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Antiviral Research 55 (2002) 397–412

Antiviral Research

www.elsevier.com/locate/antiviral

Review

Nucleotide triphosphatase/helicase of hepatitis C virus as a target for antiviral therapy

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Received 8 April 2002; accepted 13 June 2002

Abstract

The RNA nucleoside triphosphatase (NTPase)/helicases represent a large family of proteins that are detected in almost all biological systems where RNA plays a central role. The enzymes are capable of enzymatically unwinding duplex RNA structures by disrupting the hydrogen bonds that keep the two strands together. The strand separating activity is associated with hydrolysis of nucleoside triphosphate (NTP). Because of this, potential specific inhibitors of NTPase/helicases could act by one or more of the following mechanisms: (i) inhibition of NTPase activity by interference with NTP binding, (ii) inhibition of NTPase activity by an allosteric mechanism and (iii) inhibition of the coupling of NTP hydrolysis at the unwinding reaction. There are also other inhibitory mechanisms conceivable, which may involve a modulation of the interaction of the enzyme with its RNA substrate, for example, (iv) the competitive inhibition of RNA binding and (v) the inhibition of the unwinding by sterical blockade of the translocation of the NTPase/helicase along the polynucleotide chain. NTPase/helicase has also been identified in the viral genome of hepatitis C virus (HCV) which is a member of the Flaviviridae family. It is conceivable that the inhibition of the unwinding activity of the enzyme leads to the inhibition of virus replication and this may represent a novel antiviral strategy. This review updates the current spectrum of inhibitors targeting different mechanisms by which the NTPase and/or helicase activities of the HCV NTPase/helicase are inhibited. Consequently, some of the compounds might be important as antiviral agents against HCV.

Keywords: Hepatitis C virus; Nucleoside triphosphatase helicase; Flaviviridae; Target for antiviral therapy

1. Introduction

Hepatitis C virus (HCV) was identified in 1989 as the causative agent of non-A, non-B hepatitis (Choo et al., 1989, 1990). The virus has infected approximately 170 million people worldwide. At least 85% of the infected patients develop a chronic hepatitis ending up as liver cirrhosis and end-stage liver disease in 20–30% (McHutchison et al., 1998; Poynard et al., 1998). Phylogenetic analyses have classified HCV in six major genotypes, numbered 1–6. The genotypes distinguish in their geographic distribution, response to therapy and severity of the disease (Mizokami and Orito, 1999; Gordon et al., 1997).

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0166-3542/02/$ - see front matter
PII: S0166-3542(02)00096-7

Until now, an effective antiviral drug therapy for infections with HCV does not exist. Ribavirin, that has been successfully used for the treatment of pathogenic DNA and RNA viruses like influenza virus (Gilbert et al., 1985), respiratory syncytial virus (Taber et al., 1983), Lassa fever virus (McCormick et al., 1986) and Hantaan viruses (Huggins et al., 1986), was not more effective in reducing or eliminating the RNA of HCV than a placebo (Zoulim et al., 1998; Lee et al., 1998). Nevertheless, a viral clearance of 40% of the patients with chronic hepatitis C infection was obtained when the compound was used in combination with interferon-α2 (IFNα). It should be mentioned that the long-term application of IFNα as monotherapy cured only 10–30% of the patients (McHutchison et al., 1998; Poynard et al., 1998; Quin, 1997).

HCV, a member of the Flaviviridae family, is a plus-stranded RNA virus with a linear genome of approximately 9.6 kb. It possesses a single open reading frame (ORF) that translates to a single polyprotein. The ORF of the Flaviviridae is flanked by 5′- and 3′- terminally located untranslated regions (5′UTR and 3′ respectively). The 5′UTR of HCV is approximately 340 bases long and reveals features of an internal ribosomal entry site, mediating the binding to the ribosome (Tsukiyama-Kohara et al., 1992; Poole et al., 1995). The function of 3′UTR remains to be established. There are some indications that it may be important for the initiation of minus strand synthesis (Ito et al., 1998) or that the adenosine- and/or uridine-rich stretches, present in the 3′UTR, are involved in the regulation of the nucleoside triphosphatase (NTPase) activity of the NS3 associated NTPase/helicase (Blight and Rice, 1997).

In the course of the infection, the polyprotein is co- and post-translationally cleaved by both virus-encoded and host cellular proteases (signalases) releasing 10 known structural and nonstructural (NS) proteins. The proteins occur in the following sequence: NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Westeway, 1987; Monath and Heinz, 1996; Rice, 1996; Pryor et al., 1998). The initial cleavages, which are catalyzed by host proteases, liberate the individual envelope and capsid proteins and a strong hydrophobic peptide, called p7, function of which is unknown (Bartenschlager, 1997; Zhong et al., 1999).

For the cleavage of the NS2/NS3 junction, an activity of the Zn^{2+}-dependent protease is responsible that is associated with the adjacent segments of the NS2 and NS3 protein (Pallaoro et al., 2001). For the cleavage of the NS region at sites that lie COOH-terminally to NS3, an activity of a further viral serine-type protease is required. This activity is associated with the NH2- terminal domain of the NS3-protein (Monath and Heinz, 1996; Rice, 1996; van Doorn, 1994). The cleavage results in a NS3-protease cofactor (NS4A), two proteins of unknown function (NS4B and NS5A) and a RNA-dependent RNA polymerase (RdRp) (NS5B).

Among the viral proteins, the most promising antiviral targets in HCV infection are the enzymes of the replication complex. The replication complex of Flaviviridae includes the NTPase/helicase located in the COOH-terminus of the NS3 protein, the above mentioned RdRp and some further viral proteins capable of anchoring the enzymes at the membranes of the endoplasmatic reticulum by their hydrophobic amino acid motifs (Bartenschlager, 1997). A range of knock out experiments, in which the helicase or polymerase activities of viruses of the Flaviviridae family were switched off, demonstrated the key function of these enzymes for virus replication (Gu et al., 2000; Matusan et al., 2001). Consequently, the compounds that block or at least reduce the activity of the NTPase/helicase or RdRp could act as inhibitors of virus replication.

A number of inhibitors of the viruses of Flaviviridae family, like derivatives of 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide, tiazofurin or selenazofurin, tested mainly with yellow fever virus, have been developed (De Clerq et al., 1991; Neyts et al., 1996; Balzarini et al., 1993). However, the specific modes of action of these compounds are not precisely determined. Recently, an attempt to develop of HCV inhibitors acting by inhibition of the NTPase/helicase has been described. A group of such compounds, reported only as patents by ViroPharma (Diana et al., 1998; Diana and Bailey, 1996), is based on two benzimidazoles linked by spacers of different

length. The inhibitory potential of some related compounds was investigated and reported in a later structure–activity relationship study (Phoon et al., 2001). Despite the great attraction of the NS3 NTPase/helicase as antiviral target, there is no such drug applicable for chemotherapy until now.

As demonstrated below, several compounds, possessing inhibitory potential against the NTPase and/or helicase activities of the HCV NTPase/helicase, have been identified and their possible targets within the enzyme molecule are discussed. Some of the compounds could be indeed viewed as potential anti-HCV agents.

2. Structure and enzymatic properties of HCV NTPase/helicase

The sequence comparisons of DNA and RNA NTPase/helicases and of other nucleoside triphosphate (NTP) consuming enzymes revealed a range of conserved motifs, which are associated with NTP binding (Kadare and Haenni, 1997; Gorbalenya and Koonin, 1989, 1993). These include the Walker motif A, which binds the terminal phosphate groups of the NTP, and the Walker motif B, responsible for the chelation of the Mg^{2+} of the Mg-NTP complex (Walker et al., 1982). Based on the altered Walker motif A the NTPase/helicases are arranged in three superfamilies (SF's): the SF1 that is characterized by the classic Walker motif A (G-X-X-X-X-G-K-S/T) and the SF2 and SF3 revealing variations of the domain ((A-X-X-G-X-G-K-S/T) and (G-X-G-X-G-K-S) respectively) (Kadare and Haenni, 1997; Lüking et al., 1998; Fuller-Pace, 1994). The viral NTPase/helicases classified in SF1 are represented by alphavirus-like (nsP2-like) proteins; SF2 includes polypeptides, similar to the NS3 protein (NS3-like proteins), encoded by potyviruses and the members of the family of Flaviviridae; SF3 includes the picornavirus-like (2C-like) proteins (Gorbalenya et al., 1989; Lain et al., 1989). The SF2 NTPase/helicases are further divided, according to the sequences surrounding the conserved D-E residues (Walker motif B) in four different subgroups of proteins. The first is formed by the classic D-E-A-D box proteins. The second and third one are named D-E-A-H and D-E-X-H, based on their deviating Walker motif B sequences (Kadare and Haenni, 1997; Lüking et al., 1998; Fuller-Pace, 1994). The last subgroup is more heterogenous than the D-E-A-D box proteins with respect to its sequence and biochemical function (Lüking et al., 1998; Wassarman and Steitz, 1991). The NTPase/helicase, associated with the NS3 protein of HCV, possesses the D-E-C-H motif and is, therefore, member of the D-E-X-H box subgroup (Miller and Purcell, 1990; Yao et al., 1997). The structure of the HCV enzyme has been solved to 2.1 Å (Yao et al., 1997) and 2.3 Å (Cho et al., 1998) resolution in the absence and to 2.2 Å resolution in the presence of bound oligonucleotide (Kim et al., 1998). The protein consists of three equally-sized structural domains separated by series of clefts. The domains 1 and 3 share with each other a more extensive interface than either of them shares with the domain 2. In the consequence, the clefts between the domains 1 and 2 and the domains 2 and 3 are the largest. The domain 2 is flexibly linked to the other two and undergoes a rigid-body movement relative to domains 1 and 3. Domains 1 and 2 contain all of the conserved helicase sequence motifs (Yao et al., 1997; Cho et al., 1998; Kim et al., 1998).

The mentioned Walker motifs A and B (motifs I and II of the SF2 helicases) are localized on the surface of the domain 1. In the absence of substrate the residues of the Walker motifs bind to each other and additionally to the residues of the conserved T-A-T sequence (motif III). The motif III is a part of the flexible switch region that connects the first and the second domain of the enzyme (Kadare and Haenni, 1997; Yao et al., 1997). The conformational changes of the molecule induced by the NTP hydrolysis are transmitted by this switch sequence (Yao et al., 1997; Matson and Kaiser-Rogers, 1990).

The role of the highly conserved arginine-rich motif (G-R-X-G-R-X-G-R; motif VI), which is localized on the surface of domain 2, is discussed controversially. On the base of the X-ray crystallographic data, it was suggested that the motif is required for RNA binding (Yao et al., 1997; Cho et al., 1998). On the other hand, the structure of

the HCV NTPase/helicase with bound dU8 (Kim et al., 1998) suggests a direct involvement of the arginine residues of this motif in ATP binding.

Based on the structures of the enzyme and biochemical analyses, two alternative mechanisms of the unwinding reaction have been proposed. According to the first one, so called 'passive' mechanism, the NTPase/helicase molecule binds single stranded (ss) regions of the substrate and does not actively participate on the separation of the duplex RNA or DNA structures (Lüking et al., 1998; Yao et al., 1997; Matson and Kaiser-Rogers, 1990). Corroborating with this model is the observation that some proteins, like eukaryotic replication protein A (Georgaki et al., 1992), herpes simplex virus (HSV) type 1 ICP8 protein (Boehmer and Lehman, 1993), T4gp32 (Chase and Wiliams, 1986) and the protein vasa from *Drosophila melanogaster* (Hay et al., 1988) are capable of unwinding the duplex DNA structures in a NTP-independent manner. In a recent study a strand separating activity of the HCV NTPase/helicase was identified, which is also present in the absence of ATP (Porter and Preugschat, 2000).

The second, so called 'active', mechanism of the unwinding, predicts an NTP-dependency of the reaction and at least two nucleic acid binding sites on the surface of the enzyme (Yao et al., 1997; Matson and Kaiser-Rogers, 1990). According to this model the NTP-triggered conformational changes facilitate the binding of RNA or DNA substrate to the alternate sites (Yao et al., 1997). The energy resulting from the NTP hydrolysis is transferred to the fork where it is consumed for the disruption of the hydrogen bonds and/or is used for the translocation of the enzyme along the RNA or DNA strand (Yao et al., 1997; Cho et al., 1998; Kim et al., 1998). This mode of the unwinding requires the presence of multiple substrate binding sites on a single polypeptide (Yao et al., 1997). Alternatively, as postulated for the majority of NTPase/helicases, the multiple substrate binding sites could result from the polymerisation of the enzyme. Indeed, an oligomeric status of the HCV NTPase/helicase could be demonstrated (Levin and Patel, 1999) and recently we could show the same for the related West Nile virus (WNV) enzyme (Borowski et al., 2001a).

Although the X-ray crystallography data provide insight into the mode of the unwinding reaction, the basic information regarding the biochemical properties of the enzyme has come from kinetic studies and mutational analyses. The HCV NTPase/helicase has been cloned, expressed and purified to homogenity in numerous laboratories (Levin and Patel, 1999; Gallinari et al., 1998; Preugschat et al., 1996; Kim et al., 1997; Hesson et al., 2000; Morgenstern et al., 1997; Tai et al., 1996). The following common biochemical properties have been observed by using the purified enzyme and various in vitro assay systems.

2.1. The length of the enzyme

The minimal amino acid stretch of NS3, displaying the helicase activity, is approximately 400 residues in length and is situated between residues 1209 and 1608 of HCV polyprotein (Kim et al., 1997). Nevertheless, the overwhelming biochemical and inhibitory studies are performed with an enzyme, that consists of the entire COOH-terminally localized NTPase/helicase domain of NS3 or of the full-length NS3 protein. The studies, that compare the biochemical properties of full-length with the truncated NS3, supply rather contrasting results. For example Morgenstern et al. (1997) demonstrated significant differences between the proteins regarding the pH value and polyU concentration required for optimum ATPase activity. In contrast to this, an other comparative study, using recombinant full-length NS3 and its isolated COOH-terminal domain (Gallinari et al., 1998), did not reveal significant differences in the strand separating activity of the enzymes analyzed in independent in vitro assays. In this context, it appears to be important to verify the inhibitory potential of NTPase/helicase inhibitors evaluated with the COOH-terminal NTPase/helicase domain, with full-length enzyme or even in the presence of other components of the replication complex.

The information about the intracellular processing of HCV NS3 is limited. Numerous proteases cleave NS3 of HCV at the site connecting the protease and NTPase/helicase regions and in the 'hinge region' between the domains 1 and 2 of the

NTPase/helicase in vitro (Borowski et al., 1999b). There is, however, no evidence that such cleavages occur during the virus life cycle. On the other hand, there are indications for an in vivo cleavage of NS3 of HCV mediated by cellular proteases, occurring directly adjacent to the motif VI of the enzyme and abolishing its helicase activity (Shoji et al., 1999).

2.2. The NTPase activity

The NTPase activity of the HCV NTPase/helicase was characterized in numerous previous studies (Gallinari et al., 1998; Preugschat et al., 1996; Hesson et al., 2000; Morgenstern et al., 1997). The enzyme displays low selectivity towards the nucleobase of the NTP. It hydrolyzes all NTP's, dNTP's and even acyclovir triphosphate, acyclic ATP and tri-polyphosphate, which lack the ribosyl functionality of natural nucleosides (Preugschat et al., 1996; Warrener and Collett, 1995; Tamura et al., 1993). The hydrolysis of the NTP's and dNTP's is stimulated by ribohomopolymers, 2′-deoxyribohomopolymers, RNA and DNA (Morgenstern et al., 1997; Suzich et al., 1993). The response of the NTPase/helicase to the polymers depends on their concentration, length and nucleotide forming the polymer (Preugschat et al., 1996; Morgenstern et al., 1997; Suzich et al., 1993). The ATPase activity of the HCV enzyme is stimulated with the following order of efficiency: poly(U) = poly(dU) > > poly(A) > poly(dT) = poly(C) > poly(dI) > poly(I) > poly(dA) > poly(dC) > poly(G) (Suzich et al., 1993). As shown in numerous studies, performed with full-length NS3 or the COOH-terminal NTPase/helicase domain, the extension of the activating effect corresponds closely to the rate of the binding of the polynucleotides to the protein (Bartenschlager, 1997; Gwack et al., 1996). Both, the binding of the polynucleotide and the polynucleotide mediated NTPase activation require a minimum length of nucleic acid (15 nucleobases) (Preugschat et al., 1996). It has been demonstrated that this length of the polynucleotide may contribute to the binding energy which is needed for the complex formation (Preugschat et al., 1996).

The preference for the polymer that acts as activator of the NTPase reaction may be related to the presence of the homopolymeric motifs within the 3′UTR. Indeed, in a recent study a specific interaction of the HCV NS3 protein with the 3′-terminal sequences consisting of the viral 3′UTR was demonstrated (Banerjee and Dasgupta, 2001). The mechanistic significance of the activation mediated by the polynucleotide for virus replication remains unclear.

2.3. The helicase activity

For the processing of the genome of the members of the Flaviviridae family the negative-stranded RNA must be synthesized by using the parental positive-stranded RNA as template. Then the resulting negative-stranded RNA is used as template for the synthesis of the positive-stranded progeny RNA that is assembled in viral particles. Since the negative and positive orientated RNA strands are complementary, the NS3 associated helicase activity appears to be necessary for the strand separation. Recent genetic knock out experiments support the essential role of the NS3 associated helicase activity for the replication of Dengue fever virus and Bovine viral diarrhea virus (Matusan et al., 2001; Gu et al., 2000).

The helicase activity of HCV NS3 was experimentally documented and characterized by many authors (Porter and Preugschat, 2000; Levin and Patel, 1999; Gallinari et al., 1998; Preugschat et al., 1996; Tai et al., 1996). The enzyme exhibits a 3′–5′-directionality with respect to the template strand and in contrast to the majority of the NTPase/helicases it is capable of unwinding DNA/DNA and RNA/RNA homoduplexes and RNA/DNA heteroduplexes (Tai et al., 1996 and own observations). The explanation for this lack of specificity is the fact that the interaction between the protein molecule and the DNA or RNA substrate is mediated by phosphate groups and not by the nucleotide base or sugar moieties (Yao et al., 1997; Kim et al., 1998).

Despite numerous kinetic analyses and the solution of the structure of several NTPase/helicases, it remains unclear how the NTP binding and the hydrolysis are coupled to the unwinding of

double-stranded substrate. Numerous data suggest that the activities of the enzyme are not necessarily coupled: (i) The enzyme functions as polynucleotide-stimulated and not as polynucleotide-dependent NTPase. The stimulation of ATPase activity of HCV NTPase/helicases by ss nucleic acids is not related to the procession of the enzyme along the RNA or DNA substrates (Hesson et al., 2000; Tai et al., 1996). (ii) A broad range of structurally unrelated compounds like 5-fluoro-2-selenocytosine or derivatives of O^6-benzylguanine are able to inhibit or enhance the ATPase activity of the HCV and of the related WNV NTPase/helicase without affecting the respective helicase activity. On the other hand, some chloroethylguanine derivatives stimulate the helicase activity of the enzyme without apparently effecting its ATPase activity (Borowski et al., 2001a and unpublished data). (iii) The HCV NTPase/helicase has different optimum conditions like pH-value, concentrations of salt or detergent for NTPase and helicase reactions (Gallinari et al., 1998; Preugschat et al., 1996; Hesson et al., 2000; Morgenstern et al., 1997; Tai et al., 1996).

The observations indicate that the inhibitors designed against the NTPase/helicase, should rather be evaluated by the two separate tests: NTPase and helicase assays.

3. Inhibition of specific targets within the NTPase/helicase molecule

The mentioned lack of selectivity of the HCV NTPase/helicase for the binding of the polynucleotide, the hydrolysis of the NTP's or for the sequence of the double-stranded substrates suggests it would be difficult to design specific and selective inhibitors based on the solved crystal structure of the enzymes. Screening of the chemical libraries followed by structural modification of active compounds might be more promising for the development of such inhibitors. In this context, some of the mechanisms of the enzyme action could serve as targets for the compounds.

3.1. Inhibition of NTPase activity by interference with NTP binding

In general, the NTP hydrolysis supplies the energy for the unwinding reaction. Thus, the reduction of the accessibility of the NTP-binding site for the NTP may lead to a decreased NTPase activity and therefore to a respective reduction of the unwinding rate. A wide range of competitive NTPase inhibitors like ribavirin-5′triphosphate (RTP), ribavirin-5′diphosphate (RDP) (Fig. 1), adenosine-5′-γ-thiotriphosphate (ATP-γ- S) or ADP (Borowski et al., 2000) has been tested as inhibitors of the helicase activity. Surprisingly, although the IC_{50} values, measured for the inhibition of the ATPase activity, lie in a low micromolar range, the compounds were only moderate inhibitors of the unwinding activity of the HCV enzyme (Fig. 1). Considering the low specificity mentioned above of the HCV NTPase/helicase and related enzymes towards nucleotides and/or nucleosides (Preugschat et al., 1996; Warrener and Collett, 1995; Tamura et al., 1993; Lain et al., 1991) one could speculate that compounds like RTP, RDP, ATP-γ- S or ADP might underlie a partial hydrolysis to less active derivatives. However, non-hydrolysable 5′-adenylimidodiphosphate (AMP-PNP) and β,γ-methylene-adenosine 5′-triphosphate (AMP-PCP) acting as classical competitive inhibitors of the ATPase activity of the HCV enzyme did not inhibit the helicase activity up to a concentration of 1.5 mM (P.B. and H.S., unpublished data).

The results of inhibitory studies obtained with paclitaxel agree with the findings. This compound, the structure of which is not related to NTP, is known for interaction with the Walker motif A of the nucleotide binding pocket of efflux proteins, members of the ABC (ATP-binding cassette) superfamily of membrane proteins (Wu et al., 1998). Also using the HCV NTPase/helicase, paclitaxel was able to block the ATP-binding site and to inhibit the ATPase activity of the enzyme with a similar efficacy (22 and 17 μM respectively) in a competitive manner (Borowski et al., 1999b). However, when tested as an inhibitor of the helicase activity of the enzyme, paclitaxel was not

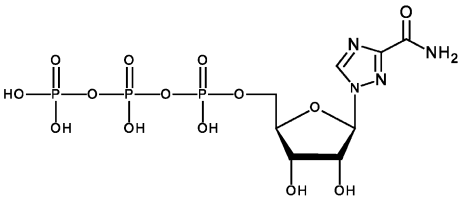
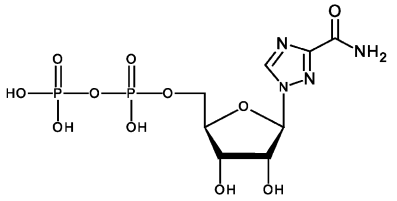
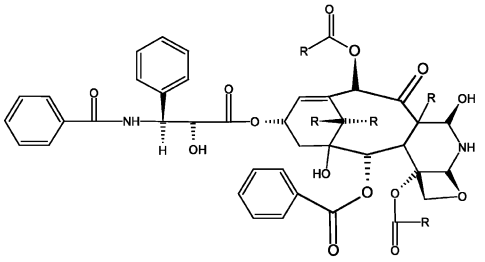
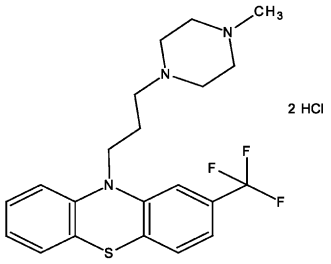
Structure		Inhibition Potency	
		ATPase	Helicase
	RTP	IC_{50} = 40 μM Competitive	IC_{50} = 180 μM
	RDP	IC_{50} = 90 μM Competitive	IC_{50} = 250 μM
	Paclitaxel	IC_{50} = 17 μM Competitive	IC_{50} > 1 mM
	Trifluoperazine	IC_{50} = 105 μM Noncompetitive	IC_{50} = 0.6-0.7 mM

Fig. 1. Structures of inhibitors of the ATPase activity of the HCV NTPase/helicase; ribavirin-5′-triphosphate (RTP), ribavirin-5′-diphosphate (RDP), paclitaxel and trifluoperazine, their potency and inhibition mechanism. The ATPase and helicase assays were performed at ATP concentration corresponding to the K_m value determined in the ATPase reaction in the presence of DNA substrate and increasing amounts of RTP according to the methods described previously (Borowski et al., 1999b, 2000, 2001a). The inhibition parameters were expressed as the concentration of the compound at which the half-maximal inhibition was observed (IC_{50}).

capable of inhibiting up to the concentration of 1 mM (unpublished data) (Fig. 1).

The basis of the phenomenon remains unclear. A common property of the inhibition mediated by these compounds is an only incomplete reduction of the NTPase activity. The maximum of the inhibition was obtained at concentrations of the inhibitor exceeding the IC_{50} value by 3–5 times. At these concentrations the NTPase activity reached 10–35% of the control (Borowski et al., 1999b, 2000, 2001b). In this context, it might be interesting to test whether the remaining NTPase activity is sufficient to support the helicase activity, or if the 'passive' (NTP-independent) mode of unwinding is switched on in this situation of limited energy supplied.

3.2. Inhibition of NTPase activity by allosteric mechanisms

Some of the problems raised above could be bypassed by using compounds chemically unrelated to NTP, which reduce the accessibility of the NTP-binding site by a non-competitive mechanism. In our previous study we have found that trifluoperazine (Fig. 1), a calmodulin antagonist (Ganapathi et al., 1991), was capable of inhibiting the NTP binding and NTPase activity of HCV NTPase/helicase. The inhibition occurred with a similar efficacy: using 98 and 105 μM respectively the half-inhibition of the ATP binding and ATPase activity was achieved (Borowski et al., 1999b). When the helicase activity was investigated as function of the trifluoperazine concentration, the inhibition of the unwinding reaction occurred with an IC_{50} value of 600–700 μM (data not shown). Whether the helicase activity was inhibited as a consequence of blocking the NTP-binding site is unknown. Although trifluoperazine acts on the level of the domain 1 of the HCV NTPase/helicase a second allosteric site binding trifluoperazine could not be excluded. At increasing concentrations of the compound, the site becomes more and more occupied. Consequently, there is a conformational change occurring in the enzyme, which ultimately leads to inhibition of its helicase activity.

3.3. Inhibition of the coupling of NTP hydrolysis to unwinding reaction

At present, structural divergent molecules that are capable of uncoupling the NTPase and helicase activity have been characterized including a range of macromolecules like modified polynucleotides, positively or negatively charged polymers such as dextrane sulfate, heparin and polylysine of defined length (Hesson et al., 2000; Tai et al., 1996; Borowski et al., 2000 and unpublished data). The compounds are potent modulators (inhibitors or activators) of the helicase activity without corresponding influence on the NTPase activity. Although in many cases the mechanism by which the compounds act remains unknown, there are indications that some of the mentioned macromolecules disturb the energy transfer within the enzyme molecule.

As we had demonstrated before the lysine-rich histone H1 and core histones H2B and H4 may form stable complexes with HCV NTPase/helicase (Borowski et al., 1996). This binding induces changes of the conformation of the histone molecule that lead to an alteration of its properties as a substrate for some protein kinases (Borowski et al., 1999a) and reduces its DNA-binding capacity (Borowski et al., 1999c). On the other hand, the binding of the histone changes the conformation of the HCV NTPase/helicase. This results in a strong inhibition of the unwinding activity of the enzyme with an IC_{50} lying in a nanomolar range (Fig. 2). It should be mentioned that the histones

Fig. 2. Inhibition of the helicase activity of the HCV NTPase/helicase as function of the histone III-S concentration. The helicase reaction was carried out under standard conditions as described in the legend of Fig. 1, in the presence (lanes 1–4), or absence (lane 5) of histone III-S. The histone concentration was adjusted to 1 nM (lane 1), 10 nM (lane 2), 100 nM (lane 3) and 1000 nM (lane 4).

did not influence the NTPase activity of the enzyme up to a concentration as high as 0.3 mM.

Microsequencing of proteolytically generated small, histone binding fragments of the NTPase/helicase revealed that the interaction is mediated by a linear stretch of amino acids located between the residues 1343 and 1379 of the HCV polyprotein (Borowski et al., 1999c). According to the crystallisation studies, cited above, this region of the enzyme corresponds to the 'hinge region' connecting the domains 1 and 2 and is responsible for the transmission of the energy between the domains. Moreover, it guarantees the mobility of the domain 2 necessary for the unwinding (Yao et al., 1997). The currently running modelling studies should help to find out structural similar compounds, peptidomimetics, that could simulate the action of histone.

3.4. Competitive inhibition of RNA binding

In the first reports, in which the HCV NTPase/helicase was characterized, it was pointed out that numerous polynucleotides, although activating the NTPase activity, were inhibitory towards the helicase activity of the enzyme (Tai et al., 1996). These inhibiting effects resulted from the competition of the polynucleotides with DNA or RNA substrates for the binding site. Based on this information, macromolecular inhibitors that block HCV helicase activity were developed. For example, RNA aptamers rich in secondary structures were found to interact with the NS3 protein and to inhibit the helicase activity (Kumar et al., 1997). Also some small molecular inhibitors acting apparently on the level of the nucleic acid binding site were reported. Two chemically related compounds, piperidine derivative and heterocyclic substituted carboxamide (Fig. 3), have been demonstrated to inhibit the helicase activity of the HCV NTPase/helicase in vitro. Both compounds showed IC_{50} values lying in low micromolar range (Diana et al., 1998; Diana and Bailey, 1996). Analysis of a chemical library of related compounds performed recently (Phoon et al. 2001), cleared the role of hydrophilic and hydrophobic residues for the interaction of the compounds with the substrate binding site of the enzyme.

In our own investigations, we could observe that some benzimidazole and benzoxazole derivatives chemically related to the compounds mentioned above, with attached (directly or over by a spacer) amino acid moieties (referred in Deluca and Kerwin, 1996) inhibit effectively the unwinding activity of the HCV enzyme without affecting its NTPase activity. This inhibition could be partly abolished by increase of the DNA or RNA substrate concentration (unpublished data).

3.5. Inhibition of the unwinding by sterical blockage of translocation of the helicase along the polynucleotide chain

To our knowledge, there are no reports addressing the influence of compounds that modulate the structure of the DNA or RNA substrates on the procession of the unwinding reaction mediated by any of the viral NTPase/helicase. Nevertheless, a broad range of DNA or RNA binding or covalently intercalating compounds has been found and characterized as anti-cancer or immunosuppressant drugs (Lun et al., 1998; Zhu et al., 1999; Bachur et al., 1992). There is increasing evidence that DNA or RNA, together with a bound/intercalated agent is more stable and that the energy necessary for its unwinding is higher. Therefore, the one of the important targets of these compounds although indirect could be the NTPase/helicases (Lun et al., 1998; Zhu et al., 1999; Bachur et al., 1992). In addition, the inhibitory activity of the intercalating agents may result from an enhanced rigidity and deformation of the structure of the DNA or RNA substrates caused by the compounds (Lown et al., 1984).

These observations challenged us to test a range of commercially available, established DNA and RNA binding/intercalating agents as inhibitors of the NTPase and helicase activities of HCV NTPase/helicase. As demonstrated in Fig. 4 the compounds belonging to the group of anthracycline anticancer antibiotics are very effective inhibitors of the unwinding activity of the enzyme. Noteworthy, none of the examined antibiotic reduces the NTPase activity of the enzyme up to milimolar concentrations.

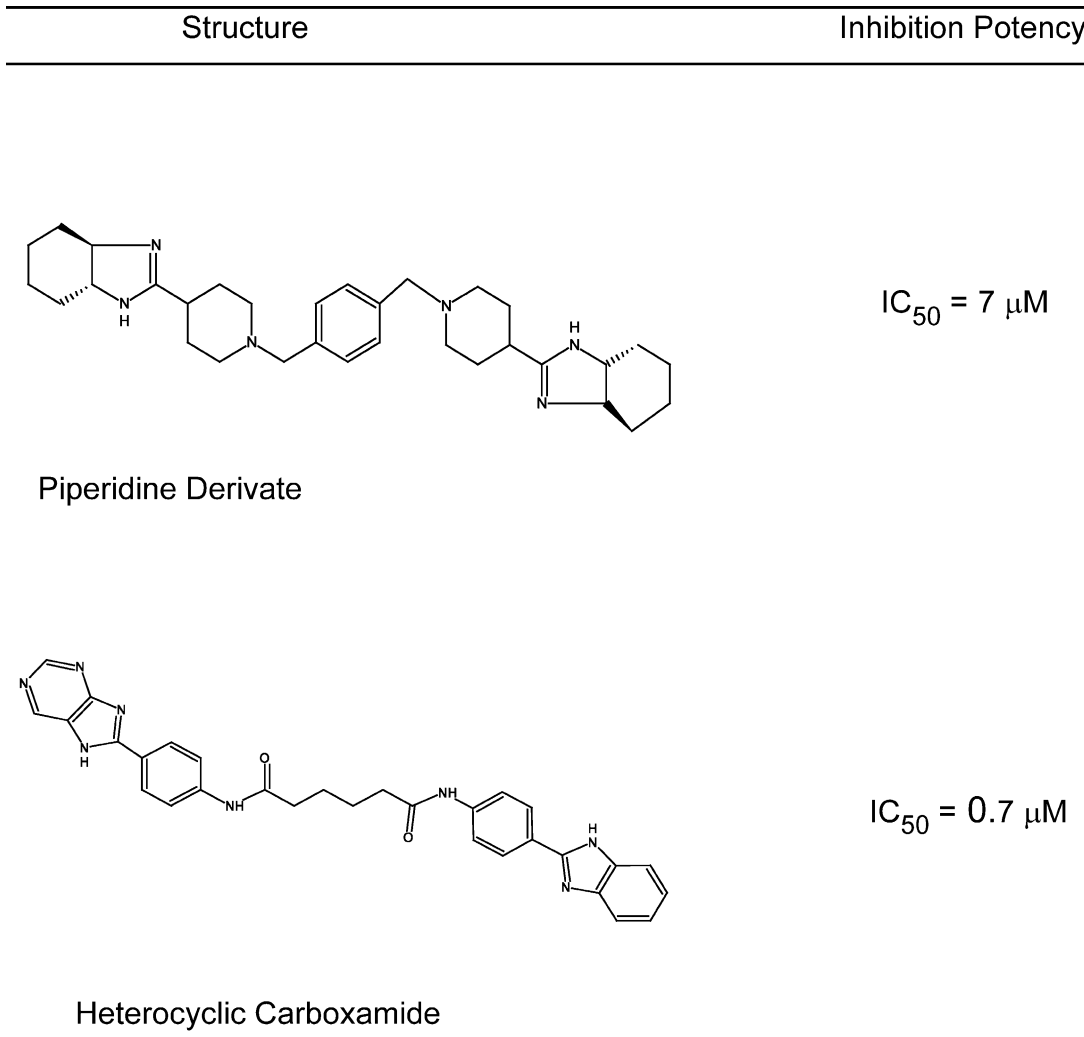


Fig. 3. Structures of inhibitors of helicase activity of the HCV NTPase/helicase and their inhibition potency.

A further DNA and RNA intercalator, the mitoxantrone that is chemically related to the antracycline antibiotics (Zhu et al., 1999; Bachur et al., 1992) inhibited also the helicase activity of the HCV NTPase/helicase (Fig. 4).

The high cytotoxicity of the compounds and their weak penetration into the cell limit their application in the cell culture or in an animal model. Nevertheless, the mode of action of the agents, at least in part, by intercalative modulation of the DNA or RNA substrates, promises a high inhibiting potential of the compounds. This might make the group of substances attractive antivirals. Further search for less toxic and more selective derivatives is necessary.

4. Other strategies

Recently, we have reported that the helicase activity of a highly purified preparation of the WNV NTPase/helicase obtained from the native source is significantly activated by N^7-chloroethylguanine and N^9-chloroethylguanine. Under standard reaction conditions, the activation reached a maximum of 850 and 220% of the control at

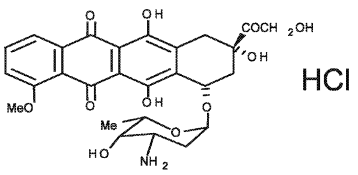
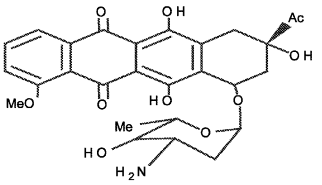
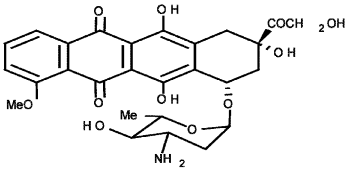
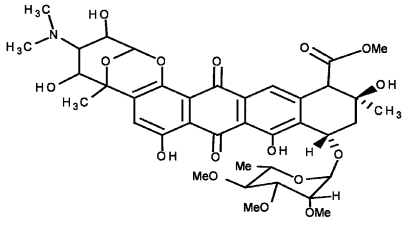
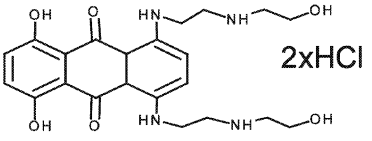
Inhibitor	Structure	Potency
Doxorubicin		IC_{50} = 5.0 μM
Daunomycin		IC_{50} = 57 μM
Epirubicin		IC_{50} = 0.75 μM
Nogalamycin		IC_{50} = 0.1 μM
Mitoxantrone		IC_{50} = 6.7 μM

Fig. 4. Structures of Doxorubicin, Daunomycin, Epirubicin, Nogalamycin and Mitoxantrone and comparison of their inhibitory potential towards helicase activity of HCV NTPase/helicase. The helicase reaction was performed under standard conditions described in the legend of Fig. 1.

concentrations of 200 and 250 μM respectively for both compounds whereas the NTPase activity of the enzyme was influenced only marginally (Borowski et al., 2001a). A similar activating effect on the unwinding activity was measured when the HCV NTPase/helicase was investigated (Fig. 5).

Our detailed investigations performed with the HCV NTPase/helicase and with its isolated domains revealed, that the chloroethylguanines do not influence the binding of ATP neither to the domain 1 nor to the entire protein. This observation agrees with the fact that the ATPase activity of the HCV enzyme remained unchanged up to the concentrations of the compounds lying in high milimolar range. The compounds act as 'competitive activators' of the helicase activity; an enhancing of the concentration of the RNA or DNA substrate leads to a corresponding increase of the unwinding activity (unpublished data).

When we tested the replication of the WNV in the cell culture as a function of increasing concentrations of N^7-chloroethylguanine and of N^9-chloroethylguanine, a strong antiviral effect was observed. The efficacy of the inhibition of the virus replication corresponds closely to the extend of the activation of the unwinding reaction induced by the compounds. Thus, at the concentrations of N^7-chloroethylguanine or N^9-chloroethylguanine at which twofold increase of the unwinding activity was measured, a comparable reduction of the viral RNA could be detected (unpublished data). Because of their low toxicity in the cell culture the compounds could be applied at high concentrations (300–500 μM). The mechanism by which the chloroethylguanines inhibit the virus replication remains unclear. One could speculate that the activation of the helicase activity leads to an overproduction of ssRNA that is a substrate for

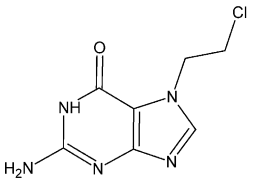
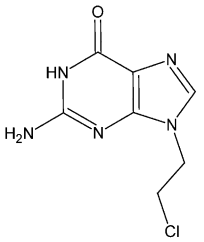
Structure	Activation Potency	
	ATPase	Helicase
 N^7-chloroethylguanine	ED_{200} > 1 mM	ED_{200} = 18 μM
 N^9-chloroethylguanine	ED_{200} > 1 mM	ED_{200} = 120 μM

Fig. 5. Structures of N^7-chloroethylguanine and N^9-chloroethylguanine and their activating effect on the ATPase and helicase activities of HCV NTPase/helicase. The ATPase and helicase assays were performed under standard conditions described in the legend of Fig. 1 in the presence of increasing amounts of N^7-chloroethylguanine or N^9-chloroethylguanine. The activation parameters were expressed as the effective dose of the compound at which 200% activation was measured (ED_{200}).

the viral polymerase, and thus down-regulates its activity by substrate-inhibition.

5. Perspectives

In view of the importance of modulation of RNA structures in diverse metabolic processes, RNA NTPase/helicases have probably a key position in the life cycle of viruses whose genomes are composed of RNA. Indeed, previously mentioned knock out experiments, in which the helicase and/or NTPase activities of flavi- or pestiviruses were switched off, demonstrated the essential role of the enzymatic activities for the virus replication (Gu et al., 2000; Matusan et al., 2001). Thus compounds reducing the activity of the enzymes could act as inhibitors of the virus replication. Our recent observations with some imidazo[4,5-D]pyridazine nucleosides (referred in Chen and Hosmane, 2001) are corroborating with this hypothesis. When 1-(2′-*O*-methyl-β-D-ribofuranosyl)imidazo-[4,5-d]pyridazine-4,7(5H,6H)-dione) was tested as inhibitor of the helicase activity of WNV NTPase/helicase, it caused a moderate inhibition of the enzyme with an IC_{50} of approximately 30 μM. A similar inhibitory potency was also observed in tissue cultures of the virus. In contrast to this, closely related compounds: 1-(β-D-ribofuranosyl)imidazo[4,5-d]pyridazine-4,7-(5H,6H)dione and 1-(2′-deoxy-α-D-ribofuranosyl)imidazo[4,5-d]-pyridazine-4,7(5H,6H)dione, that did not inhibit the helicase activity, exerted no effect on the virus replication (Borowski et al., 2002). This suggests that blocking of the viral helicase activity may be predominantly responsible for the observed antiviral activity. Since neither of the substances affected the cell proliferation, up to concentrations as high as 300 μM, the derivatives of imidazo[4,5-d]-pyridazine may represent a starting point for the development of a new class of helicase-specific antivirals.

A similar observation has been made, by using high throughput screening, two independent groups who identified the same class of aminothiazole derivatives, to inhibit the helicase activity of the HSV UL5/8/52 helicase/primase complex (Crute et al., 2002; Spector et al., 1998). The compounds inhibited HSV growth in cell culture and in an animal model (Crute et al., 2002). Although crystallization studies performed with HCV NTPase/helicase helped to determine the potential sites for inhibitor binding and the mode of their action, numerous molecular details of the mechanism of this class of enzymes remain unresolved. In particular it has to be studied, how the enzyme recognizes (or not) the nucleotide base, the mechanism by which the NTP hydrolysis is coupled to the unwinding reaction or which residues (motifs) are involved in the RNA binding. Thus, as mentioned above, a random screening of chemical libraries, combined with the designing of molecules based on the structure of the enzyme, may be the optimal way to discover effective inhibitors.

However, on the way to establish cellular and/or animal models that allow the verification of the effectiveness of the compounds in vivo with a high throughput, there are still technical hurdles to overcome. All strategies developed up to date provide only a starting point towards the resolution of the problem.

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