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Review

Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase

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Abstract

The treatment of chronic disease caused by the hepatitis C virus (HCV) is an unmet clinical need, since current therapy is only partially effective and limited by undesirable side effects. The viral serine protease and the RNA-dependent RNA polymerase are the best-studied targets for the development of novel therapeutic agents. These enzymes have been extensively characterized at the biochemical and structural level and thus used to set up screening assays for the identification of selective inhibitors. These efforts lead to the discovery of several classes of compounds with potential antiviral activity. The hepatitis C virus does not replicate in the laboratory. The formidable challenge posed by the difficulty of developing cell-based assays and preclinical animal systems has been partially overcome with several alternative approaches. The development of new assays permitted the optimization of enzyme inhibitors leading eventually to molecules with the desired drug-like properties, the most advanced of which are being considered for clinical trials.

Keywords: Hepatitis C; HCV; Protease inhibitor; RNA-dependent RNA polymerase; RdRp; NNI; Nucleoside analogue; Pyrophosphate mimic

1. Introduction

Hepatitis C virus (HCV), the major etiological agent of the non-A non-B hepatitis, was identified at the molecular level at the end of the 1980s (Choo et al., 1989; Houghton, 1996). Presently, it is estimated that HCV infects more than 170 million persons worldwide and thus represents a viral pandemic that is about five times more widespread than infection by the human immunodeficiency virus (HIV) (Wasley and Alter, 2000). The number of new infections has been significantly reduced by the introduction of tests to identify contaminated blood in the early 1990s, and currently new cases are acquired mostly as the result of injection-drug use and percutaneous or mucous-membrane transmission. Acute infection by HCV is only seldom diagnosed because of the vague clinical manifestations. However, more than 50% of infected individuals develop a slowly progressive chronic disease characterized by liver fibrosis and relatively aspecific symptoms that, although often not life-threatening, have adverse effects on the quality of life (Kenny-Walsh, 2001). Spontaneous healing is rare once chronic infection has been established. Cirrhosis develops in 15–20% of the infected individuals and is accompanied by severe complications, leading eventually to liver failure and occasionally hepatocellular carcinoma. Extrahepatic manifestations of HCV infections, such as cryoglobulinemia and non-Hodgkin's lymphomas, are mostly associated with autoimmune or lymphoproliferative states and are possibly related to the ability of HCV to replicate also in lymphoid cells.

Current HCV therapies are based on interferon alpha (IFN-α) and have undergone impressive advancement in the last decade (Di Bisceglie and Hoofnagle, 2002). The combination of pegylated IFN-α and ribavirin is now the standard of care and yields a sustained virologic response in more than 40% of patients infected by genotype 1 viruses and about 80% of those infected by genotypes 2 and 3 (Chander et al., 2002). Despite this remarkable progress and the forthcoming use of improved forms of ribavirin (Watson, 2002), it is unlikely that current treatment will succeed in curing all patients. In fact, besides the incomplete efficacy on genotypes 1, combination therapy has significant side effects and is poorly tolerated in particular by individuals

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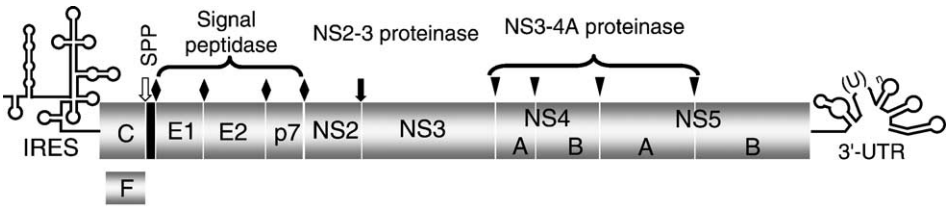
affected by other diseases (Fried, 2002). Consequently, many patients cannot be treated with current combination therapy and overall chances of cure for patients are probably below 50%. Given the high prevalence of the disease, the development of more effective, convenient and tolerated treatments is a major public health objective. Similar to the HIV antiviral research, efforts to develop antiviral agents for HCV have mostly focused on inhibition of key viral enzymes. In this review, we summarize the progress in the identification of inhibitors of the NS3-4A serine protease and NS5B polymerase, the most advanced of which are being evaluated in early human clinical trials.

2. Virus genome structure and replication

The molecular characterization of HCV became possible with the cloning of the viral genome, 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). This seminal finding spurred an outburst of molecular investigations that have rapidly advanced our knowledge of HCV (Rosenberg, 2001). Nonetheless, many fundamental aspects of the virus structure, replication, and pathogenesis are still largely hypothetical and based on analogies with other viruses of the same or related family. The low abundance of HCV in biological samples, 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. As described below, the identification of molecular clones infectious in the chimpanzee, the development of a robust cell culture replication system, along with the description of mouse models for HCV infection have changed the scenario of HCV research and accelerated drug discovery efforts.

The viral genome is a 9.6-kb long positive-polarity RNA molecule that comprises a single open reading frame flanked by two untranslated regions (UTR) (Choo et al., 1989; Kato et al., 1990; Takamizawa et al., 1991). The 5′-UTR is 341-b long and is highly conserved between different virus genotypes. This region contains an internal ribosome entry site (IRES) responsible for cap-independent translation, as well as indispensable *cis*-acting replication elements (Friebe et al., 2001; Rijnbrand and Lemon, 2000; Tsukiyama-Kohara et al., 1992). The 3′-UTR is more variable in length and encompasses an approximately 50 nucleotides variable domain, a poly-U stretch of variable length and a highly conserved 98 bases element essential for replication (Friebe and Bartenschlager, 2002; Kolykhalov et al., 1997; Kolykhalov et al., 1996; Tanaka et al., 1995). The open reading frame encodes a >3000 amino acid polyprotein. Individual viral proteins are released from the precursor polyprotein by an orchestrated series of co- and posttranslational endo-proteolytic cleavages (Fig. 1). Host proteases associated with the endoplasmic reticulum (ER) membrane cause the release of the structural proteins: the core protein C, the two glycosylated envelope proteins E1 and E2, and a small membrane protein of unknown function, p7 (Hijikata et al., 1991; Lin et al., 1994; Santolini et al., 1994). Another protein of unknown function, termed alternative reading frame protein (ARFP) or frameshift protein (F), is encoded by an alternative ORF that overlaps with the core-protein gene (Walewski et al., 2001; Xu et al., 2001). The remaining part of the precursor contains the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The NS proteins are believed to function as enzymes or accessory factors that catalyze and regulate the replication



–C	Nucleocapsid protein
–E1	Envelope glycoprotein 1
–E2	Envelope glycoprotein 2
–p7	Unknown function
–NS2	Component of Zn^{++}-activated NS2/3 auto-proteinase
–NS3	Serine proteinase (aa 1-180) and RNA helicase (aa 181-631)
–NS4A	Cofactor of the NS3 serine proteinase
–NS4B	Integral membrane protein of unknown function
–NS5A	Phosphoprotein, putataive component of replication complex
–NS5B	RNA-dependent RNA polymerase

Fig. 1. Scheme of the genetic organization and processing of HCV polyprotein; F, frameshift protein; C, core protein; E, envelope; NS, nonstructural. Symbols indicate cleavage by host and HCV encoded proteases: empty arrow, signal peptide peptidase (SPP); diamonds, signal peptidase; solid arrow, NS2-3 protease; inverted triangles, NS3-4A seine protease. The black bar indicates portion of the E1 signal sequence removed from C by SPP cleavage.

of the HCV genome and are released from the nascent precursor by the concerted action of two viral proteases. The NS2-3 protease is a zinc-dependent auto-protease associated with the C-terminal half of NS2 and the N-terminus of NS3. It is responsible for a single cut between NS2 and NS3 (Grakoui et al., 1993b; Hijikata et al., 1993). The NS3-4A complex encompasses a distinct serine protease, comprising the N-terminal domain of NS3 and the central domain of NS4A, and cleaves the polyprotein at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (Bartenschlager et al., 1993; Grakoui et al., 1993a; Tomei et al., 1993). The C-terminal domain of NS3 contains a NTPase/helicase necessary for translation and replication of the HCV genome (Kim et al., 1995). NS4B is an integral membrane protein for which no function has yet been identified (Huge et al., 2001). NS5A is a highly phosphorylated protein, possibly involved in the resistance to the antiviral effect of interferon (Tan and Katze, 2001). NS5B is the RNA-dependent RNA polymerase (RdRp) and is the catalytic core of the viral replicase (Behrens et al., 1996; De Francesco et al., 1996a).

HCV follows a replication strategy similar to that of other positive strand RNA viruses. Viral entry is possibly mediated by one or more surface cellular receptors. Candidate receptors for HCV binding to host cells include the E2-binding tetraspannin CD81, the low-density lipoprotein receptor (LDLR) and the B-I scavenger receptor (Flint et al., 2001; Pileri et al., 1998; Scarselli et al., 2002). Following entry and uncoating of the viral genome, the IRES promotes translation of the polyprotein that is coupled to processing and release of mature viral proteins. The structural proteins become associated (C) or integrated (E1, E2 and p7) in the ER membrane and form functional oligomers that will eventually promote virus budding. The NS proteins are also associated with the cytoplasmic side of the ER membrane where they interact with each other and with host proteins to form the viral replication machinery. This machinery uses the genome itself as template for the transcription of a complementary minus stranded RNA molecule. The minus strand and/or the double stranded replicative intermediate serves, in turn, as a template for the synthesis of new genomic positive stranded RNA molecules, that can be used for translation, or replication, or can be packaged into progeny virions. These probably bud in intracellular membranes and reach the extracellular milieu through the cell secretory apparatus.

3. Approaches for identifying and evaluating inhibitors of the NS3-4A serine protease and NS5B RNA dependent RNA polymerase

While in theory all steps of the virus replication cycle could be considered for therapeutic intervention, the absence of suitable assays has delayed progress in many areas. Thus far, the most widely established strategy for the development of novel anti-HCV therapeutics hinges on the identification of low molecular weight inhibitors of essential viral enzymes. Conventionally, enzyme inhibitors are identified by screening of chemical compound libraries using biochemical assays based on recombinant proteins. For this reason, efforts to develop new anti-HCV agents initially focussed on viral enzymes, namely the NS3-4A serine protease and the NS5B RdRp. Both enzymes were later shown by genetic means to be essential for viral replication, thus validating their choice as targets for therapeutic intervention (Kolykhalov et al., 2000; Lohmann et al., 1999). Undoubtedly, the success of protease and polymerase inhibitors in controlling HIV infection reinforced the conviction that the corresponding HCV enzymes were the choice drug targets.

Several laboratories expressed recombinant forms of the HCV serine protease and RdRp in heterologous systems and used purified enzymes to establish biochemical assays in order to identify selective inhibitors. These efforts were accompanied by in-depth investigations of the enzymology of these proteins and by the determination of their three-dimensional structures that provided the necessary background for deciphering the mechanism of action of the inhibitors and for optimization of their activity.

As described in the next sections, several inhibitors of both enzymes have been reported in the scientific and in the patent literature. However, the absence of an efficient cell culture system for HCV delayed the assessment of the effect of these inhibitors on viral replication. Infection of primary cell cultures and certain human cell lines had been reported by several laboratories, but the levels of HCV replication were too low to permit the use of these system for testing antiviral agents (reviewed in Bartenschlager and Lohmann, 2001). A revolutionary leap forward came in 1999 when Lohman et al. described the first robust cell culture HCV replication system (Lohmann et al., 1999). This system is based on engineered subgenomic HCV RNAs, or “replicons,” that are capable of limited replication in a human hepatoma Huh-7 cell line. The inclusion in the replicons of the selectable *neomycin* resistance marker allowed the selection of adaptive variants that replicated to higher levels in these cells (Blight et al., 2000; Krieger et al., 2001), as well as the selection of cell clones that are highly permissive for subgenomic and genomic HCV RNA replication (Blight et al., 2002). Despite being restricted to this cell line and genotype 1 isolates, this system provides a cell-based model for evaluating inhibitory compounds identified in biochemical screens and facilitates the development of high throughput assays for the identification of novel antiviral compounds (Bartenschlager, 2002).

The development of animal models for HCV has been equally slow and difficult. Besides humans, chimpanzees represent the only host for HCV and for obvious ethical and economic reasons cannot be considered for evaluating antiviral drugs. Only recently, two interesting murine models for HCV infection and antiviral studies have been described. One system relies on the use of immunodeficient transgenic mice, whose liver can be repopulated with human hepatocytes (Mercer et al., 2001). After transplantation,

human hepatocytes engraft into the mouse liver and form foci of regenerating cells that sustain HCV infection. The second system is based on lethally irradiated, immunocompromised mice that support the ectopic engraftment of ex vivo infected fragments of human liver (Ilan et al., 2002b). The usefulness of the latter model for evaluation of anti-HCV agents was demonstrated by the ability of a small molecule (an HCV internal ribosomal entry site inhibitor) and an anti-HCV human monoclonal antibody to reduce virus loads in a dose-dependent manner (Ilan et al., 2002a). While these systems are very laborious, require special expertise and are of limited throughput, they are certainly very promising for testing the effects of antiviral agents.

4. The NS3-4A serine protease

The HCV NS3-4A serine protease is a heterodimeric enzyme comprising the N-terminal domain of the NS3 protein as well as the NS4A protein (for review see De Francesco and Steinkuhler, 2000). This enzyme belongs structurally to the trypsin superfamily, but is unique in requiring a noncatalytic, structural zinc atom and a second viral protein as a cofactor (Fig. 2). The protease cofactor, NS4A, is a relatively small protein, consisting of only 54 residues. The first ~20 residues of NS4A are hydrophobic and predicted to form a transmembrane α-helix involved in membrane anchoring of the NS3-4A protease/helicase complex. The function of the hydrophilic 20 C-terminal residues of the cofactor is currently unknown, while the central domain of NS4A, amino acids 21–34, are directly implicated in the interaction with NS3 and absolutely required for the enhancement of its serine proteinase activity (Lin et al., 1995). The role of NS4A as a cofactor became clearer when the three-dimensional structure of the NS3-4A protease was solved by X-ray crystallography (Kim et al., 1996; Love et al., 1996; Yan et al., 1998) and NMR spectroscopy (Barbato et al., 1999; McCoy et al., 2001). Similar to other trypsin-like serine

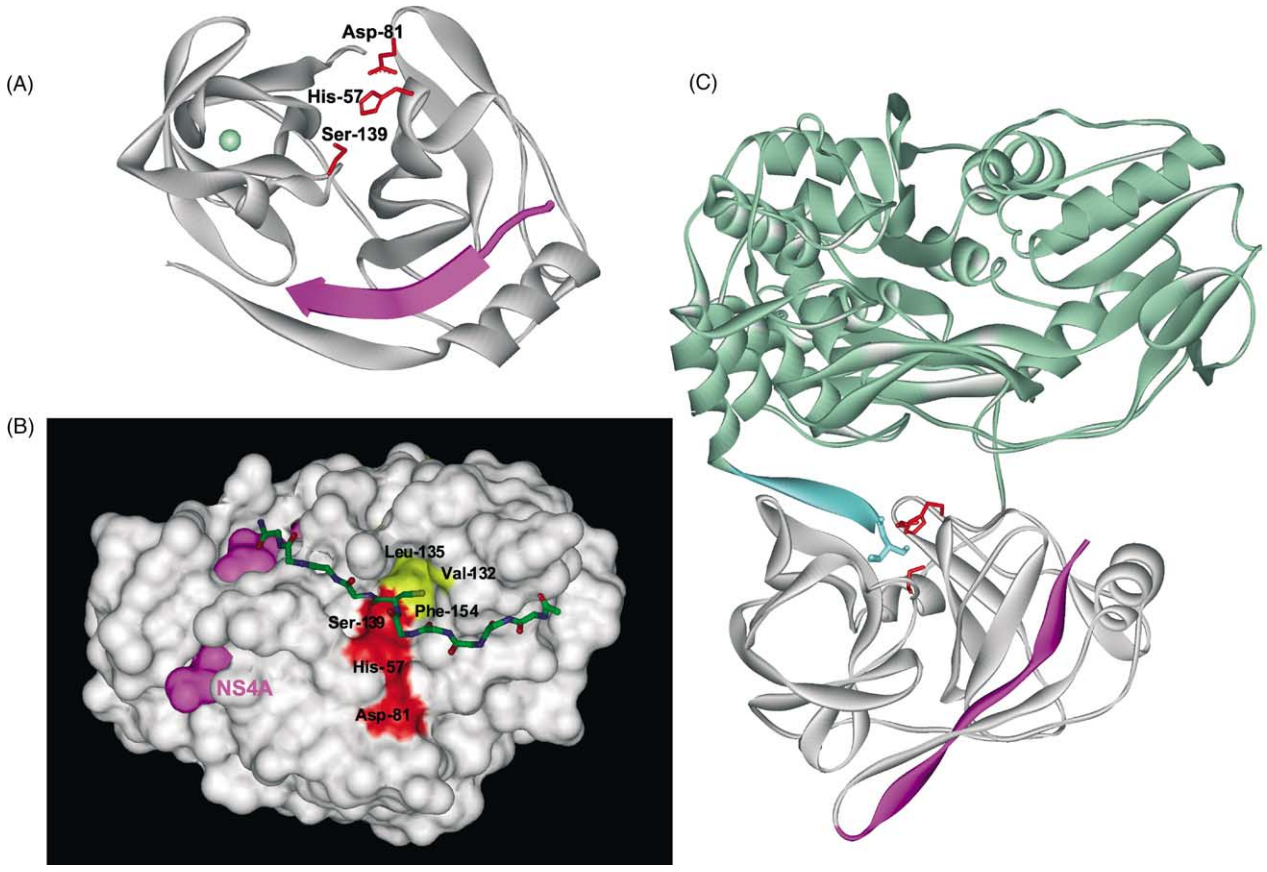


Fig. 2. X-ray crystallographic structures of the HCV NS3-4A protease. (A) NS3-4A protease domain. The side chains of the residues forming the catalytic triad (His-57, Asp-81, and Ser-139) are shown in red. The NS4A cofactor is shown in purple, the bound zinc ion is modeled in light green. (B) A decapeptide substrate (backbone only) is modeled in the NS3-4A protease active site. The NS4A cofactor is shown in purple. The side chains of the residues forming the catalytic triad are shown in red. The side chains of the residues forming the S1 specificity pocket of the enzyme (Val-132, Leu-135, and Phe-154) are shown in yellow. (C) Product inhibition in the NS3-4A complex. The NS4A peptide (red) has been artificially linked to the N-terminus of NS3 protease domain (gray) in the single-chain construct used for crystallization. The side chains of the residues forming the protease catalytic triad are shown in red. The helicase domain (top portion of the molecule) is depicted in light green. The C-terminus of NS3 generated as product of the NS3/NS4A cleavage reaction, forms a β-strand that occupies the proteinase active site (shown in light blue).

proteases, NS3-4A is made of two domains both composed of a β-barrel and two short α-helices, with the catalytic triad located between the two domains. The central region of NS4A is an integral part of the amino-terminal domain and forms one strand of an eight-stranded β-barrel (Fig. 2A). Comparison of the NS3 protease structures, with or without cofactor, suggests that NS4A increases the enzymatic activity by stabilizing the N-terminal domain of the protease, by optimizing the orientation of the residues of the catalytic triad (Fig. 2A), and by contributing to the formation of the substrate binding site on the protein surface (Fig. 2B).

A peculiar feature of the HCV NS3-4A serine protease is the requirement of zinc ion for activity (De Francesco et al., 1998). Zn^{2+} was initially proposed to have an essential structural role, as its removal caused unfolding and precipitation of the protein (De Francesco et al., 1996b). X-ray crystallography showed that the metal was located opposite to the active site and coordinated tetrahedrally by three cysteines and an histidine (Fig. 2A) (Kim et al., 1996; Love et al., 1996; Yan et al., 1998). The metal ligating residues are located in a long loop connecting the two β-barrels and a short loop in the C-terminal domain and therefore metal binding may affect the relative position of the two β-barrels. Since the catalytic triad residues are also distributed between the two domains, the relative position of the two β-barrels is expected to affect the orientation of the catalytic residues and consequently enzyme activity (Urbani et al., 1998).

The NS3-4A serine protease cleaves the viral polyprotein at four junctions: NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. Cleavage at the NS3/NS4A site is an intramolecular event (*cis*-cleavage), while the processing at the other three junctions occurs at the intermolecular level (*trans*). The junctions have the consensus sequence Asp/Glu-$(Xaa)_4$-Cys/Thr ↓ Ser/Ala-$(Xaa)_2$-Leu/Trp/Tyr (we follow the nomenclature of Schechter & Berger (Schechter and Berger, 1967) in designating the cleavage sites as P_6-P_5-P_4-P_3-P_2-P_1...P'_1-P'_2-P'_3-P'_4, with the scissile bond between P_1 and P'_1 and the C-terminus of the substrate on the prime side; the relative binding pockets on the enzyme are termed S_6-S_5-S_4-S_3-S_2-S_1...S'_1-S'_2-S'_3-S'_4), with cleavage occurring after cysteine or threonine (Grakoui et al., 1993a; Pizzi et al., 1994). The NS3-4A protease specificity has been further characterized by mutational analysis (Bartenschlager et al., 1995; Kolykhalov et al., 1994) as well as by studies with synthetic peptides (Urbani et al., 1997; Zhang et al., 1997) and selection of optimized cleavage sites using peptide libraries (Kim et al., 2000; Pacini et al., 2000). A cysteine residue is invariably found in the P_1 position at all the junctions that are cleaved in *trans*, while the *cis*-cleaved NS3/NS4A junction of HCV is the only natural cleavage site with threonine in the P_1 position. Although the P_1 position determines the primary specificity of the NS3-4A protease, additional residues within the substrate consensus sequence are crucial for efficient processing. An acidic residue is always present at the P_6 position of all cleavage sites and its substitution or ablation is detrimental for cleavage. P_3, P'_1 and P'_4 residues contribute to efficient substrate recognition, although in a less dramatic way, the preferred residues being those most frequently found in the natural cleavage sites. Analysis of the cleavage kinetics of different peptide substrates has shown that the minimum length required for a synthetic substrate is a decamer incorporating all of these conserved features and spanning P_6 to P'_4. The rather unusual requirement for large peptide substrates can be explained by the characteristics of the substrate binding site revealed by the analysis of the enzyme three-dimensional structure (Fig. 2B). The NS3-4A protease lacks several surface loops that form the substrate binding cleft in other serine proteases. As consequence of this, the substrate binding cleft is solvent-exposed and relatively featureless (De Francesco and Steinkuhler, 2000). Thus, selective recognition of the substrate is derived from a series of weak interactions that are distributed along an extended contact surface and that involve all of the evolutionarily conserved features of the cleavage site sequences.

5. Inhibitors of the HCV NS3-4A serine protease

Based on the enzyme features described above, three alternative strategies for developing inhibitors of the HCV serine protease were initially envisioned: tampering with the NS3/NS4A interaction, interfering with zinc binding and preventing substrate binding in the active site. However, the first two strategies are currently viewed as extremely difficult (De Francesco et al., 1999) and the development of active site inhibitors of the NS3-4A enzyme is considered the most promising approach.

The development of potent competitive inhibitors of the HCV serine protease has initially been hampered by the structure of its active site: the absence of a well defined substrate binding site made the design of low molecular weight competitive inhibitors a daunting job. Nevertheless, a number of active site inhibitors have thus far been described for the NS3-4A protease and at least one of those is currently being investigated in early clinical trial as an antiviral agent.

5.1. Covalent NS3-4A protease inhibitors

Covalent serine proteinase inhibitors are typically derived from known substrates by replacing the scissile amide bond with an electrophilic moiety that is able to form a covalent adduct with the catalytic serine residues (Edwards and Bernstein, 1994). Compounds of this mechanistic class are referred to as “transition-state analogues” or “serine-trap inhibitors.” Several biopharmaceutical groups have reported a series of electrophile-based peptidic or peptidomimetic inhibitors, with electrophilic groups that included aldehydes (Ede et al., 2000), boronic acids (Priestley et al., 2002; Priestley and Decicco, 2000), lactams (Slater et al., 2002), azapeptides (Zhang et al., 2002), alpha-keto amides (Bennett

et al., 2001; Han et al., 2000), and alpha-keto acids (Colarusso et al., 2002a; Narjes et al., 2000; Nizi et al., 2002). This approach, reviewed in Steinkuhler et al. (2001), and Fischmann and Weber (2002), has led to potent and selective inhibitors. Although compounds of this class have significantly contributed to our understanding of the requirements for efficient inhibition of the NS3-4A protease, electrophile-based covalent inhibitors may ultimately be of limited use in a clinical setting because of their intrinsic chemical reactivity (Sanderson, 1999).

In the present review, we focus our discussion to noncovalent inhibitors of the NS3-4A serine protease.

5.2. Noncovalent NS3-4A protease inhibitors

5.2.1. Product analogues

An important class of peptidomimetic inhibitors derives from the finding that the NS3-4A protease is susceptible to feedback inhibition by the N-terminal products released from the polyprotein substrate after enzymatic cleavage (Llinas-Brunet et al., 1998b; Steinkuhler et al., 1998). The distinctive feature of these product-based inhibitors is the presence of a free carboxylic acid on the C-terminal P_1 residue. This carboxylic group, which is liberated by the cleavage of the substrate peptide bond, is believed to establish crucial and unique interactions with the enzyme active site and has been recognized as an essential feature imparting selectivity with respect to other serine proteases (Llinas-Brunet et al., 1998a). The relevance of product inhibition for the NS3-4A protease is underlined by the three-dimensional structure of the full-length NS3/NS4A protein complex (Yao et al., 1999). In this complex, the C-terminal threonine of the NS3 helicase domain, which represents the P-side product of the cleavage between NS3 and N4A, occupies the active site of the protease domain (Fig. 2C). Capitalizing on this seminal observation, two groups have systematically modified the natural amino acids of the hexapeptide N-terminal products derived from the NS4A/NS4B and the NS5A/NS5B junction, respectively (Fig. 3). This effort resulted in the synthesis of very potent hexapeptide inhibitors of the NS3 proteinase (Ingallinella et al., 1998; Llinas-Brunet et al., 2000a) (Fig. 3, compounds 1 and 2). Optimal active site binding by these peptides requires a dual anchor: a "P_1 anchor" and an "acid anchor" at the N-terminal end of the molecule. It is well known for peptide-based inhibitors of serine proteases that the P_1 residue contributes considerably to the potency and specificity of the ligand (Lu et al., 1997). In the case of product inhibition, the P1 residue contributes to the binding energy through both its side chain and its terminal carboxylic acid (Steinkuhler et al., 1998). The preference displayed by the NS3 serine proteinase for a cysteine residue in the P_1 position can be rationalized on the basis of the structure of the enzyme S_1 specificity pocket (De Francesco and Steinkuhler, 2000). The S_1 specificity pocket of the NS3 proteinase is small and lipophilic, lined by the hydrophobic side chains of valine 132, leucine 135, and phenylalanine 154 (Fig. 2B). The shape of the relatively small and lipophilic cysteine side chain is complementary to this pocket. In addition, the sulfhydryl group can interact in a rather specific way with the aromatic ring of phenylalanine 154. The second anchor of the product inhibitors resides in the P_5-P_6 acidic pair. These residues are believed to establish productive electrostatic interaction with a region of the protein characterized by a cluster of basic amino acids, most prominently lysine 165, arginine 161 and arginine 123 (Koch et al., 2001; Steinkuhler et al., 2001).

The presence of a thiol-containing side chain on the preferred P_1-residue constituted a major obstacle for the medicinal chemists and thus a major effort has been devoted to designing suitable and chemically stable replacements for the P_1 sulfhydryl group. Amino acids with small hydrophobic side chains, such as alanine or α-amino butyric acid, are tolerated but lead to a loss of potency due to the reduction of the lipophilic contact surface area. Amino acid with larger side chains, also lead to significant loss of potency possibly due to steric incompatibility (Llinas-Brunet et al., 1998b). Narjes et al. (2002a) used the following rationale for the design of a surrogate P_1 residue. The cysteine sulfhydryl is lipophilic, but possesses lone-pair electrons as well as a polarized S–H bond, which confer the potential for electrostatic interaction in the enzyme active site. An analysis of the steric and electrostatic properties of the thiol group suggested a difluoro methyl group as a mimetic of the canonical thiol. Thus, incorporation of (S)-4,4-difluoro-2-aminobutyric acid as a cysteine replacement (Fig. 3, hexapeptide 3) produced an inhibitor equipotent to the initial hexapeptide 1.

An alternative approach to thiol replacement within NS3-4A protease inhibitors was taken by the group at Boehringer-Ingelheim (Llinas-Brunet et al., 2000a). Interestingly, when the natural P_1 residue was replaced with 1-aminocyclopropylcarboxylic acid in a hexapeptide series, an inhibitor nearly equipotent to the parent compound was obtained (Fig. 3, hexapeptide 4) (Beaulieu and Llinas-Brunet, 2002). Subsequently, stepwise optimization of the product inhibitors has lead to a new generation of potent, selective carboxylic acids inhibitors of smaller size (Llinas-Brunet et al., 2000b). Alterations of the P_1 through P_4 positions were explored in order to compensate for the subsequent truncation of the N-terminal P_5 and P_6 acidic residues. In particular, addition of large lipophilic aromatic systems to the P_2 proline ring significantly enhance inhibitor potency (reviewed in Fischmann and Weber, 2002). Subsequent removal of the P_5-P_6 fragment led to tetrapeptides with IC_{50} values in the low or submicromolar range (Fig. 3, compound 5) (Beaulieu and Llinas-Brunet, 2002). The limitations of peptides as drug candidates are well documented (Lipinski et al., 2001). Therefore, much effort has been devoted to reducing the peptidic nature of the peptidyl carboxylic acid inhibitors just described. Very potent macrocyclic peptidyl carboxylic acids were thus obtained by designing a macrocyclic ring in order to connect the side chain of the P_1

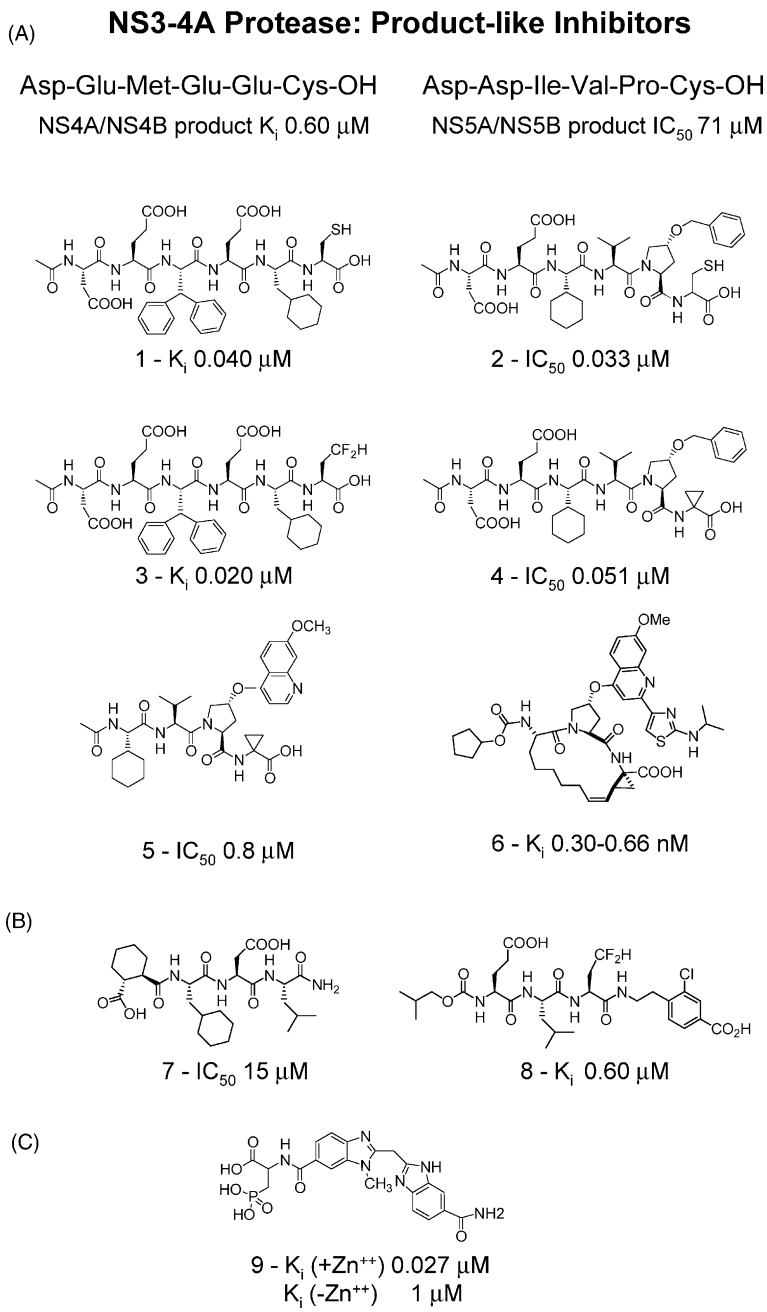


Fig. 3. Chemical structures of selected inhibitors of the NS3-4A proteinase activity. (A) Product-like peptide and peptidomimetic inhibitor. (B) Inhibitors that exploit the prime region of the substrate binding cleft. (C) A benzimidizole-based Zn^{2+}-dependent nonpeptidic active site inhibitor.

and the P_3 residues (Lamarre et al., 2002). Very recently, researchers from Boehringer-Ingelheim reported the discovery, preclinical and clinical profile of a compound of these series, BILN-2061 (Fig. 3, compound 6). BILN-2061 is a very potent competitive inhibitor of the NS3-4A protease that inhibits HCV replication in human cells and is orally bioavailable in experimental animals. A phase I clinical trial with BILN-2061 showed favorable pharmacokinetics in healthy volunteers (Narjes et al., 2002b). No influence on liver enzymes and no serious adverse events were observed, only minor gastrointestinal disturbances being observed at the highest dose (2400 mg). Based on this study, an oral single dose of 2000 mg was considered as the maximally tolerated dose in healthy volunteers. Two proof-of-concept clinical studies addressed the effect of 2-days oral therapy with BILN-2061 (Benhamou et al., 2002; Hinrichsen et al., 2002). In an open trial, 31 patients with HCV genotype 1 and minimal liver fibrosis were treated with BILN-2061 (25, 200 or 500 mg, twice daily for 2 days), administered orally as solution with PEG400. All patients were followed up for 10–14 days. Viral load decreased by at least 10-fold in seven out of nine, eight out of eight, and eight out of

eight patients treated with 25, 200 and 500 mg, respectively, without difference between naïve and pretreated patients. No response was seen in the placebo group. After the end of the treatment, viral load returned to baseline levels within 1–7 days. Liver enzymes did not increase during treatment and the drug was well tolerated (Hinrichsen et al., 2002). In a double-blind, placebo-controlled, randomized trial in 10 patients with HCV genotype 1 and significant liver fibrosis, placebo or BILN-2061, 200 mg, was administered orally, twice daily, for two days. All patients treated with BILN-2061 had a decrease in serum HCV RNA levels of at least two orders of magnitude and two patients showed a decrease of greater than three orders of magnitude. Viral levels returned to baseline after stopping therapy. No safety issues were identified in the latter trial as well (Benhamou et al., 2002). These early studies of BILN-2061 show that the drug may be safe and effective in the treatment of hepatitis C. Larger trials of prolonged BILN-2061 treatment are required in order to confirm efficacy and safety.

5.2.2. Noncleavable substrate mimetics and P′-inhibitors

In contrast to the P region, the P′ region of the substrate has received comparably less attention. However, pockets that contribute to substrate recognition do exist in the prime region of the substrate binding site (Fig. 2B). These pockets could also be explored for the design of competitive inhibitors of the enzyme.

Landro et al. (1997) first exploited the S′ region of the substrate binding site in order to design potent and specific inhibitors of the NS34-4A protease. These authors observed that P'_1 substitutions with the amino acids proline, tetrahydroisoquinoline-3-carboxylic acid (Tic) or pipecolinic acid (Pip), respectively, resulted in noncleavable substrate analogues that retained high binding affinity for the enzyme active site. Thus, the decapeptide Glu-Asp-Val-Val-Leu-Cys-Tic-Nle-Ser-Tyr was reported to be a potent, competitive inhibitor of the NS3-4A proteinase. The S′-site binding portion of noncleavable decapeptides, spanning P_6-P'_4, was independently optimized by Ingallinella et al. (2000). Their effort led to a substrate-derived peptide inhibitor of the NS3-4A proteinase with the amino acid sequence Asp-(D)Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu. The affinity displayed by this peptide inhibitor for the protease active site is more than three orders of magnitude greater than that of the starting substrate peptide. Although a great deal of selectivity and potency can thus be obtained through the design of substrate analogues, their relatively large molecular weight limits cell membrane permeability and bioavailability, preventing further development as clinical candidates. Bearing this in mind, Ingallinella and colleagues developed a new class of NS3-4A inhibitors that bind to the prime site in the absence of any contacts with the nonprime region of the enzyme (Ingallinella et al., 2002). The strategy combined key binding elements of the two previously described classes, namely the optimized prime-site binding sequence (Cha-Asp-Leu-NH2), and an N-terminal carboxylic acid, suitably positioned in the active site to engage in interactions similar to those previously established for the C-terminal carboxylate of peptide product inhibitors. The success of this design required selecting the proper linkage between these two elements from a small combinatorial library. This effort has resulted in capped tri-peptides that are low micromolar competitive inhibitors of the NS3-4A protease. The most potent compound obtained thus far in this series is shown in Fig. 3 (compound 7). More recently, a novel series of reversible, competitive NS3-4A peptide-based inhibitors has been reported that bind to the enzyme substrate cleft across the active site (Colarusso et al., 2002b). These inhibitors are characterized by the presence of a C-terminal phenetyl amide group, which extends to make contacts into the S′ side of the enzyme, possibly with the side chain of lysine 136. It was possible to evolve tripeptide phenetyl amide inhibitors that inhibit the purified NS3/NS4A protease with potencies in the submicromolar concentration range (Fig. 3, compound 8). This finding suggests that the phenetyl amide moiety might constitute a novel active site anchor and a potentially very valid alternative to the C-terminal free carboxylic group present in the product analogue series.

5.3. Nonpeptidic NS3-4A protease inhibitors

Significant efforts of several biopharmaceutical groups have been devoted to the identification of nonpeptidic small molecule inhibitors of NS3-4A enzyme (reviewed in Steinkuhler et al., 2000, and Beaulieu and Llinas-Brunet, 2002). Nonpeptidic molecules have emerged mainly through random screening and have displayed a noncompetitive mechanism of action. More recently, benzimidazole-based compounds have been reported that inhibit the enzyme via the formation of a ternary complex between the serine protease catalytic residues, the heterocycle and a Zn^{2+} ion (Sperandio et al., 2002; Yeung et al., 2001). Bisbenzimidazole derivatives belonging to this mechanistic class (Fig. 3, compound 9) are potent Zn^{2+}-dependent inhibitors of the enzyme. The potential of this class of compounds to inhibit HCV replication in cell culture, that is, under physiological concentrations of free Zn^{2+}, needs to be established before these compounds could be considered as candidates for further development.

6. NS5B RdRp

NS5B is the viral RdRp, an enzyme that is pivotal in viral genome replication. This enzyme is required for the synthesis of both the negative stranded RNA intermediate, complementary to the viral genome, and of the positive stranded RNA genomes complementary to the negative stranded intermediate. Uninfected cells normally do not express an RNA-dependent RNA. It is therefore possible that specific inhibitors of this enzyme could be found

that block HCV replication with negligible associated toxicity.

The RdRP activity of the NS5B gene product was initially predicted from the presence of hallmark sequence Gly-Asp-Asp motif (GDD) common to the reverse transcriptases and other viral polymerases (Choo et al., 1989) and subsequently demonstrated in vitro (Behrens et al., 1996). The full-length enzyme was purified from a variety of expression systems (Al et al., 1998; Behrens et al., 1996; Lohmann et al., 1997) and some of its biochemical properties characterized. In vitro, the purified NS5B catalyzes the elongation reaction using a variety of RNA templates in a primer-dependent fashion and with no apparent specificity for the HCV genomic sequences. However, the full-length protein displayed a rather poor catalytic activity (Lohmann et al., 1997, 1998; Tomei et al., 2000) and was found to be poorly soluble, in line with the notion that NS5B is a component of a membrane-bound replication complex (Ivashkina et al., 2002). These technical difficulties slowed considerably the discovery of novel inhibitors of this enzyme. A major breakthrough came from the observation that systematic deletion of the NS5B hydrophobic C-terminal protein domain led to the production of proteins with enhanced solubility and activity (Ferrari et al., 1999; Lohmann et al., 1997; Tomei et al., 2000). Interestingly, while the C-terminal 21 residue-long hydrophobic tail of NS5B is necessary and sufficient to target NS5B to the ER membrane (Schmidt-Mende et al., 2001), this sequence is totally dispensable for the in vitro activity of the purified NS5B polymerase.

The availability of soluble forms of the enzyme also allowed considerable progress toward the determination of the enzyme three-dimensional structure. The unliganded crystal structure of NS5B has been reported by several groups (Ago et al., 1999; Bressanelli et al., 1999; Lesburg et al., 1999), and has revealed unique structural features. As other polymerases, the NS5B folds acquiring the classic "right hand" shape, in which the characteristic fingers, palm and thumb subdomains can be recognized (Fig. 4A). However, different from many cellular and viral polymerases that adopt an "half-open right hand" architecture, the HCV polymerase has a more compact shape due to the presence of two extended loops that span the fingers and thumb domains at the top of the active site cavity. The fingers, thumb and palm subdomains completely encircle the active site cavity to which the RNA template and the NTP substrates have access through two positively charged tunnels (Bressanelli et al., 2002). The strong interaction between fingers and thumb domains limits their freedom to change conformation independently from each other. Structural studies on other RdRps reveal that all of them share this same global architecture (Butcher et al., 2001; Hansen et al., 1997; Ng et al., 2002). The residues responsible for the nucleotidyl transfer reaction are found within the palm domain that, in the case of RdRps, contains an Asp-$(Xaa)_4$-Asp motif and the signature GDD motif. The first aspartate of each motif provides the carboxylate side chains required for ligating the two magnesium ions involved in catalysis. The geometry of the palm domain is conserved in virtually all polymerases; comparison of their three-dimensional structure reveals a conserved "two-metal-ion" catalytic center that is required for the catalysis of a phosphoryl transfer reaction at the polymerase active site (Fig. 4B).

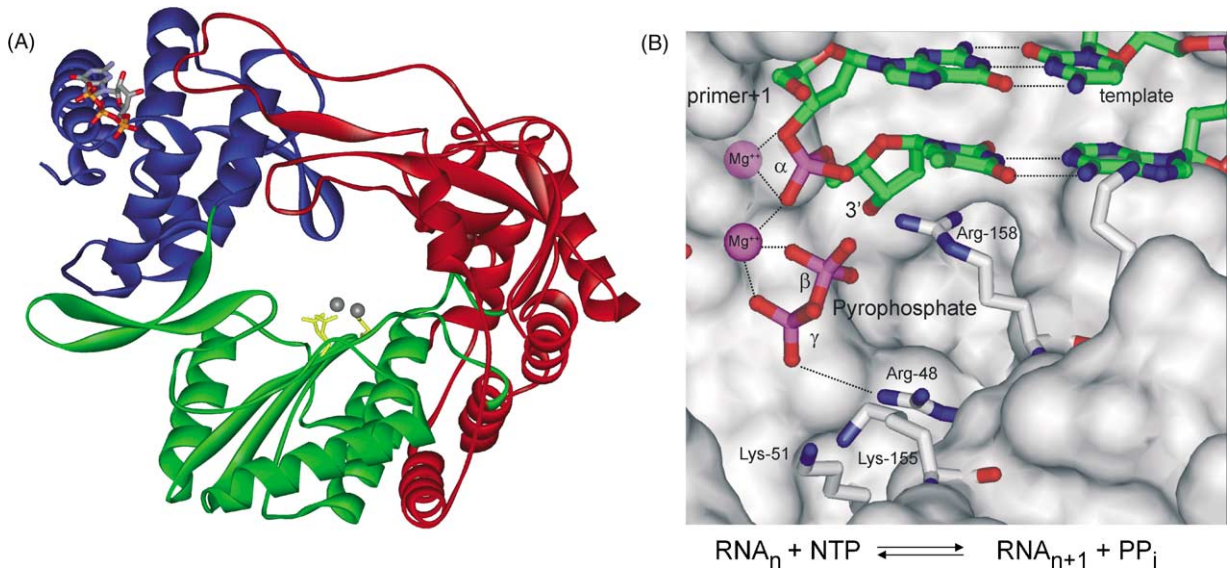


Fig. 4. X-ray crystallographic of the HCV NS5B RdRp. (A) RdRp (right): the thumb, palm, and fingers domains are indicated in blue, green, and red, respectively. The two Mg^{2+} ions (grey) and the active site aspartates (yellow) are shown in the active site. A GTP molecule is bound at the allosteric site on the surface of the thumb domain. (B) The "two-metal-ion" catalytic center of the polymerase after the formation of a phosphodiester bond. The RNA template and the nascent RNA chain (primer + 1) are shown. The leaving pyrophosphate group is coordinate by a Mg^{2+} ion in the enzyme catalytic center. The polymerase reaction scheme is shown at the bottom.

An additional feature of the NS5B polymerase structure is the presence of a unique β-hairpin in the thumb subdomain, which protrudes toward the active site cleft and may thus restrict binding of the template/primer at the enzyme's active site. This structural element is becoming more and more interesting as its role in the initiation mechanism of RNA synthesis is being demonstrated by both structural and biochemical studies (Bressanelli et al., 2002; Hong et al., 2001; Laurila et al., 2002). Initiation is one of the most critical steps in the replication of viral genomes since the precise terminal sequences have to be strictly maintained during each cycle. Some viruses have evolved a mechanism through which the precise terminal initiation is ensured in a primer-dependent reaction. In these cases, as for poliovirus, often a protein acts as the primer to which the polymerase adds nucleotides in a template-complementarity manner (Paul et al., 1998). Most viral RNA polymerases, however, are capable of de novo initiation of RNA synthesis. According to the de novo initiation model of RNA polymerization, complementary RNA synthesis is initiated at the genome 3′-end by a nucleotide triphosphate rather than by a nucleic acid or a protein primer. Purified NS5B is capable of such a primer-independent mechanism of initiation (Luo et al., 2000; Shim et al., 2002; Zhong et al., 2000). The C-terminal β-loop has been proposed to play an important role in the correct positioning of the 3′-end of the RNA template functioning as a gate that impedes slippage of the RNA 3′-end through the polymerase active site (Hong et al., 2001). In vitro, in the absence of an RNA primer, NS5B appears to initiate RNA synthesis preferentially via a "copy back" mechanism. According to this mechanism, the 3′-terminal-OH group of the template is used as a primer for polymerization leading to the synthesis of a double stranded molecule in which template and product RNA remain covalently linked (Behrens et al., 1996). By using this mechanism, the purified enzyme is able to copy the entire HCV genome in a highly processive manner (Lohmann et al., 1997). It is unlikely that the copy back initiation mode is the initiation mechanism through which viral replication takes place, since it would lead to the loss of some terminal sequences and an additional enzymatic function would be required to separate the covalently linked template and product RNA molecules. It is, therefore, generally accepted that HCV in infected cells does utilize the de novo initiation of RNA synthesis to replicate its genome (Butcher et al., 2001). The recently reported structure of the NS5B polymerase in complex with nucleotides (Bressanelli et al., 2002) has landed further support to the de novo initiation hypothesis. In this structure, three distinct nucleotide-binding sites were in fact observed in the catalytic center of the HCV RdRP whose geometry was remarkably similar to that observed in the de novo initiation complex of the phi 6 polymerase (Butcher et al., 2001). De novo initiation must then be followed by RNA elongation, termination of polymerization and release of the nascent strand. At least in principle, each of these steps could be seen as a target for therapeutic intervention.

7. Inhibitors of the NS5B polymerase

The majority of approved antiviral drugs exploit the inhibition of a viral polymerase as the primary mechanism of action (De Clercq, 2001a,b). It is therefore not surprising that a major effort by several pharmaceutical companies has focussed on identifying novel inhibitors of the HCV NS5B polymerase. Existing inhibitors of viral polymerases can be tentatively classified into three categories according to their chemical structure and their mechanism of action: (i) nucleoside (substrate) analogues, (ii) non-nucleoside inhibitors (NNI), and (iii) pyrophosphate mimics. The detailed mechanism of action of each individual member of the various classes of antiviral agents was reviewed recently (De Clercq, 2001a).

Nucleoside analogues are substrate analogues that need to be phosphorylated to their corresponding nucleoside triphosphate (nucleotide) in the cytoplasm of infected cells in order to become active against the viral polymerases. The nucleotide may be incorporated by the polymerase during processive nucleic acid synthesis, leading to early termination of the elongation reaction and thus inhibition of the virus life cycle. Nucleoside inhibitors of the viral polymerase are used therapeutically for HIV, hepatitis B and herpes viruses. The NNI class comprises structurally heterogeneous compounds, which usually bind to a site on the enzyme surface away from the enzyme active site, such as an allosteric site. NNI of therapeutic interest have been described for the HIV-1 reverse transcriptase. In this case, the NNI binding site does not exist in the enzyme without ligand. When the NNI binds to the enzyme it induces the formation of its own pocket, possibly distorting the precise geometry of the transcriptase active site so that the enzymatic function is impaired. Lastly, the drug Foscarnet (phosphonoformic acid) is the prototypic member, and the only approved member, of the pyrophosphate (product) analogue. These agents are thought to interact directly with the pyrophosphate-binding site of the viral polymerases (Crumpacker, 1992) (Fig. 4B).

In the last few years, several research groups in the biopharmaceutical industry have reported a number of inhibitors of NS5B enzymatic activity that are undergoing preclinical development. Selected examples are reported below.

7.1. Nucleoside inhibitors of NS5B

The only nucleoside analogue that thus far was shown to be therapeutically useful against HCV infection is the broad-spectrum antiviral agent D-ribavirin (1-β-D-ribofuranosyl-1-1,2,4-triazole-3-carboxamide; compound 10 in Fig. 5A) (McHutchison and Patel, 2002). The mechanism of action of D-ribavirin is currently debated (Lau et al., 2002). Some authors have proposed that D-ribavirin triphosphate is in fact incorporated by NS5B into the nascent viral genome (Maag et al., 2001). This leads, in turn, to an increased error frequency of the viral polymerase that might be responsible for the antiviral activity of this agent (Crotty et al., 2000).

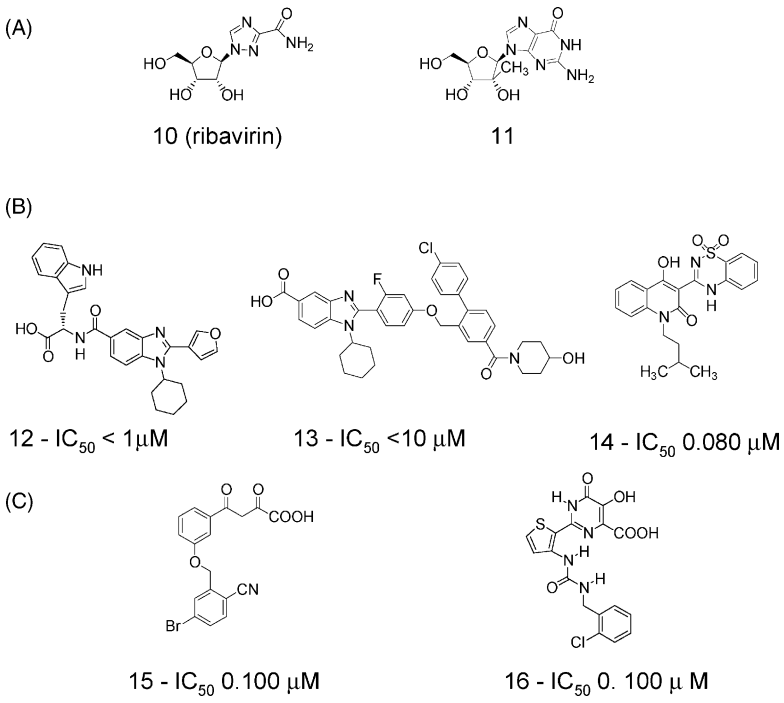


Fig. 5. Chemical structures of selected inhibitors of the NS5B RdRp activity. (A) Nucleoside inhibitors, (B) non-nucleoside inhibitors, and (C) active site metal-ion chelating inhibitors (pyrophosphate mimics).

However, this model has not yet been proven relevant in a clinical setting and it should be remembered that monotherapy with D-ribavirin does not lead to a decrease in HCV titers.

Dioxolane-triphosphate- and nucleoside-triphosphate-based inhibitors of recombinant HCV polymerase have also been described in the recent patent literature (Ismaili et al., 2002; Storer, 2001) and have been reviewed elesewhere (Beaulieu and Llinas-Brunet, 2002). Notably, the discovery of a novel series of oral, once-daily nucleosides, potentially useful for the treatment of all HCV genotypes, was recently reported (Sommadossi and Lacolla, 2001). Among these, β-D-2′-methyl-ribofuranosyl-guanosine (Fig. 5A, compound 11) is phosphorylated in cultured, uninfected cells and orally bioavailable in primates.

Recent structural work has lead to the identification of both catalytic and regulatory nucleotide binding sites in the HCV RdRP (Bressanelli et al., 2002). The structural details of nucleotide binding at the catalytic site may provide some guidance for the design of novel nucleotide analogues that would be inhibitors for HCV RdRp-catalyzed RNA synthesis.

7.2. Non-nucleoside inhibitors of NS5B

Several NS5B inhibitors have been reported in the patent literature, some of which are likely to be the subject of future preclinical and clinical investigations. Selected examples of non-nucleoside inhibitors of the HCV RdRP are illustrated in Fig. 5B: two benzimidazole derivatives (Beaulieu et al., 2002; Hashimoto et al., 2001) (compounds 12 and 13), and a benzothiadiazine derivative (Dhanak et al., 2001) (compound 14). Compounds of the benzothiadiazine series have been thoroughly characterized biochemically and in cell culture models of HCV replication (Dhanak et al., 2001). Submicromolar concentrations of compound 14 effectively reduced HCV RNA in the replicon system with no apparent cell toxicity. Agents of this class interact directly with the viral polymerase and inhibit RNA synthesis noncompetitively with respect to GTP. It has been suggested that benzothiadiazine NNI block the HCV RdRP prior to the formation of an elongation complex (Dhanak et al., 2001). Similarly, the mechanism of action of the benzimidazole NNIs has been suggested to be antagonistic with respect to the formation of a productive enzyme-template complex (Kukolj et al., 2002). The site(s) where the various NS5B NNIs bind on the enzyme is not known at the molecular level. Interestingly, an allosteric, possibly regulatory nucleotide binding site has been recently identified in the HCV RdRP (Bressanelli et al., 2002) at the interface between the fingers and the thumb. In the crystal, this site can be occupied by GTP, but not ATP, CTP or UTP (Fig. 4A). Bressanelli et al. (2002) suggested that this unique nucleotide-binding site could provide an attractive target for potential allosteric inhibitors of the enzymatic reaction. Future research will need to address whether any of the NNI reported so far act by binding to this allosteric pocket on the enzyme surface.

NNI inhibitors of the HCV polymerase are receiving a great deal of interest as potential antiviral agents: an orally bioavailable analogue of compound 13 (Fig. 5) termed JTK-003, is currently being studied in a phase Ib multicenter, placebo-controlled clinical study on HCV patients who

were refractory to interferon-based therapy (McHutchison and Patel, 2002).

7.3. Pyrophosphate mimics: active site metal-ion chelating inhibitors of NS5B

A series of diketo acids was reported to selectively and potently inhibit the HCV RdRP elongation activity in vitro (Altamura et al., 2000). An example of this class of compounds is given in Fig. 5C (compound 15). The mechanism of action of this and related compounds was found to be noncompetitive with respect to both the RNA template and to the nucleotides. Binding of diketo acids to the HCV RdRP is mediated by active site divalent cations, such as magnesium or manganese (R.D.F., L.T. and S.A., unpublished observation). One attractive hypothesis to explain inhibition by diketo acid compounds is that the diketo acid fragment could inhibit the RdRP activity through an interaction with the catalytic metal-ions found in the enzyme active site (Fig. 4B). A similar mechanism for the inhibition of the viral polymerase has been invoked to explain inhibition by canonical pyrophosphate analogues such as Foscarnet and phosphonoacetic acid (Sundquist and Oberg, 1979). These latter compounds are believed to act as product-like inhibitors of the polymerase reaction. Viral polymerases that are inhibited by these classical pyrophosphate analogues include the HIV RT and the HBV and herpes virus DNA polymerases (Crumpacker, 1992; Hess et al., 1980). Interestingly, differentially substituted diketo acids were characterized, with each inhibiting HIV-RT or HBV polymerase in a highly selective manner (Altamura et al., 2000). Furthermore, experiments aimed at measuring the combined effect of diketo acids and Foscarnet on HCV and other viral polymerases indicated that the two classes of inhibitors interact with the enzymes in a mutually exclusive fashion, suggesting interaction with a common binding site, that is, the pyrophosphate binding site (S.A. and RD. F., unpublished observation). Compound 16 (Fig. 5) represents a novel class of substituted dihydroxy-pyrimidine carboxylates (Gardelli et al., 2002) that is also believed to bind to the “two-metal-ion” catalytic center of the HCV polymerase as a pyrophosphate product mimic (L.T. and S.A., unpublished observation).

Although the therapeutic potential of diketo acids and dihydroxy-pyrimidine carboxylates has yet to be demonstrated for HCV, classical pyrophosphate analogues, such as Foscarnet, possess well documented antiviral activity (De Clercq, 2001b). Moreover, diketo acid inhibitors of HIV integrase (Hazuda et al., 2000) and influenza transcriptase (Tomassini et al., 1994) have been reported to inhibit viral replication in cell culture and in animal models.

8. Conclusions and future prospects

Current approved therapeutic approaches for HCV infection (interferon or interferon plus ribavirin) are not very efficacious (Chander et al., 2002) and plagued with many unwanted side effects (Fried, 2002). Therefore, the HCV epidemic represents a largely unmet medical need that has triggered intensive research effort toward the development of new, safer and more effective drugs. The viral NS3-4A serine protease and the NS5B RdRp constitute the most promising targets for the development of novel anti-HCV compounds. The discovery of inhibitors of these enzymes have therefore been the focus of intense research of many groups operating in the biopharmaceutical industry. Despite the inherent difficulties due to the lack of a laboratory HCV infection system, tremendous progress has been made toward the discovery of effective such agents. As of today, inhibitors of both enzymes are in various stages of preclinical development and two of them have entered early clinical trials. The prospects for new HCV treatments in the near future are bright. The next years will be very exciting as more drug candidates move through clinical trials and hopefully into widespread clinical use.

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