. These data are currently used to tailor therapy and also to assist the development of new drugs. In comparison, the field of HCV resistance to antiviral agents is still in its infancy, though it is growing up at a rapid rate. Considering that much of the information reported in this review has yet to appear in press, we suspect that the results we have seen so far represent only the tip of the iceberg and we expect to see HCV resistance studies increasingly published in the near future. Hopefully, these forthcoming results will contribute to deciphering the molecular determinants of inhibitor activity and will provide the key for predicting the probability of therapeutic response to the NS5B inhibitors.

The documented emergence of *in vitro* resistance to inhibitors of the NS5B polymerase – as well as to inhibitors of the NS3-4A protease (Lin *et al*., 2004) - will probably have decisive bearing on the clinical development pathways of direct anti-HCV agents. In the near future these agents will probably be developed in combination with IFN or IFN and ribavirin. This is already the case for the NS5B inhibitor NM283. Once more agents are available, it will become possible to conceive new combination schemes. Considering that one of the major limitations of current therapies is the severity of the side effects, it would be desirable to shift away from the current drugs and develop combinations of therapies based entirely on novel antiviral agents. Whether these combinations will be sufficient to effectively treat HCV patients remains an open question that will hopefully be answered soon.

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