Acta Biochimica Polonica

Vol. 52 No. 1/2005

57–70

QUARTERLY

Review

Existing and future therapeutic options for hepatitis C virus infection✪

Maria Bretner✉

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland

Received: 31 December, 2004; accepted: 08 March, 2005

Key words: hepatitis C virus, antiviral agents

Hepatitis C virus (HCV) infection is an important cause of chronic hepatitis, cirrhosis, hepatocellular carcinoma and liver failure worldwide. Chronic hepatitis C virus infection is treated with interferon-α (IFN-α), pegylated interferon-α (PEG-IFNα) alone or in combination with ribavirin; however, a significant fraction of patients either fail to respond or relapse after cessation of therapy. Efforts to identify and develop highly specific and potent HCV inhibitors have intensified recently. Each of the virally encoded replication enzymes has been a focus of studies as well as viral receptors and the host immune system. This review summarizes recent progress in the search for novel anti-HCV agents.

Hepatitis C virus (HCV) infection is a serious disease and can develop into chronic hepatitis, liver cirrhosis or hepatocellular carcinoma. After the identification of the virus in 1989, reliable diagnostic methods have led to the realisation that infection afflicts about 300 million people worldwide (Nishikawa *et al.*, 2003). Interferons (IFNs) are the foundation of antiviral therapy. Pegylated interferons have an improved efficacy compared to standard IFN because of longer half-lives and decreased clearance but current combination treatment with an antiviral nucleoside, ribavirin, is still not sufficiently effective.

✪This paper was written with financial support from the Commission of the European Communities, specific RTD programme „Quality of Life and Management of Living Resources", QLK2-CT-2002-01079 „HCV Inhibitors"

✉Correspondence to: M. Bretner, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawińskiego 5A, 02-106 Warszawa, Poland; e-mail: majka@ibb.waw.pl

Abbreviations: CC, cytotoxic concentration; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome-entry site; NNI, non-nucleoside inhibitor; NS, non-structural; NTPase, nucleoside triphosphatase; NTR, nontranslated region; ORF, open-reading frame; RdRp, RNA-dependent RNA polymerase; RR, relative risk; SVR, sustained virological response.

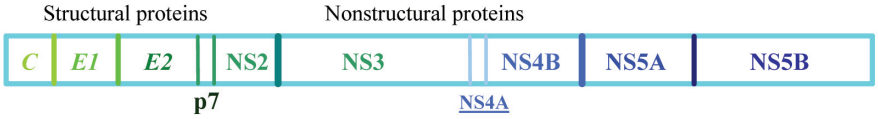
These drugs work non-specifically by inhibiting HCV replication and stimulating the immune response, but they have numerous side effects. The efforts in developing new therapeutics are hampered by difficulties with replication of HCV in cell cultures. Animal infection models are limited to chimpanzee (Lanford & Bigger, 2002) or immunodeficient mice carrying engrafted human liver cells (Mercer *et al.*, 2001). The understanding of genomic organisation, availability of the three-dimensional structures of virally encoded enzymes and the recent development of an HCV-replicon system in Huh-7 cells have led to an advance in the development of antivirals, many of which are currently in clinical trials.

GENOMIC ORGANISATION AND POLYPROTEIN PROCESSING

Hepatitis C virus is a positive-sense single-stranded RNA virus with a genome of about 9.6 kb (Takamizawa *et al.*, 1991). It consists of a single open-reading frame (ORF) flanked by 5′ and 3′ non-translated regions (NTR) (Tanaka *et al.*, 1995).

The 5′NTR contains the internal ribosome-entry site (IRES) which mediates the initiation of viral RNA translation. The HCV genomic RNA encodes a polyprotein of 3010–3011 amino acids which undergoes cotranslational and posttranslational proteolytic processing in the cytoplasm or in the endoplasmic reticulum of the infected cell to give rise to four structural and six non-structural (NS) proteins (Hijikata *et al.*, 1991). Figure 1 summarises the information concerning the identification and the function of individual gene products.

The structural proteins consist of the capsid or core protein C, two envelope glycoproteins E1 and E2, and small hydrophobic polypeptide p7. The non-structural proteins are NS2, NS3, NS4A and B, and NS5A and B. NS2 has a zinc-stimulated protease activity that cleaves the NS2/NS3 junction (Love *et al.*, 1996). NS3, a 70 kDa protein, has been subject of intensive study because it is a multifunctional molecule with a trypsin-like serine protease catalytic domain at the N-terminal 181 residues (Tomei *et al.*, 1993), and a nucleoside triphosphatase (NTPase)/RNA helicase domain at the C-terminal two-thirds. NS4A is a small protein (8 kDa) that is an NS3 protease cofactor (Failla *et al.*, 1994). NS4B is a hydrophobic, 27 kDa protein, and it has been suggested that it may function as an anchor to secure part of the HCV replication



Amino acids	Protein	Function
1-191	C	nucleocapsid
192-383	E1	envelope glycoprotein
384-746	E2	envelope glycoprotein
747-809	p7	virioporin
810-1026	NS2	Zn-activated NS2/3 auto-protease
1027-1657	NS3	Ser protease (aa 1-180) RNA helicase (aa 181-631)
1658-1711	NS4A	NS3 Ser protease cofactor (aa 21-34)
1712-1972	NS4B	Induces the formation of intracellular membrane vesicle
1973-2420	NS5A	α-interferon resistance
2420-3010	NS5B	RNA dependent RNA polymerase

Figure 1. Organisation of HCV polyprotein.

apparatus to the endoplasmic reticulum (ER) membrane (Lundin *et al.*, 2003). Two cytoplasmic phosphoproteins, p56 and p58 (56 kDa and 58 kDa, respectively) are produced from the NS5A region of the HCV genome (Kaneko *et al.*, 1994; Neddermann *et al.,* 2004). Both proteins are phosphorylated at serine residues in the region between amino acids 2200 and 2250 and in the C-terminal region of NS5A. Although the function of NS5A in viral replication is unknown, it is possible that NS5A might be involved in the resistance of HCV to α-interferon therapy (Tan & Katze, 2001). The NS5B protein of HCV has been overexpressed both in insect cells infected with recombinant baculovirus and in *E. coli* (Lohmann *et al.,* 1997). The purified enzyme exhibits a highly processive primer-independent RNA-dependent RNA polymerase (RdRp) activity capable of copying *in vitro* transcribed full-length genomic HCV RNA (Oh *et al.*, 1999).

HCV REPLICATION

The infected cells are predominantly hepatocytes. Detailed information about the molecular mechanism of HCV replication is not available, but it is thought that it may be similar to that of other positive-stranded RNA viruses (De Francesco, 1999). HCV infection begins with attachment, which is mediated by a specific interaction between cell surface molecules on the target cells and the viral envelope proteins. The mechanisms of virus entry remain unknown, as are the receptors for HCV entry are not known at present. The virus is probably engulfed by receptor-mediated endocytosis, and the positive-strand RNA genome is delivered to the cytoplasm. The positive-strand RNA is translated to produce a single large polyprotein which is then cleaved into structural and non-structural proteins important for replication. In order for HCV to replicate, negative-strand RNA must be synthesised using the incoming positive-stranded RNA as a template. The negative-strand replicative intermediate is then used as a template to synthesise positive-strand progeny RNA, which is packed into viral capsids. Because of the high replication rate and a lack of a proof-reading function of NS5B, the HCV genome has high genetic variability (Tan *et al.,* 2002). A schematic model of HCV replication is shown in Fig. 2.

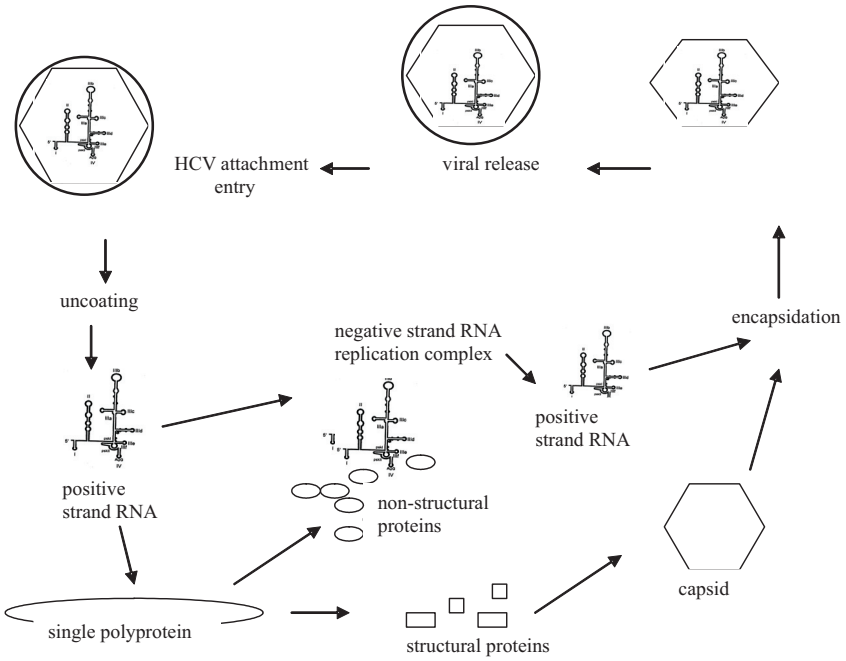


Figure 2. Model of HCV replication.

CURRENT THERAPIES

IFN-α is a member of the interferon family of multifunctional proteins involved in antiviral defence, cell growth regulation and immune activation. The efficacy of IFN-α monotherapy was unsatisfactory resulting in a sustained virological response (SVR) in only 14% of patients infected with HCV genotype 1 (HCV-1) (Shepherd *et al.*, 2004). Semi-synthetic protein-polymer conjugates of interferon with polyethylene glycol have been developed (Wang *et al.*, 2002). These conjugates protect the protein from degradation, reduce its immunogenicity, and prolong exposure to the drug by sustained absorption, restricted volume of distribution and sustained high serum concentration. Therapy with pegylated interferon is associated with significantly greater SVR compared to the non-pegylated formulation (SVR 31%) (Lake-Bakaar, 2003).

Ribavirin is a guanosine analog with minimal antiviral activity against HCV. It demonstrates, however, significant clinical synergism when administered in combination with interferon. In the trials that tested pegylated interferon plus ribavirin against non-pegylated interferon plus ribavirin the combined percentage of sustained virological response was 55%. The relative risk (RR) for remaining infected was reduced by 17% for pegylated interferon plus ribavirin compared with non-pegylated interferon plus ribavirin. Response to therapy varied according to the viral genotype. Amantadine blocks entry of influenza A virus into cells. Used in combination with ribavirin and interferon as triple therapy, it may have some benefit compared to dual or monotherapy. Recently it has been shown that amantadine blocks the viral ion channel formed by p7 protein (Griffin *et al.*, 2003). Current treatment with pegylated interferons combined with weight-based ribavirin provides the highest sustained virological response rates.

FUTURE ANTIVIRAL THERAPIES

Several potential antiviral targets have been identified from the knowledge of the structure of the HCV genome. These include (a) the internal ribosome entry site (IRES) which could be blocked by molecules that inhibit the binding to ribosomes; (b) core protein and E1/E2 proteins, which could be attacked by antisense RNA, (c) protease or helicase susceptible to inhibition by specific inhibitors, (d) RNA-dependent RNA polymerase that could by attacked by specific inhibitors. The recently developed subgenomic HCV replicon which allows high-level replication of HCV RNAs in the human hepatoma cell line Huh-7 is widely used to unravel the principles of HCV replication and for drug screening (Lohmann *et al.*, 1999; 2001). The first subgenomic HCV replicon consists of 5′-NTR which directs expression of neomycin phosphotransferase II from its gene (neo) cloned downstream, followed by the encephalomyocarditis virus internal ribosome entry site (IRES) which drives expression of HCV non-structural proteins NS3-NS5B from their gene (Zhu *et al.*, 2003). The replicon-containing cells support only replication of HCV RNA encoding structural proteins, not the entire viral replication. A variety of modified replicons have been produced to improve the efficiency of colony formation, and to include different subtypes and strains of HCV (Huang & Deshpande, 2004). Replication can be measured using a variety of techniques: by counting the number of cells in which replication is established (neo selection experiments) by measuring viral RNA (Northern blotting or RNAase protection assay) or proteins (Western blotting or enzyme-linked immunosorbent assay), or by using reporters such as luciferase expressed from the replicon (Murray *et al.*, 2003).

Inhibitors of RNA-dependent RNA polymerase

Several structural classes of NS5B RdRp inhibitors that are active in the cell-based replicon assay have been identified (Carroll *et al.*, 2003; Gu *et al.*, 2003; Tomei *et al.*, 2004). Inhibitors can be classified into three categories according to their chemical structure: nucleoside analogues, pyrophosphate mimics and non-nucleoside inhibitors (NNIs). Among the nucleoside analogues, 2'- or 3′-substituted nucleosides act as chain terminators and effectively inhibit replication of HCV subgenomic replicon. Merck Research Laboratories and Isis Pharmaceuticals Inc. described a detailed structure-activity relationship (SAR) study of 2′- and 3′-substituted ribonucleosides (Eldrup *et al.*, 2004). However, only a few of the analogues have show activity in cell-based subgenomic replicon assay. Triphosphates of 2′-C-methyladenosine (2′-C-MeA, **1**; Fig. 3) and 2′-O-methylcytidine (2′-O-MeC, **2**; Fig. 3) demonstrated similar inhibition of NS5B *in vitro* (IC_{50} 2.5 μM and 3.5 μM), but significantly higher concentrations of **1** than **2** were detected in cells (Carroll *et al.*, 2003). This is consistent with the greater potency of 2′-C-methyladenosine (EC_{50} = 0.3 μM) *versus* 2′-O-methylcytidine (EC_{50} = 21 μM) in cells. Valeant Pharmaceuticals patented adenine analogues, one of them (**3**, Fig. 3) in pharmacokinetic studies in mice showed a bioavailability of 100% and did not exhibit significant acute toxicity at doses of up to 160 mg/kg (Ni & Wagman, 2004). Non-chain-terminating nucleoside analogues are also feasible, especially those with mutation-inducing abilities. Recent studies suggest that once misincorporated they cause errors in viral replication (Stuyver *et al.*, 2003), as is also suggested for the ribavirin (Maag *et al.*, 2001). The introduction of the amino acid valine at the 3′-position of ribose gave NM-283 (**4**, Fig. 3), currently in development by Idenix Pharmaceuticals Inc., which is now into clinical trials (Standring, 2003). NM-283 has low toxicity *in vitro* (CC_{50} > 100 μM) and *in vivo* (no observed adverse effect level (NOAEL) = 166 mg/kg for monkeys). In early clinical studies oral dosing of NM-283 (200 mg/day for 7 days) in HCV infected patients reduced the viral titer by up to 0.49 log (Ni & Wagman, 2004).

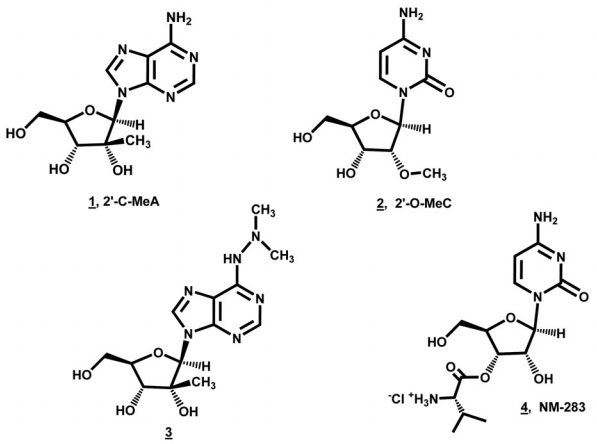


Figure 3. Chemical structures of HCV NS5B RdRp inhibitors.

Compounds interfering with the binding of phosphoryl groups of the nucleotide substrates at the active site have been identified. Over 200 compounds including alkyl-, phenyl-, pyrrole- and thiophene-substituted diketoacids were evaluated against HCV NS5B polymerase activity (Altamura *et al.,* 2000). Among them, several phenyldiketoacids and 2-aryl-4,5-dihydroxy-carboxypyrimidines demonstrated low nanomolar IC_{50} values (**5** and **6**, Fig 4.) in the cell-based HCV subgenomic replicon assay (De Francesco, 2000). The benzo-1,2,4-thiadiazine derivative of hydroxyquinoline [1-butyl-3-(1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)-4-hydroxyquinolin-2-(1*H*)-one (**7**) and 1-isopentyl-3-(1,1-dioxido-4*H*-1,2,4-benzothiadiazin-3-yl)-4-hydroxyquinolin-2-(1*H*)-one (**8**), Fig. 4] were shown to be potent inhibitors of the synthesis of the positive and negative strand RNA by HCV RdRp with IC_{50} 0.17–0.14 μM (**7**) and, respectively, 0.06–0.04 μM (**8**) (Dhanak *et al.*, 2002).

A significant advance in the understanding of the NS5B polymerase was provided by crystallographic studies of the apoenzyme

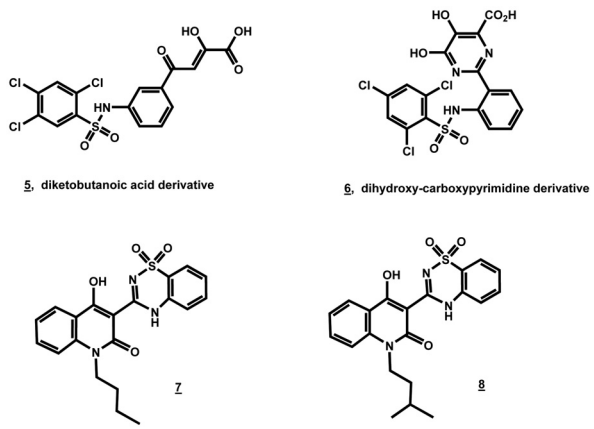


Figure 4. Chemical structures of HCV NS5B RdRp inhibitors.

and of complexes with nucleotides or RNA template (Ago *et al.*, 1999; Bressanelli *et al.*, 2002). In all structures reported the fingers, palm, and thumb subdomains characteristic of all known RNA and DNA polymerases may be seen (Fig. 5). The unique feature of HCV NS5B is its encircled active site with overall globular shape instead of the typical U shape found in other polymerases (Lesburg *et al.*, 1999). Among the numerous non-nucleotide compounds documented to have inhibitory activity, (2*S*)-2-[(2,4-dichloro-benzoyl)-(3-trifluoromethyl-benzyl)-amino]-3-phenyl-propionic acid has been shown to bind on the protein surface in a narrow cleft in the thumb domain (Wang *et al.*, 2003) (**9**, Fig. 5).

Inhibitors of NS3/4A serine protease

The second enzyme very important for viral replication is the NS3 serine protease which is responsible for processing the HCV polyprotein into smaller functional proteins. NS3 becomes fully activated as a serine protease when bound in a heterodimeric complex with NS4A. X-ray crystallography (Love & Parge, 1996) and nuclear magnetic resonance (NMR) spectroscopy (McCoy *et al.*, 2001) have shown that the structure consists of two domains, both composed of a β-barrel and two α-helices. The catalytic triad comprises histidine 57, aspartate 81 and serine 139 and is located between the two domains (Fig. 6).

Initial investigations revealed that the N-terminal peptide products derived from the sequences of the HCV NS3 protease substrates

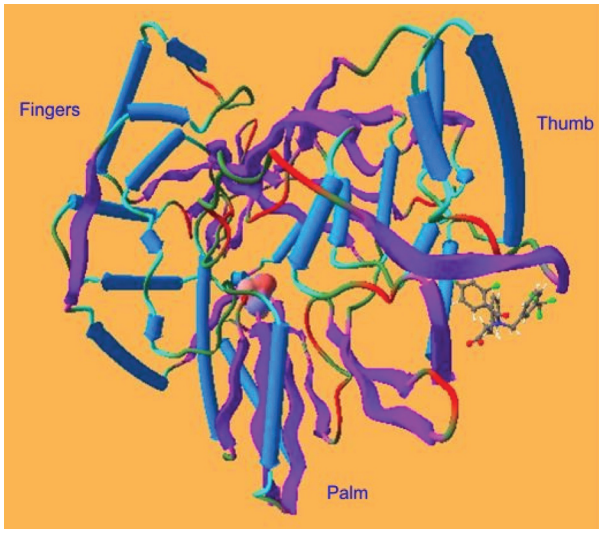


Figure 5. A ribbon diagram of HCV NS5B/inhibitor (9) complex structure.

The protein is colored according to the secondary structure. Asp^{318} in active site coordinating Mg^{2+} ions during the polymerization reaction is shown with red and blue surface. Inhibitor is shown as stick model. Figure generated with MDL Sculpt using 1NHU coordinates from PDB.

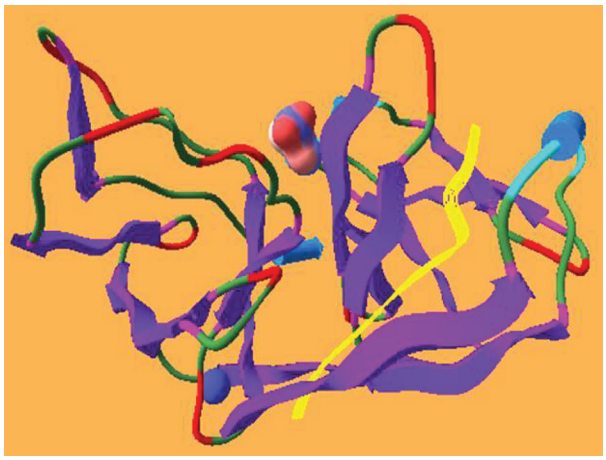
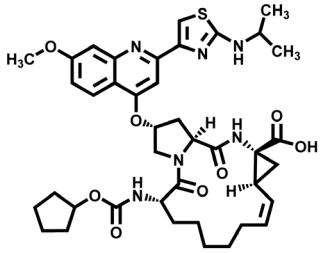


Figure 6. A ribbon diagram of HCV NS3–$NS4A_{protein}$ complex structure.

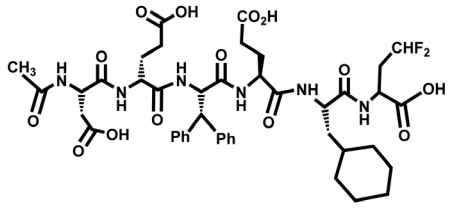
The protein is colored according to the secondary structure, and NS4A protein is yellow. Asp^{81} in the active site is shown with red and blue surface; a blue boll represents Zn^{2+}. Figure generated with MDL Sculpt using 1JXP coordinates from PDB.

are competitive inhibitors of this enzyme, so these peptides became leads for the design of peptidomimetic inhibitors (Llinas-Brunet *et al.,* 2000). Lamarre and his team started with a weak hexapeptide enzyme inhibitor Asp-Asp-Ile-Val-Pro-Cys and K_i of 79 μM (Steinkuhler *et al.,* 1998). Next they shortened the size to a tripeptide and finally stabilized the compounds by intramolecular linking to obtain BILN-2061 (**10**, Fig. 7; Lamarre *et al.,* 2003) patented by Boehringer Ingelheim Corp. In cell-free assays the K_i was 0.3 nM, and in the subgenomic replicon model the compound was effective at penetrating cells and displayed an EC_{50} value of 4 nM. The 50% cytotoxic concentration (CC_{50}) in Huh-7 cells was acceptable at 33 μM (Lamarre *et al.,* 2002). BILN-2061 was not significantly active against human proteases such as leukocyte elastase and liver cathepsin B. In a phase 1B study of eight genotype 1 infected patients with advanced liver fibrosis, oral treatment with 200 mg BILN-2061 for two days was well tolerated and led to one log decrease in virus titre (Benhamou *et al.,* 2002). Another class of inhibitors was designed by replacing the carboxylic acid moiety of the peptide inhibitors with an α-ketoacid moiety (**11**, Fig. 7), which improved potency, with the overall K_i values between 10 pM and 67 nM (Narjes *et al.,* 2000).

Three new series based on the novel cores, such as the bicyclic aminopyridone derivative (**12**, Fig. 8) IC_{50} 0.12 μM (Zhang *et al.,* 2002), the pyrimidinone derivative (**13**, Fig. 8) IC_{50} 0.10 μM (Glunz *et al.,* 2003) and the pyrazinone derivative (**14**, Fig. 8) IC_{50} 20 nM (Zhang *et al.,* 2003) were developed by Bristol-Myers Squibb. The successful clinical use of protease and polymerase inhibitors still has to overcome the hurdle of the emergence of resistant HCV variants. Bearing in mind the high mutation rate observed for HCV it is highly likely that viruses expressing a mutant protease or polymerase with decreased sensitivity could emerge during therapy.



10, BILN-2061



11

Figure 7. Chemical structures of HCV NS3 serine protease inhibitors.

Inhibitors of nucleoside triphosphatase (NTPase)/RNA helicase

The C terminal region of NS3 is a member of a large class of helicases that unwind dsRNA in a nucleoside triphosphate (NTP)-dependent fashion in a 3′ to 5′ direction and plays a key role in viral replication. Different three-dimensional structures of the isolated NS3 helicase domain have been determined (Yao *et al.,* 1997; Cho *et al.,* 1998; Kim *et al.,* 1998). HCV helicase consists of three structural domains separated by clefts, together forming Y-shaped molecule (Fig. 9).

The active site for NTP hydrolysis can be identified as the $APTG^{207}SGKT$ sequence, known as the NTP binding motif I, which is involved in binding the γ-phosphate of NTP. Motif II $DECH^{293}$ is proximal to motif I and is involved in binding of the Mg^{2+}-NTP substrate. His^{293} is located at the bottom of the interdomain cleft and is essential for the coupling of the NTPase activity to polynucleotide binding. Domain 1 is connected to domain 2 *via* a flexible linker region corresponding to

motif III T^{322}ATPP. The second domain contains the highly conserved Q^{460}RRGRTGRGRRG sequence identified as motif VI. Studies of several helicases have evaluated the effects of mutations in this motif; however, a role of this motif has not been clearly defined. Gln^{460} lies at the bottom of the cleft and is thought to interact with His^{293} of the $DECH^{293}$ box found on the opposite side of the cleft on the inner face of domain 1. Arginines –461, 464, and 467 have been proposed by Yao *et al.,* (1997) to be involved in binding single-stranded RNA in the cleft between domains 1 and 2. However, the structure published by Kim *et al.* (1998), where the helicase is complexed with dU_8 shows that Arg^{461} points away from the cleft and is hydrogen bonded to Asp^{412} and Asp^{427} is not consistent with this interpretation. In the X-ray structure of the nucleic acid-bound HCV helicase (Kim *et al.,* 1998; Fig. 9), the oligonucleotide binds in the orthogonal binding site and contacts relatively few conserved residues. The cleft is large enough to bind ssDNA (or ssRNA) but is too small to bind a double-stranded DNA (or RNA; De la Cruz *et al.,* 1999).

Serebrov and Pyle (2004) used a novel combinatorial time-resolved approach to gain insight into the mechanism of unwinding of duplex RNA by HCV helicase. They suggest that the helicase has a leading edge and a lagging edge. The leading edge binds to a duplex region of RNA, while the lagging edge binds to the single stranded 3′ tail. The conformational changes associated with the binding of ATP and its hydrolysis provide the energy for separation of 18 bp of the duplex.

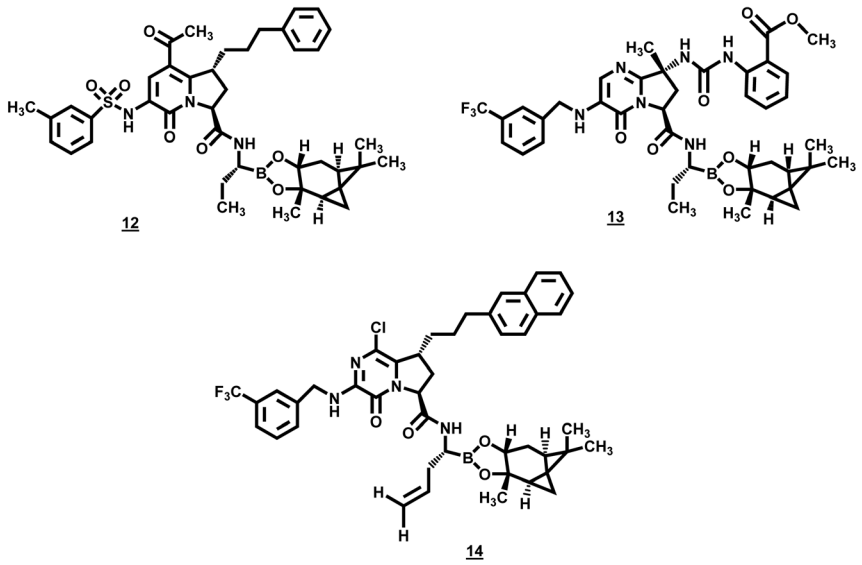


Figure 8. Chemical structures of HCV NS3 serine protease inhibitors.

Because the ATPase activity is coupled to strand separation, researchers are pursuing potential specific inhibitors that target the ATPase and helicase activities of the NS3 protein. Non-hydrolysable ATP analogues do not substitute for ATP in the RNA unwinding reaction. It was pointed out that using adenosine-5′-(3-thio)-triphosphate (ATP-γ-S) or β,γ-methylene ATP (AMPPCP) only a low level or inhibition of the unwinding activity of the NTPase/helicase of HCV was detected (Gallinari *et al.,* 1998). The adenosine analogue 5′-O-(4-fluorosulphonylbenzoyl)-adenosine (FSBA) (**15**, Fig. 10) has been found to react irreversibly with many enzymes which use NTPs as substrates, such as kinases, ATPases, and polymerases (Colman, 1983). This compound might be considered an analogue of ATP or ADP. The structure and size of FSBA is such that it resembles ATP, and

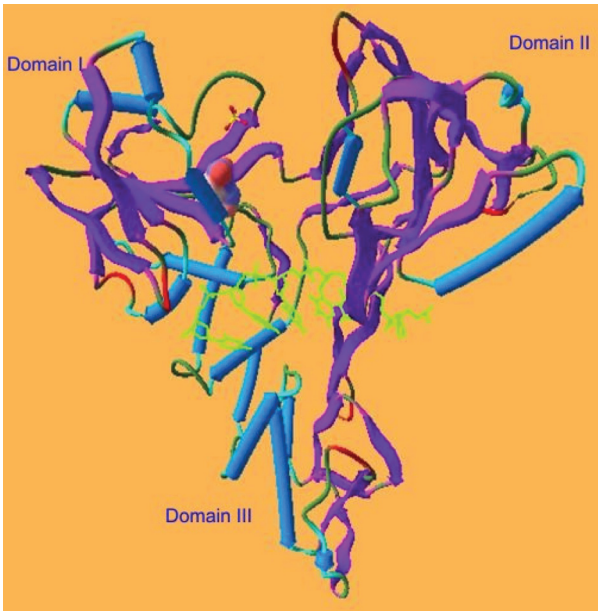


Figure 9. A ribbon diagram of HCV NS3/dU8 complex structure.

The protein is colored according to the secondary structure. Asp^{290} in the DExH motif is shown with red and blue surface. The sulphate ion, shown as stick model appears to occupy the position of β-phosphate of ADP. The DNA is colored green. Figure generated with MDL Sculpt using 1A1V coordinates from PDB.

its reactive sulphonyl fluoride moiety may occupy a position similar to that of the γ-phosphate in NTP. The 5′-O-(4-fluorosulphonylbenzoyl)-esters of ribavirin and purines were synthesized and tested as inhibitors of the nucleotide triphosphatase/helicase activities of HCV and related Flaviviruses (Bretner *et al.,* 2004a). Whereas the ATPase activity of the West Nile virus enzyme could be completely blocked by all tested compounds at 500 μM, they did not cause any measurable inhibition of the ATPase activity of HCV. When the unwinding activity of the viral enzymes was tested under standard conditions, only weak inhibition was obtained with 5′-O-(4-fluorosulphonylbenzoyl)-inosine (FSBI, $IC_{50} \geq 120$ μM). Preincubation of the HCV enzyme with 5′-O-FSB esters increased the inhibitory effect. The influence on the activity of HCV polymerase was also examined, and FSBI exhibited the highest inhibitory activity with $IC_{50} = 80$ μM.

During the course of a random screening of a wide range of unrelated small-molecule compounds in a search for lead inhibitors of HCV NTPase/helicase activity it was noted that 4,5,6,7-tetrabromo-1*H*-benzotriazole (**16**, TBBT) and 5,6-dichloro-1-(β-D-ribofuranosyl)-benzotriazole (**17**, DCBTR) were good inhibitors of the helicase activity with an IC_{50} = 20 and 1.5 μM, respectively (Borowski *et al.,* 2003), while the closely related 5,6-dichloro-1-(β-D-ribofuranosyl)-benzimidazole was practically inactive (IC_{50} 450 μM). The synthesis of new 1*H*-benzotriazole and 1*H*-benzimidazole derivatives was undertaken. N-alkyl and N1-ribofuranosyl derivatives of brominated analogues of 1*H*-benzotriazole and 1*H*-benzimidazole were synthesized and tested for their anti-helicase activity against the HCV enzyme (Bretner *et al.,* 2004b). 1- and

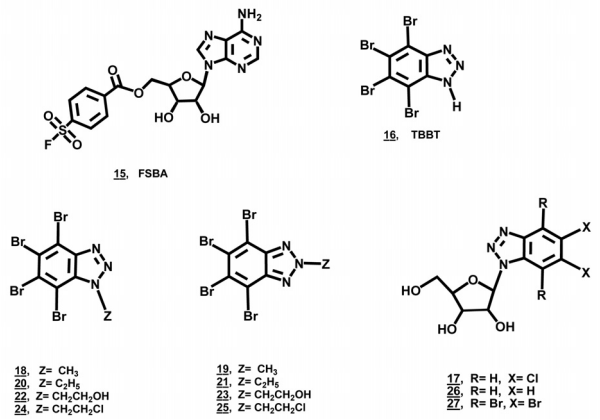


Figure 10. Chemical structures of HCV NS3 NTPase/helicase inhibitors.

2-alkyl derivatives of 4,5,6,7-tetrabromo-1*H*-benzotriazole (**18**–**21**) were obtained by alkylation of 4,5,6,7-tetrabromo-1*H*-benzotriazole (**16**) with the use of respective alkyl halides. 1- and 2-hydroxyethyl (**22**, **23**) and 1- and 2-chloroethyl (**24**, **25**) derivatives were synthesized with the use of 2-bromoethanol and 1-bromo-2-chloroethane as alkylating agents respectively. 1-β-D-Ribofuranosyl derivatives of benzotriazole (**26**, **27**) were synthesized by condensation using the method of Vorbrüggen. The halogenation of benzimi-

dazole or benzotriazole, when combined with N-alkylation induced the inhibitory activity and selectivity towards the helicase activity of HCV NTPase/helicase. The most active were the N2-methyl (**19**), and N2-ethyl (**21**) derivatives of 4,5,6,7-tetrabromo-1*H*-benzotriazole (**16**) (IC_{50} = 6.5 μM in the presence of DNA as a substrate). Derivatives of **16** in which hydroxyethyl or chloroethyl replaced the alkyl substituents lost their inhibitory activity. Among the ribofuranose derivatives of analogues of benzotriazole, the highest anti-helicase activity was shown by DCBTR previously reported by us (Borowski *et al.,* 2003). When DCBTR and TBBT were tested in the HCV subgenomic replicon system in Huh-5-2 cells at 11 μM, reduction of viral RNA to 65% was observed (Bretner & De Clercq, unpublished results). Tests in Vero and HeLa Tat cells showed a decrease of cytotoxicity of N-alkyl derivatives as compared to the parent compound – TBBT. A series of ring-expanded heterocycles, nucleoside and nucleotide analogues (RENs) containing the imidazo-[4,5-e][1,3]diazepine ring system have been synthesized and tested as potential inhibitors of HCV and some related Flaviviridae NTPase/helicases (Zhang *et al.,* 2003). Some of them inhibited the viral helicase activity with IC_{50} values in the micromolar range. The RENs-5′-triphosphates did not influence the unwinding reaction, but exerted an inhibitory effect on the ATPase activity of the enzymes (IC_{50} 0.55 μM). A large series of 2,3,5-trisubstituted-1,2,4-thiadiazol-2-ium salts were claimed to exhibit anti-NS3 helicase activity with low micromolar activity *in vitro* (Janetka *et al.,* 2000), and the group with the benzimidazole ring was patented (Diana & Bailey, 1997). *In vitro* analysis of these compounds in the subgenomic replicon system may yield important information as to their ultimate potential. Future studies of NS3 will probably focus on obtaining the co-crystal structure (enzyme-compound) to allow lead optimization and improved specificity.

CONCLUDING REMARKS

The current standard treatment for chronic HCV infection, interferon α and ribavirin, succeeds in only 50% of patients and can cause serious side effects. The development of new antiviral agents with minimum side-effects, designed to inhibit the activities of proteins essential to HCV proliferation, is thus crucial to curbing this viral pandemic. Inhibitors in Phase II clinical development include those that target the HCV NS3 protease, NS5B polymerase and IRES. Some companies are focusing their efforts on developing ribavirin analogues, whereas others try to develop an effective and safe prophylactic vaccine. Correlations between compound potency in replicon assays with enzymatic activity, and ultimately efficacy in man, will significantly accelerate the progression of drug discovery and development, leading to novel treatments for HCV infection.

I am grateful to Professor T. Kulikowski (Institute of Biochemistry and Biophysics, PAS, Warszawa, Poland) for many helpful discussions.

REFERENCES

Ago H, Adachi T, Yoshida A, Yamamoto M, Habuka N, Yatsunami K, Miyano M. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Struct Fold Des.*; **7**: 1417–26.

Altamura A, Tomei L, Koch U, Neuner PJS, Summa V. (2000) *Diketoacid-derivatives as Inhibitors of Polymerases.* WO 00/06529.

Benhamou Y, Hinrichsen H, Sentijens R, Reiser M, Manns MP, Forns X, Avendano C, Cronlein J, Nehmiz G, Steinmann G. (2002) Safety, tolerability and antiviral effect of BILN 2061, a novel HCV serine protease inhibitor, after oral treatment over 2 days in patients with chronic hepatitis C genotype 1, with advanced liver fibrosis. *Hepatology.*; **36**: 304A.

Borowski P, Deinert J, Schalinski S, Bretner M, Ginalski K, Kulikowski T, Shugar D. (2003) Halogenated benzimidazoles and benzotriazoles as inhibitors of the NTPase/helicase activities of hepatitis C and related viruses. *Eur J Biochem.*; **270**: 1645–53.

Bressanelli S, Tomei L, Rey FA, De Francesco R. (2002) Structural analysis of the hepatitits C virus RNA polymerase in complex with ribonucleotides. *J Virol.*; **76**: 3482–92.

Bretner M, Schalinski S, Haag A, Lang M, Schmitz H, Baier A, Behrens SE, Kulikowski T, Borowski P. (2004a) Synthesis and evaluation of ATP-binding site directed potential inhibitors of nucleoside triphosphatases/ helicases and polymerases of hepatitis C and other selected *Flaviviridae viruses*. *Antivir Chem Chemother.*; **15**: 35–42.

Bretner M, Najda A, Podwińska R, Baier A, Paruch K, Lipniacki A, Piasek A, Borowski P, Kulikowski T. (2004b) Inhibitors of the NTPase/helicase of hepatitis C and related flaviviridae viruses. *The fourth multidisciplinary conference on drug research*, Gdańsk-Sobieszewo, Poland, May 17–19, K-14.

Carroll SS, Tomassini JE, Bosserman M, Getty K, Stahlhut MW, Eldrup AB, Bhat B, Hall D, Simcoe AL, La Femina R, Rutkowski CA, Wolanski B, Yang Z, Migliaccio G, De Francesco R, Kuo LC, MacCoss M, Olsen DB. (2003) Inhibition of hepatitis C virus RNA replication by 2′-modified nucleoside analogs. *J Biol Chem.*; **278**: 11979–84.

Cho H-S, Ha N-C, Kang L-W, Chung KM, Back SH, Jang SK, Oh B-H. (1998) Crystal structure of RNA helicase from genotype 1b hepatitis C virus. *J Biol Chem.*; **273**: 15045–52.

Colman RF. (1983) Affinity labeling of purine nucleotide sites in proteins. *Annu Rev Biochem.*; **52**: 67–91.

De Francesco R. (1999) Molecular virology of the hepatitis C virus. *J Hepatol.*; **31** (Suppl 1): 47–53.

De Francesco R. (2000) Diketobutanoic acids as HCV polymerase inhibitors. *7th International Meeting on hepatitis and related viruses*, Gold Coast, Queensland, Australia, 3–7 December.

De la Cruz J, Kressler D, Linder P. (1999) Unwinding RNA in *Saccharomyces cerevisiae:* DEAD-box proteins and related families. *Trends Biochem Sci.*; **24**: 192–8.

Dhanak D, Duffy KJ, Johnston VK, Lin-Goerke J, Darcy M, Shaw AN, Gu B, Silverman C, Gates AT, Nonnemacher MR, Earnshaw DL, Casper DJ, Kaura A, Baker A, Greenwood C, Gutshall LL, Maley D, Del Vecchio A, Macarron R, Hofmann GA, Alnoah Z, Cheng H-Y, Chan G, Khandekar S, Keenan RM, Sarisky RT. (2002) Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem.*; **277**: 38322–7.

Diana GD, Bailey TR. (1997) Compounds, compositions and methods for treatment of hepatitis C. *Patent US5633388*.

Eldrup AB, Allerson CR, Bennett CF, Bera S, Bhat B, Bhat N, Bosserman MR, Brooks J, Burlein C, Carroll SS, Cook PD. (2004) Structure-activity relationship of purine ribonucleosides for inhibition of hepatitis C virus RNA-dependent RNA polymerase. *J Med Chem.*; **47**: 2283–95.

Failla C, Tomei L, De Francesco R. (1994) Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J Virol.*; **68**: 3753–60.

Gallinari P, Brennan D, Nardi C, Brunetti M, Tomei L, Steinkühler C, De Francesco R. (1998) Multiple enzymatic activities associated with recombinant NS3 of hepatitis C virus. *J Virol.*; **72**: 6758–69.

Glunz PW, Douty BD, Decicco CP. (2003) Design and synthesis of bicyclic pyrimidinone-based HCV NS3 protease inhibitors. *Bioorg Med Chem Lett.*; **13**: 785–8.

Griffin SDC, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, Harris MPG, Rowlands DJ. (2003) The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett.*; **535**: 34–38.

Gu B, Johnston VK, Gutshall LL, Nguyen TT, Gontarek RR, Darcy MG, Tedesco R, Dhanak D, Duffy KJ, Kao CC, Sarisky RT. (2003) Arresting initiation of hepatitis C virus RNA synthesis using heterocyclic derivatives. *J Biol Chem.*; **278**: 16602–7.

Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohno K. (1991) Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc Natl Acad Sci USA.*; **88**: 5547–51.

Huang M, Deshpande M. (2004) Hepatitis C drug discovery: *in vitro* and *in vivo* systems and drugs in the pipeline. *Expert Rev Anti-infect Ther.*; **2**: 375–88.

Janetka JW, Ledford BE, Mullican MD. (2000) *Pentacyclic Compounds Useful as Inhibitors of Hepatitis C Virus NS3 Helicase*. pp 1–60. Vertex Pharmaceuticals Inc.

Kaneko T, Tanji Y, Satoh S, Hijikata M, Asabe S, Kimura K, Shimotohno K. (1994) Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. *Biochem Biophys Res Commun.*; **205**: 320–6.

Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C, Caron PR. (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure.*; **6**: 89–100.

Lake-Bakaar G. (2003) Current and future therapy for chronic hepatitis C virus liver disease. *Curr Drug Targets Infect Disord.*; **3**: 247–53.

Lamarre D, Bailey M, Bolger G, Cameron DR, Cartier M, Faucher A-M, Goudreau N, Kukolj G, Lagace L, Pause A, Rancourt J, Thibeault D, Tsantrizos Y, Llinas-Brunet M. (2002) The discovery of BILN-2061 — an orally available small molecule inhibitor of the HCV serine protease and a promising antiviral for treatment of hepatitis C. *Hepatology.*; **36**: 464A.

Lanford RE, Bigger C. (2002) Advances in model systems for hepatitis C virus research. *Virology.*; **293**: 1–9.

Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, Boes M, Cameron DR, Cartier M, Cordingley MG, Faucher AM. (2003) An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature.*; **426**: 186–9.

Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol.*; **6**: 937–43.

Llinas-Brunet M, Bailey M, Fazal G, Ghiro E, Gorys V, Goulet S, Halmos T, Maurice R, Poirier M, Poupart MA, Rancourt J, Thibeault D, Wernic D, Lamarre D. (2000) Highly potent and selective peptide-based inhibitors of the hepatitis C virus serine protease: towards smaller inhibitors *Bioorg Med Chem Lett.*; **10**: 2267–70.

Lohmann V, Körner F, Herian U, Bartenschlager R. (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol.*; **71**: 8416–28.

Lohmann V, Körner F, Koch JO, Herian U, Theilmann L, Bartenschlager R. (1999) Replication of subgenomic hepatitis C virus RNAs in hepatoma cell line. *Science.*; **285**: 110–13.

Lohmann V, Körner F, Dobierzewska A, Bartenschlager R. (2001) Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol.*; **75**: 1437–49.

Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomaw EW, Adachi T, Hostomska Z. (1996) The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell.*; **87**: 331–42.

Lundin M, Monne M, Widell A, Von Heijne G, Persson MA. (2003) Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol.*; **77**: 5428–38.

Maag D, Castro C, Hong Z, Cameron CE. (2001) Hepatitis C virus RNA-dependent

RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. *J Biol Chem.*; **276**: 46094–8.

McCoy MA, Senior MM, Gesell JJ, Ramanathan L, Wyss DF. (2001) Solution structure and dynamics of the single-chain hepatitis C virus NS3 protease NS4A cofactor complex. *J Mol Biol.*; **305**: 1099–110.

Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP, Churchill TA, Lakey JR, Tyrrell DL, Kneteman NM. (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat Med.*; **7**: 927–33.

Murray EM, Grobler JA, Markel EJ, Pagnoni MF, Paonessa G, Simon AJ, Flores OA. (2003) Persistent replication of hepatitis C virus replicons expressing the beta-lactamase reporter in subpopulations of highly permissive Huh7 cells. *J Virol.*; **77**: 2928–35.

Narjes F, Brunetti M, Colarusso S, Gerlach B, Koch U, Biasiol G, Fattori D, De Francesco R, Matassa VG, Steinkuhler C. (2000) Alphaketoacids are potent slow binding inhibitors of the hepatitis C virus NS3 protease. *Biochemistry.*; **39**: 1849–61.

Narjes F, Koch U, Steinkuehler C. (2003) Recent developments in the discovery of hepatitis C virus serine protease inhibitors — towards a new class of antiviral agents? *Expert Opin Investig Drugs.*; **12**: 153–63.

Neddermann P, Quintavalle M, Di Pietro Ch, Clementi A, Cerretani M, Altamura S, Bartholomew L, De Francesco R. (2004) Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J Virol.*; **78**: 13306–14.

Ni Z-J, Wagman AA. (2004) Progress and development of small molecule HCV antivirals. *Curr Opin Drug Discovery Dev.*; **7**: 446–59.

Nishikawa F, Kakiuchi N, Funaji K, Fukuda K, Sekiya S, Nishikawa S. (2003) Inhibition of HCV NS3 protease by RNA aptamers in cells. *Nucleic Acids Res.*; **31**: 1935–43.

Oh J-W, Ito T, Lai MC. (1999) A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J Virol.*; **73**: 7694–702.

Serebrov V, Pyle AM. (2004) Periodic cycles of RNA unwinding and pausing by hepatitis C virus NS3 helicase. *Nature.*; **430**: 476–80.

Shepherd J, Brodin H, Cave C, Waugh N, Price A, Gabbay J. (2004) Pegylated interferon alpha-2a and -2b in combination with ribavirin in the treatment of chronic hepatitis C: a systematic review and economic evaluation. *Health Technol Assess.*; **8**: 1–140.

Standring D. (2003) NM283 has potent antiviral activity against chronic hepatitis C virus genotype-1 in the chimpanzee. *HEP DART 2003*; *Frontiers in Drug Development for Viral Hepatitis*, Kauai, Hawaii, HI USA, Abs. 28.

Steinkuhler C, Biasiol G, Brunetti M, Urbani A, Koch U, Cortese R, Pessi A, De Francesco R. (1998) Product inhibition of the hepatitis C virus NS3 protease. *Biochemistry.*; **37**: 8899–905.

Stuyver LJ, Whitaker T, McBrayer TR, Hernandez-Santiago BI, Lostia S, Tharnish PM, Ramesh M, Chu CK, Jordan R, Shi J, Rachakonda S, Watanabe KA, Otto MJ, Schinazi RF. (2003) Ribonucleoside analogue that blocks replication of bovine viral diarrhea and hepatitis C viruses in culture. *Antimicrob Agents Chemother.*; **47**: 244–54.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H. (1991) Structure and organisation of the hepatitis C virus genome isolated from human carriers. *J Virol.*; **65**: 1105–113.

Tan S-L, Katze MG. (2001) How hepatitis C virus counteracts the interferon response: The jury is still out on NS5A. *Virology.*; **284**: 1–12.

Tan S-L, Pause A, Shi Y, Sonenberg N. (2002) Hepatitis C therapeutics; current status and emerging strategies. *Nat Rev Drug Discov.;* **1**: 867–81.

Tanaka T, Kato N, Cho M-J, Shimotohno K. (1995) A novel sequence found at the 3′ter-

minus of hepatitis C virus genome. *Biochem Biophys Res Commun.*; **215**: 744–9.

Tomei L, Failla C, Santolini E, De Francesco R, La Monica N. (1993) NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol.*; **67**: 4017–26.

Tomei L, Altamura S, Bartholomew L, Bisbocci M, Bailey C, Bosserman M, Cellucci A, Forte E, Incitti I, Orsatti L, Koch U, De Francesco R, Olsen DB, Carroll SS, Migliaccio G. (2004) Characterization of the inhibition of hepatitis C virus RNA replication by nonnucleosides. *J Virol.*; **78**: 938–46.

Wang YS, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF. (2002) Structural and biological characterization of pegylated recombinant interferon α-2b and its therapeutic implications. *Adv Drug Deliv Rev.*; **54**: 547–70.

Wang M, Ng KK, Cherney MM, Chan L, Yannopoulos CG, Bedard J, Morin N, Nguyen-Ba N, Alaoui-Ismaili MH, Bethell RC, James MN. (2003) Non-nucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase. Crystal structures and mechanism of inhibition. *J Biol Chem.*; **278**: 9489–95.

Zhang X, Schmitt AC, Decicco CP. (2002) Design and synthesis of 6-amino-5-oxo-1,2,3,5-tetrahydro-3-indolizinecarboxylic acids as β-sheet peptidomimetics. *Tetrahedron Lett.*; **43**: 9663–6.

Zhang X, Schmitt AC, Jiang W, Wasserman Z, Decicco CP. (2003) Design and synthesis of potent, non-peptide inhibitors of HCV NS3 protease. *Bioorg Med Chem Lett.*; **13**: 1157–60.

Zhang N, Chen HM, Koch V, Schmitz H, Liao CL, Bretner M, Bhadti VS, Fattom AI, Naso RB, Hosmane RS, Borowski P. (2003) Ring-expanded ("Fat") nucleoside and nucleotide analogues exhibit potent *in vitro* activity against Flaviviridae NTPases/helicases, including those of the West Nile virus, hepatitis C virus, and Japanese encephalitis virus. *J Med Chem.*; **46**: 4149–64.

Zhu H, Zhao H, Collins CD, Eckenrode SE, Run Q, McIndoe RA, Crawford JM, Nelson DR, She JX, Liu C. (2003) Gene expression associated with interferon alfa antiviral activity in an HCV replicon cell line. *Hepatology.*; **37**: 1180–8.

Yao N, Hesson T, Cable M, Hong Z, Kwong AD, Le HV, Weber PC. (1997) Structure of the hepatitis C virus RNA helicase domain. *Nat Struct Biol.*; **4**: 463–7.