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Review

NTPase/helicase of *Flaviviridae*: inhibitors and inhibition of the enzyme✪

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RNA nucleoside triphosphatases (NTPase)/helicases represent a large family of proteins that are ubiquitously distributed over a wide range of organisms. The enzymes play essential role in cell development and differentiation, and some of them are involved in transcription and replication of viral single-stranded RNA genomes. The enzymatic activities of a NTPase/helicase were also detected in the carboxyl-terminal non-structural protein 3 (NS3) of members of the *Flaviviridae* family. The crucial role of the enzyme for the virus life cycle was demonstrated in knock out experiments and by using NTPase/helicase specific inhibitors. This makes the enzyme an attractive target for development of *Flaviviridae*-specific antiviral therapies. This review will sum-

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Abbreviations: ribavirin-TP, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate; IDA-TP, 4,6-diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo[4,5-e][1,3]diazepine-5′-triphosphate; ITA-TP, 5,8-dioxo-5,6,7,8-tetrahydro-4*H*-1-β-D-ribofuranosylimidazo[4,5-e][1,2,4]triazepine-5′-triphosphate; trifluoperazine dihydrochloride, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine dihydrochloride; paclitaxel, 5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one-4,10-diacetate-2-benzoate 13 ester with (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine; SPA, scintillation proximity assay; SAR, structure-activity relationship.

marize our knowledge about the function and structure of the enzyme, update the spectrum of inhibitors of the enzymatic activities of the NTPase/helicase and describe the different mechanisms by which the compounds act. Some of the compounds reviewed herein could show potential utility as antiviral agents against *Flaviviridae* viruses.

CLASSIFICATION OF *FLAVIVIRIDAE* AND GENOME STRUCTURE

The virus family was named after the jaundice occurring in the course of Yellow fever virus (YFV) infection. YFV was the first found virus of the *Flaviviridae* family, which caused this disease (Monath, 1987; Halstead, 1992). In humans infections with *Flaviviridae* viruses may cause fulminant, hemorrhagic diseases (YFV, dengue fever virus (DENV) and omsk hemorrhagic fever virus (OHFV)), viral encephalitis (japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV)) or hepatitis, formerly referred to as non-A, non-B hepatitis (hepatitis C virus (HCV)) (Monath & Heinz, 1996; Rice, 1996). Some representants of the *Flaviviridae* family can infect only animals causing a severe disease usually followed by death (bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BDV)) (Nettelton & Entrican, 1995).

Members of the *Flaviviridae* family could be classified into three genera: hepaciviruses, flaviviruses and pestiviruses (Westeway, 1987; Chambers *et al.,* 1990; Monath & Heinz, 1996). Recently an HCV-related virus was characterized, hepatitis G virus (HGV), formerly referred to as "GB-agent" (Muerhoff *et al.,* 1995). The phylogenetic classification of the virus is, however, not established until now (Leyssen *et al.*, 2000).

The members of the family *Flaviviridae* are small (40 to 50 nm), spherical, enveloped RNA viruses of similar structure. The genome of the viruses consists of one single-stranded, positive-sense RNA of the length 9100 to 11000 bases (i.e. 10862 for YFV (strain 17D), 10477 for Russian spring-summer encephalitis virus (RSSEV), approx. 9500 for HCV and 9143 to 9493 for HGV)). The RNA possesses a single open reading frame (ORF) flanked by 5′- and 3′-terminally located untranslated regions (5′-UTR and 3′-UTR, respectively) (Westeway *et al.,* 1985; Westeway, 1987; Monath & Heinz, 1996; Rice, 1996).

The replicative cycle of the viruses of the *Flaviviridae* family is similar. After binding to the target receptor (i.e. CD81 molecule for HCV (Pileri *et al.,* 1998) heparin sulfate for DENV (Chen *et al.,* 1997)) the virus penetrates the cell membrane and its plus-strand RNA is released from the nucleocapsid into the cytoplasm (Leyssen *et al.,* 2000). The released viral RNA is translated into a polyprotein consisting of approximately 3000 to 3500 amino acids (i.e. 3010 for HCV, 3411 for YFV and 3412 for RSSEV). In the course of infection the polyprotein is cleaved co- and post-translationally by both virus-encoded and host cellular proteases (signalases (Pryor *et al.,* 1998)). The NH_2-terminal region of the polyprotein is processed into three (hepaciviruses, flaviviruses, HGV) or into five (pestiviruses) structural proteins. The proteolytic processing of the COOH-terminal region of the polyprotein of hepaciviruses and of HGV results in six mature proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The polyprotein of the genus flavivirus is processed into seven proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and the polyprotein of pestiviruses into five fragments (NS2/NS3, NS4A, NS4B, NS5A and NS5B) (Westeway *et al.,* 1985; Westeway, 1987; Monath & Heinz, 1996; Rice, 1996; Meyers & Thiel, 1996; Leyssen *et al.,* 2000).

The viral plus-strand RNA serves further as a template for the synthesis of several copies of minus-strand RNA. A membrane associated replicase complex consisting of at least two viral proteins carries out this synthesis:

NS3 with its nucleoside-triphosphatase and helicase (NTPase/helicase) activities and NS5B with its RNA dependent RNA polymerase (RdRp) activity. The minus-strand RNA is transcribed into respective plus-strand RNA which in turn is assembled with the nucleocapsid (Westeway, 1987; Monath & Heinz, 1996; Rice, 1996; Meyers & Thiel, 1996; Leyssen *et al.*, 2000).

Although the NS proteins are not constituents of the virus particle, their intact function, particularly of the components of the replication complex, is essential for virus replication (Bartenschlager, 1997; Ishido *et al.,* 1998; Neddermann *et al.,* 1999; Koch & Bartenschlager, 1999; Leyssen *et al.,* 2000). In this context the NTPase and/or helicase activities of NS3 appear to be exceptionally attractive targets for termination of viral replication.

STRUCTURE AND FUNCTION OF NS3 NTPase/HELICASE

The first amino-acid sequence comparisons of DNA and RNA NTPase/helicases and other NTP consuming enzymes revealed a range of conserved motifs associated with NTP binding (Gorbalenya & Koonin, 1989; 1993; Kadare & Haenni, 1997). These include Walker motif A, which binds the terminal phosphate groups of the NTP, and Walker motif B responsible for chelation of the Mg^{2+} of the Mg-NTP complex (Walker *et al.,* 1982). Based on the different sequences of Walker motif A the NTPase/helicases are arranged in three superfamilies (SF's): SF1 that is characterized by the classic Walker motif A (G-X-X-X-X-G-K-S/T) and SF2 and SF3 that reveal variations of the domain (A-X-X-G-X-G-K-S/T) and (G-X-G-X-G-K-S) respectively (Kadare & Haenni, 1997; Lüking *et al.*, 1998; Fuller-Pace, 1994). In SF1 are classified alpha virus-like (nsP2-like) proteins; SF2 includes polypeptides similar to the NS3 protein (NS3-like proteins) encoded by potyviruses and the members of *Flaviviridae* family; SF3 includes picornavirus-like (2C-like) proteins (Gorbalenya *et al.,* 1989; Lain *et al.,* 1989). The SF2 NTPase/helicases are further divided, according to the sequence surrounding the conserved D-E residues (Walker motif B) in three different subgroups of proteins. The first is formed by the classic D-E-A-D box proteins, and the other are named D-E-A-H and D-E-X-H, based on their deviating Walker motif B sequences (Fuller-Pace, 1994; Kadare & Haenni, 1997; Lüking *et al.,* 1998). The latter two subgroups are more heterogeneous than the D-E-A-D box proteins with respect to their sequence and biochemical function (Wassarman & Steitz, 1991; Lüking *et al.*, 1998). The NTPase/helicases associated with the NS3 protein of the hepaciviruses and HGV possess D-E-C-H and those of pestiviruses the D-E-Y-H motif and are therefore members of the D-E-X-H box subgroup (Miller & Purcell, 1990; Gu *et al.,* 2000). On the other hand the enzymes of the flaviviruses possess the D-E-A-H motif, thus belonging to the D-E-A-H box subgroup. The membership in the D-E-X-H or D-E-A-H box subgroups has no clear predictive value regarding the key properties of the enzyme. The insights into the dependency between the structure and function have come primarily from X-ray crystallography data of a representative family member, the HCV NTPase/helicase. The structure of the HCV enzyme in the absence of a nucleic acid has been solved to 2.1 Å (Yao *et al.,* 1997) and 2.3 Å resolution (Cho *et al.*, 1998) (Fig. 1). Further, the structure of the NTPase/helicase with a bound oligonucleotide has been solved to 2.2 Å resolution (Kim *et al.*, 1998). The protein consists of three equally sized structural domains separated by a series of clefts. Domains 1 and 3 share with each other a more extensive interface than either of them shares with domain 2. As consequence, the clefts between domains 1 and 2 and domains 2 and 3 are the largest. Domain 2 is flexibly linked to the other two and may undergo a rigid-body movement relative to domains 1 and 3. Domains 1 and 2 contain all of the conserved

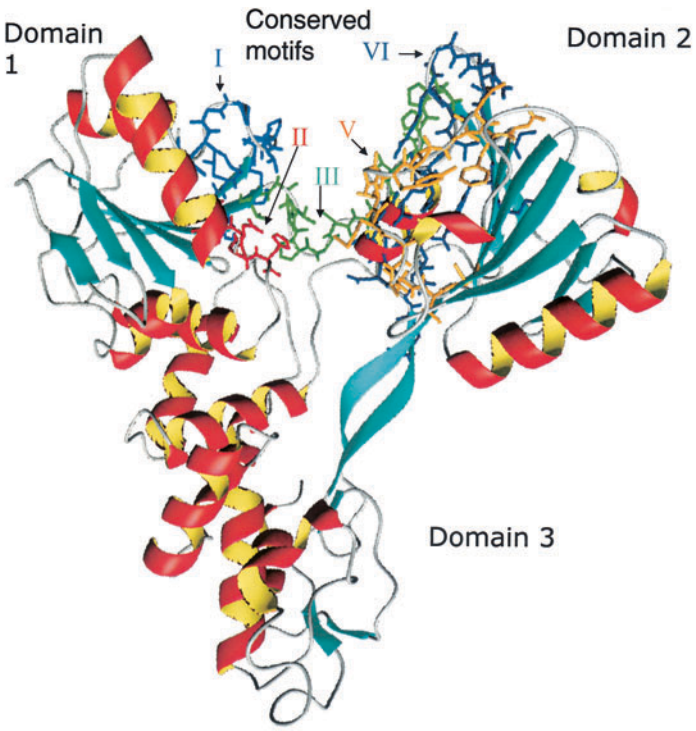

Figure 1. Ribbon diagram of the HCV RNA helicase domains (PDB accession number 1HEI).

helicase sequence motifs and, moreover, display structural similarities to the coresponding domains of PcrA helicase from *Bacillus stearothermophilus* and to Rep DNA helicase from *Escherichia coli* which are members of SF1 (Subramanya *et al.*, 1996; Korolev *et al.*, 1997). The structural comparisons made it possible to localize the mentioned Walker motifs A and B (motifs I and II of the SF2 helicases) on the surface of domain 1. In the absence of substrate residues of the Walker motifs bind to each other and additionally to the residues of the conserved T-A-T sequence (motif III). Motif III is a part of the flexible switch region, so called "hinge region", that connects the first and second domains of the enzyme (Yao *et al.*, 1997; Kadare & Haenni, 1997). As demonstrated for the DEXX DNA helicase, the conformational changes of the molecule accompanying the NTP hydrolysis are transmitted by this switch sequence (Subramanya *et al.*, 1996).

The role of the highly conserved arginine-rich motif (G-R-X-G-R-X-G-R; motif VI) localized on the surface of domain 2 is controversial. Yao *et al.* (1997) and Cho *et al.* (1998) proposed on the basis of their X-ray crystallographic data that motif VI is required for RNA binding and that a further cluster of positively charged amino acids may be involved in the interaction with the RNA substrate. This appears to be in accordance with the biochemical data obtained with the related HGV NTPase/helicase (Gwack *et al.*, 1999) and with our own data obtained with isolated domain 1 of HCV NTPase/helicase (Borowski *et al.*, 1999b). On the other hand, the structure of the HCV NTPase/helicase complexed with $(dU)_8$, proposed by Kim (Kim *et al.*, 1998), suggests rather that the arginine residues of these motifs are directly involved in the ATP binding.

Based on the structures of the enzymes and biochemical analyses, two alternative mechanisms of the unwinding reaction have been proposed. According to the first one, the so called "passive" mechanism, the NTPase/helicase molecule binds single stranded regions of the substrate and does not actively participate in the separation of the strands forming the RNA or DNA duplexes (Matson & Kaiser-Rogers, 1990; Yao *et al.*, 1997;

Lüking *et al.,* 1998). 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 & Lehman, 1993), T4gp32 (Chase & Wiliams, 1986) and the protein *vasa* from *Drosophila melanogaster* (Hay *et al.,* 1988) are capable of unwinding duplex DNA structures in an NTP-independent manner. Moreover, in a recent study Porter and Preugschat identified the strand separating activity of the HCV NTPase/helicase, that occurs in the absence of ATP, as being purely stoichiometric and not kinetic and confirmed its passive nature (Porter & Preugschat, 2000). Interestingly, our own investigations, performed on a group of closely related NTPase/helicases of flaviviruses demonstrated that the stoichiometry of the NTP-independent unwinding reaction (enzyme molecules : nucleotide bases of the substrate) vary significantly between the species of the genus (P. Borowski, A. Niebuhr, H. Schmitz, unpublished data).

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. According to this model the NTP-triggered conformational changes facilitate the binding of RNA or DNA substrate to the alternative sites (Yao *et al.,* 1997). Thus, this modus of the unwinding requires either the presence of multiple substrate binding sites on a single polypeptide, or alternatively, as postulated for the majority of NTPase/helicases, the multiple substrate binding sites could result from the polymerization of the enzyme. Indeed, an oligomeric status of the HCV NTPase/helicase was demonstrated by Levin and Patel (1999) and such of the WNV enzyme was shown recently by us (Borowski *et al.,* 2001a).

Although X-ray crystallography data supply some insights into the mechanism of the unwinding reaction, the basic information regarding the biochemical properties of NTPase/helicase has come from kinetic studies and mutational analyses.

The minimal amino-acid sequence of NS3 displaying helicase activity is approximately 400 residues in length and, for HCV, lies between residues 1209 and 1608 of its polyprotein (Kim *et al.,* 1997). Nevertheless, most of the biochemical studies are performed with an enzyme consisting of the entire COOH-terminally localized NTPase/helicase domain of NS3 or with the full-length NS3 protein. However, studies comparing the biochemical properties of the full-length and truncated NS3 do not supply uniform results. Morgenstern *et al.* (1997) demonstrated significant differences between the proteins regarding the pH value and poly(U) concentration required for optimum ATPase activity of the HCV NTPase/helicase. Further, Kuo (Kuo *et al.,* 1996) have shown that removal of the NH_2-terminal 148 amino acids enhanced the NTPase activity of JEV NTPase/helicase. On the other hand, a comparative study with recombinant full-length NS3 and its isolated NH_2- and COOH-terminal domains, performed by Gallinari *et al.* (1998), did not revealed significant differences in the enzymatic activities analyzed in independent *in vitro* assays. In this context it appears to be more appropriate 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.

Up to date the NTPase activity could be demonstrated for a wide range of the *Flaviviridae* NS3 proteins. The common property of the enzymes, thus far tested, is their low selectivity towards the nucleobase of the NTP. The enzymes hydrolyzed all NTP's, dNTP's and even acyclovir triphosphate, acyclic ATP and tri-polyphosphate, which lack the ribosyl functionality of natural nucleosides (Tamura *et al.*, 1993; Warrener & Collet, 1995; Preugschat *et al.,* 1996) This property was documented for a

wide spectrum of unrelated NTPase/helicases, like the DNA helicases from SV40 (Scheffner *et al.,* 1989), bacteriophage T7 (Notarnicola *et al.,* 1995) and *E. coli* (Moore & Lohman, 1994). The hydrolysis of the NTP's is stimulated by ribohomopolymers and 2′-deoxyribohomopolymers. The response of the NTPase/helicases to the polymers varies among the species of the *Flaviviridae* enzymes. For example 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), whereas the order of efficiency for the NTPase/helicase from YFV was the following: poly(A)>poly(C)> poly(U)>>poly(dU)>poly(I)>poly(dT)>poly(dI) >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 extent of the activating effect corresponds closely to the rate of binding of the polynucleotides to the protein (Gwack *et al.,* 1996; Bartenschlager 1997). Both, binding of the polynucleotide and polynucleotide mediated NTPase activation required a minimum length of the nucleic acid (15 nucleotides) (Preugschat *et al.,* 1996). A similar minimum length was also reported for *E. coli* Rep helicase and eukaryotic nuclear DNA helicase (Chao & Lohman, 1991; Zhang *et al.,* 1995). It has been demonstrated that this length of the polynucleotide may contribute to the binding energy 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 hepatitis C virus protease/helicase NS3 with the 3′-terminal sequences corresponding to the viral 3′-UTR was demonstrated (Banerjee & Dasgupta, 2001). The mechanistic significance of the activation mediated by the polynucleotide for virus replication remains unclear.

Recent genetic "knock out" experiments support the essentiality of the NS3 associated helicase activity for virus replication (Gu *et al.,* 2000; Matusan *et al.,* 2001). In the case of the members of *Flaviviridae* family the negative-stranded RNA must be synthesized using the parental positive-stranded RNA as a template. The resulting negative-stranded RNA is then used as a template for the synthesis of the positive-stranded progeny RNA, that is than assembled into viral particles. Since the negative and positive oriented RNA strands are complementary, the NS3 associated helicase activity appears to be necessary for strand separation.

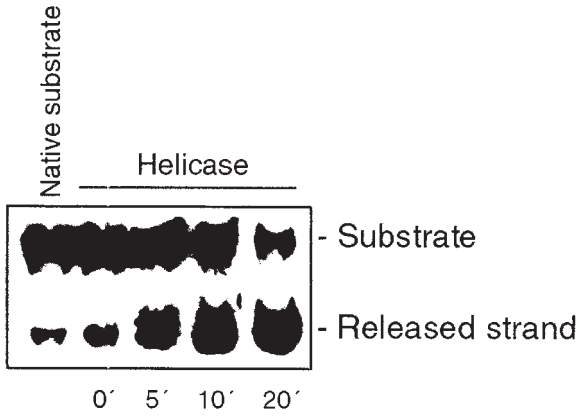

Figure 2. Time course of the unwinding reaction mediated by HCV NTPase/helicase.

The helicase assays were performed with 10 pmol of the enzyme and the unwinding reaction was terminated at the times indicated in the figure. The samples were separated on a 15% polyacrylamide gel containing 0.1% SDS for 14 h (reprinted from Borowski *et al.,* (2001) *Acta Biochim Polon.*; **48**: 739 with permission).

Nevertheless, the helicase activity could be experimentally documented only in a few members of the *Flaviviridae* family (Fig. 2) (Suzich *et al.,* 1993; Gwack *et al.,* 1999; Li *et al.,* 1999; Gu *et al.,* 2000; Utama *et al.,* 2000; Borowski *et al.,* 2001a; 2001b). All the enzymes exhibit a 3′- to 5′-directionality with respect to the template strand (Kadare & Haenni, 1997) and, in contrast to the majority of the NTPase/helicases described, they are 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 solution of the structure of several NTPase/helicases it remains unclear how the NTP binding and hydrolysis are coupled to the unwinding of double-stranded substrate. The data suggest that the activities of the enzyme are not necessarily coupled: (i) The enzymes function as polynucleotide-stimulated and not as polynucleotide-dependent NTPases and the stimulation of ATPase activity of HCV or WNV NTPase/helicases by single-stranded nucleic acids is not related to the procession of the helicase along the RNA or DNA substrates (Tai *et al.*, 1996; Hesson *et al.*, 2000; Borowski *et al.*, 2001a). (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 WNV NTPase/helicase without affecting the helicase activity (Borowski *et al.*, 2001a). On the other hand, some chloroethylguanine derivatives stimulated the helicase activity of the enzyme with no apparent effect on the ATPase activity of the enzyme (Borowski *et al.*, 2001a). (iii) The BVDV, HCV and WNV NTPase/helicases have different optimum conditions, such as pH-value, concentrations of salt or detergent, for the NTPase and helicase reactions (Preugschat *et al.*, 1996; Morgenstern *et al.*, 1997; Gallinari *et al.*, 1998; Hesson *et al.*, 2000; Borowski *et al.*, 2001a).

These observations indicate that the inhibitors designed against the helicase should be evaluated by the helicase assay rather, than by testing of the NTPase activity or of RNA binding.

HELICASE ASSAY

The assay for the detection or measurement of helicase activity is based on a substrate (DNA/DNA or RNA/RNA homo-duplexes and RNA/DNA hetero-duplex) that is susceptible to unwinding. One of the strands, with the 3′- to 5′-directionality, is referred to as template, and the other with the reverse orientation is referred to as released strain (Tai *et al.*, 1996). The substrate is exposed to the enzyme in the presence of divalent ions (Mg^{2+}, Mn^{2+}) and NTP. The single-stranded product of the reaction is separated from the substrate and quantified by a variety of methods (see below). Interestingly, the enzyme requires a short single-stranded structure of the substrate (on the template strand) to initiate the reaction (Tai *et al.*, 1996; Gallinari *et al.*, 1998).

For screening of the inhibitors of the helicase activity, a range of assays can be used: (i) The increasing signal assay developed by Kyono *et al.* (1998) using the scintillation proximity assay (SPA) system developed by Amersham. In this system, the unwinding of radiolabeled [^{3}H]DNA/RNA duplex by the NTPase/helicase is measured by hybridization of the released ss[^{3}H]DNA to a biotinylated complementary oligonucleotide. This is then bound to streptavidin-coated SPA beads, which results in a detectable scintillation signal. (ii) The assay developed by Kwong & Risano (1998), uses RNA/DNA hetero-duplex as a substrate. The radioactively labeled released strain is hybridized to a captured oligonucleotide, adsorbed to the wells coated with streptavidin (FlashPlate PLUS; NEN Life Science Products). The bound radioactivity is measured by scintillation counting. (iii) In the assay described by Hsu *et al.* (1998) an RNA/RNA homo-duplex is used as substrate. One of the strands (template) is coupled with biotin and immobilized on a streptavidin-coated solid phase. The second (released strand) is labeled with digoxigenin. After terminating the unwinding reaction and removing the separated strand the remaining du-

plex-bound digoxigenin-labeled strand is quantified with the use of tan ELISA assay employing anti-digoxigenin antibodies labeled with horseradish peroxidase.

In our studies we used as a substrate partly complementary RNA/RNA oligonucleotides and their deoxynucleotide versions of sequences reported previously (Gallinari *et al.,* 1998). In this assay the 3′-terminus of the released strand is labeled to a high specific activity with ^{32}P or ^{33}P. The product of the unwinding reaction is separated by Tris/borate/EDTA/polyacrylamide gel electrophoresis and quantified by scintillation counting of the respective parts of the gel or alternatively by scanning of autoradiography films (Borowski *et al.,* 2001a). The benefit of the method is the better quantifiability of the results due to the high reproducibility of the labeling of the released strand and the annealing procedure (Gallinari *et al.,* 1998). By using this method we were able to test a broad range of substances for their inhibitory potential towards the helicase activity.

zymes could be inhibited independently from each other. Indeed, the majority of the compounds discussed further inhibit selectively only one of the activities.

Inhibition of NTPase activity of *Flaviviridae* NTPase/helicases

According to the active mechanism of unwinding, NTP hydrolysis supplies the energy necessary for the strand displacing reaction. Thus, it could be expected that reduction of the accessibility of the NTP-binding site for NTP may lead to a decreased NTPase hydrolysis and, therefore, to a corresponding reduction of the unwinding rate. Consequently, compounds based on the structure of nucleoside-5′-triphosphates seemed to be an effective tool for inhibition of the enzyme.

However, it was found that analogues of nucleoside-5′-triphosphates, with a modified base such as ribavirin-TP (Borowski *et al.*, 2000), IDA-TP or ITA-TP (Zhang *et al.*, 2002), (Fig. 3.) when tested at ATP concentrations

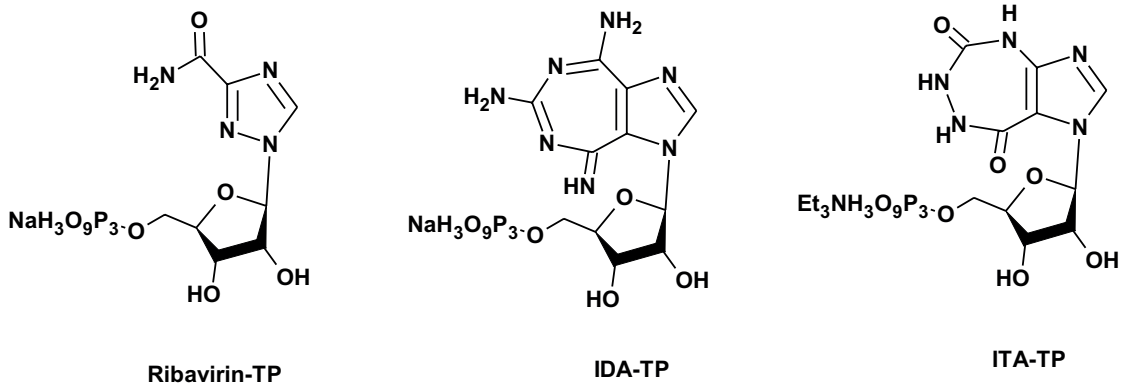

Figure 3. Structures of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate (ribavirin-TP), 4,6-diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo[4,5-e][1,3]diazepine-5′-triphosphate (IDA-TP) and 5,8-dioxo-5,6,7,8-tetrahydro-4*H*-1-β-D-ribofuranosylimidazo[4,5-e][1,2,4]triazepine-5′-triphosphate (ITA-TP).

INHIBITION OF THE ENZYMATIC ACTIVITIES OF THE *FLAVIVIRIDAE* NTPase/HELICASES

The mentioned dissociation of the NTPase and helicase activities of the NTPase/helicases suggests that both activities of the enzymes could be inhibited independently from each other. Indeed, the majority of the compounds discussed further inhibit selectively only one of the activities.

equal to the K_m values determined for the ATPase reaction of each of the viral enzymes, inhibit the ATPase reaction mediated by HCV, JEV, DENV and WNV NTPase/helicases only weakly (Borowski *et al.,* 2000; 2001a; and unpublished data; Zhang *et al.,* 2002) (Table 1). Moreover, at concentrations of ribavirin-TP,

IDA-TP or ITA-TP higher than 500 μM and >10 $\times$ K_m for ATP an activation of the ATPase activity was observed. Higher concentrations of ATP in the reaction mixture caused further dramatic increase of the hydrolytic activity of the NTPase/helicases, for example in the presence of 1 mM ATP (corresponding to 100 $\times$ K_m value) and 0.5 mM ITA-TP an increase of 1000–1100% of the hydrolytic activity of WNV NTPase/helicase was measured. On the other hand, reduction of the ATP concentration to values that are below 1/10th of K_m of the enzymes lead to an inhibition of the ATPase activity by ribavirin-TP, IDA-TP and ITA-TP. Successive lowering of ATP concentration down to values corresponding to 1×10^{-5} of K_m of each enzyme increases strongly the inhibitory effect of the compounds (Fig. 4). At very low ATP concentrations, all the nucleoside-5′-triphosphate analogues act as classical competitive inhibitors of the ATPase activity of the HCV, JEV, DENV and WNV enzymes (Borowski *et al.*, 2000; 2001a; and unpublished data; Zhang *et al.*, 2002).

Because of the mentioned lack of selectivity of the viral NTPase/helicases for the hydrolysis of NTP's, it could not be ruled out that the nucleoside-5′-triphosphate analogues tested undergo partial hydrolysis to less active derivatives. This question was addressed by using the slowly hydrolysing adenosine-5′-γ-thiotriphosphate (ATP-γ-S) and the non-hydrolysable ATP analogues 5′-adenylimidodiphosphate (AMP-PNP) and β,γ-methyleneadenosine-5′-triphosphate (AMP-PCP). All the compounds were active towards the ATPase activity of the viral NTPase/helicases; either as an activator (at higher ATP concentrations) or as an inhibitor (at lowered ATP concentrations). The ATPase modulating effects mediated by ATP-γ-S, AMP-PNP or AMP-PCP were found, however, to be considerably weaker when compared with those exerted by ribavirin-TP, IDA-TP or ITA-TP (Table 1).

Table 1. Inhibition and activation of the ATPase activity of the HCV NTPase/helicase by analogues of nucleoside-5′-triphosphate in dependency on ATP concentration

	IC_{50} (μM)		ED_{200} (μM)
	$10^{-5} \times K_m$	K_m	$10^{2} \times K_m$
Ribavirin-TP	2.5	220	350
IDA-TP	0.55	170	85.0
ITA-TP	1.5	335	110
ATP-γ-S	22.0	> 500	410
AMP-PNP	37.3	460	490
AMP-PCP	54.2	320	> 500
FSBA	> 500.0	> 500	> 500

The ATPase assay was performed at indicated ATP concentrations ($10^{-5} \times K_m$, K_m, $10^{2} \times K_m$) (Borowski *et al.*, 2000; 2001a). The inhibition was expressed as the concentration of the compound at which the half-maximal inhibition was observed (IC_{50}). The term ED_{200} reflects the effective dose of compound at which 200% activity was measured.

According to the data, it appears that unaltered conformation of the terminal phosphate group is critical for theirs recognition by the Walker motifs of the NTPase/helicases tested. In corroboration of this hypothesis are our inhibition studies with a further non-hydrolysable ATP analogue, 5′-*O*-(4-fluorosulfonylbenzoyl) adenosine (FSBA), in which the phosphate groups are substituted by the 4-fluorosulfonylbenzoyl moiety. The compound was completely devoid of inhibitory activity towards the ATPase activity of the enzymes. Noteworthy is that, in the absence of

ATP, FSBA binds covalently to the NTP-binding site of the NTPase/helicases and blocks it (Borowski *et al.,* 1999b). As a consequence, significant reduction of the hydrolytic and unwinding activities of the enzymes was observed (Borowski *et al.,* 2001a).

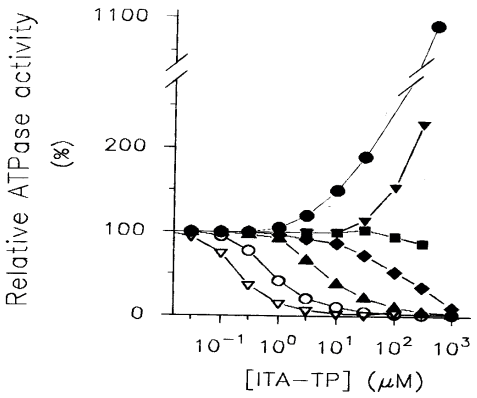

Figure 4. Modulation of the ATPase activity of the WNV NTPase/helicase by 5,8-dioxo-5,6,7,8-tetrahydro-4*H*-1-β-D-ribofuranosylimidazo[4,5-e][1,2,4]triazepine-5′-triphosphate (ITA-TP) with variations in ATP concentration. The ATPase activity was measured as function of increasing concentrations of ITA-TP.

The demonstrated assays were performed at ATP concentrations equal to (●) 100 K_m; (▼) 10 K_m; (■) 1 K_m; (◆) 0.1 K_m; (▲) 0.01 K_m; (○) 0.001 K_m; (▽) 0.0001 K_m of ATPase of WNV enzyme (9.5 μM).

Surprisingly, the nucleoside-5′-triphosphate analogues tested were not capable of significantly reducing the unwinding activity of HCV, WNV, JEV or DENV NTPase/helicase. Whereas ribavirin-TP modestly inhibited the helicase activity of the WNV enzyme (IC_{50} = 120 μM (Borowski *et al.,* 2001a)) and of the HCV enzyme (only under selected reaction conditions (IC_{50} = 30 μM; Borowski *et al.,* 2001b), it was devoid of any activity towards the JEV and DENV enzymes (unpublished data). Similarly, neither of the nucleoside-5′-triphosphates influenced the helicase activity of the viral enzymes investigated up to concentrations as high as 500 μM. Interestingly, kinetic analyses and binding studies revealed that the reduction of the helicase activity mediated by ribavirin-TP did not result from the blockade of the NTP-binding site of the enzyme (Borowski *et al.,* 2001a).

In the light of these observations the results of inhibitory studies obtained with paclitaxel, were not surprising. This compound, structurally not related to NTP, is known to interact with 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 in the case of the HCV NTPase/helicase, paclitaxel (Fig. 5A) was able to block the NTP-binding site and to inhibit the ATPase activity of the enzyme with a similar efficacy (the respective IC_{50} values were 22 μM and 17 μM) in a competitive manner (Borowski *et al.,* 1999b). Nevertheless, when tested as an inhibitor of the helicase activity of the enzyme, paclitaxel was not capable of inhibiting it, up to the concentration of 1 mM (unpublished data). It should be mentioned that paclitaxel does not influence the ATPase nor helicase activities of the WNV, JEV and DENV enzymes.

A similar observation was made when we testedtrifluoperazine dihydrochloride, as inhibitor of the NTPase activity of the HCV enzyme (Fig. 5B) . The compound, a calmodulin antagonist (Ganapathi *et al.,* 1991), is also chemically not related to nucleoside-5′-triphosphate. It was capable of inhibiting NTP binding and the NTPase activity of HCV NTPase/helicase, but in contrast to paclitaxel by a non-competitive mechanism. The inhibition occurred with a similar efficacy: 98 μM and 105 μM, respectively, for half-maximal inhibition of ATP binding and the ATPase activity (Borowski *et al.,* 1999b). Interestingly, trifluoperazine inhibits the helicase activity, however, at significantly higher concentrations (IC_{50} = 600–700 μM). Whether the helicase activity of the HCV NTPase/helicase was inhibited as a consequence of the blockade of the NTP-binding site of the enzyme is unknown. In the case of WNV NTPase/helicase trifluoperazine inhibits poorly the

helicase activity (IC_{50} = 1000–1100 μM) but does not influence its ATPase activity.

Inhibition of helicase activity of *Flaviviridae* NTPase/helicase

There is a range of mechanisms conceivable, by which the unwinding activity of NTPase/helicases could be inhibited. Tai has demonstrated previously that polynucleotides increasing the NTPase activity of HCV NTPase/helicase inhibited its unwinding activity. This inhibiting effect results from the competition of the polynucleotides with the RNA or DNA substrates for the nucleic acid binding site(s) (Tai *et al.,* 1996). Several attempts to develop small-molecular inhibitors of the helicase activity of HCV NTPase/helicase acting at the level of the nucleic acid binding site have been described. Two series of chemically related compounds, reported hitherto only as patents by ViroPharma (Diana & Bailey, 1996; Diana *et al.,* 1998), are composed of two benzimidazoles, or aminophenylbenzimidazoles attached to symmetrical linkers of variable lengths. These were reported to exhibit IC_{50} values for inhibition of the HCV helicase in the low micromolar range, subsequently confirmed and extended by an SAR study reported by Phoon (Phoon *et al.,* 2001).

Our previous studies demonstrated that the lysine-rich histone H1 and the core histones H2B and H4 form stable complexes with HCV NTPase/helicase (Borowski *et al.,* 1996). This protein–protein interaction leads to changing of the conformation of the histone molecules. As a consequence an alteration occurs of their properties as substrates for some serine/threonine protein kinases (Borowski *et al.,* 1999a) and reduction of their DNA-binding capacity (Borowski *et al.,* 1999c). On the other hand, the binding of the histones resulted in strong inhibition of the unwinding activity of the HCV NTPase/helicase with IC_{50} in the nanomolar range. The NTPase/helicase binds histones by a short stretch of amino acids located within the "hinge region" connecting domains 1 and 2 (see above). Thus, one could speculate that the bound histone molecule might affect the mobility of domain 2 and, therefore, inhibit the "march" of the NTPase/helicase along double-stranded RNA or DNA. The currently running modelling studies should help to find out structurally similar small-molecular compounds that could mimic the action of histones.

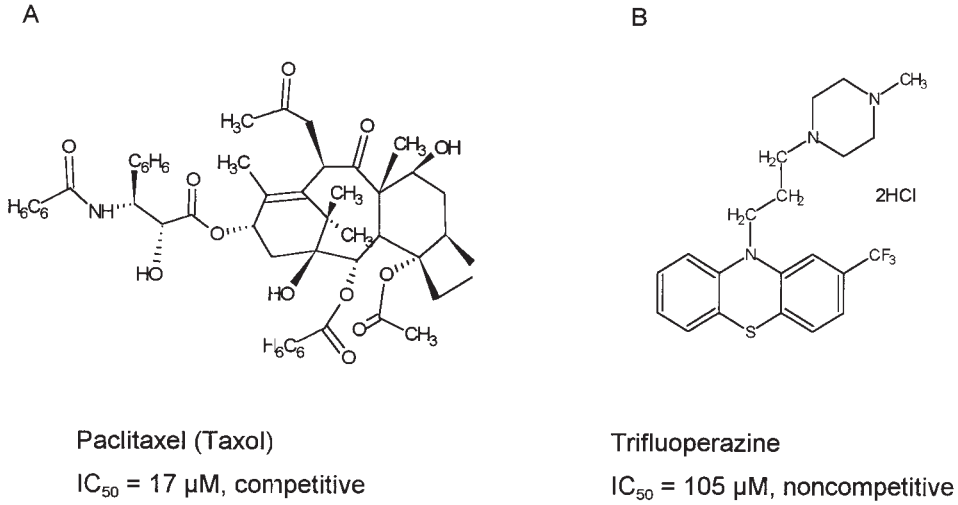

Figure 5. Structure of inhibitors of HCV helicase: paclitaxel (potent inhibitor) and trifluoperazine (weak inhibitor).

To our knowledge there are no reports addressing the influence of compounds that modulate the structure of the DNA or RNA substrate on the unwinding reaction mediated by any of the viral NTPase/helicases. This challenged us to test a range of established, commercially available DNA and RNA binding/intercalating agents as inhibitors of the NTPase and helicase activities of selected NTPase/helicases of the members of the *Flaviviridae* family. As demonstrated in Table 2, anthracycline antibiotics and mitoxantrone (Fig. 6) are very effective inhibitors of the helicase activity of the enzymes. Surprisingly, the closely related enzymes displayed significant differences in their response to the action of these compounds. Of particular interest are the parameters of the inhibition that is mediated by nogalamycin. This compound inhibited the helicase activity of the HCV enzyme with an IC_{50} = 0.1 μM, whereas the unwinding activity of the WNV and DENV enzymes were reduced only marginally. Worthy of note is that, neither of the antibiotics examined reduced the NTPase activity of the enzymes up to milimolar concentrations.

Modulators of the NTPase and/or helicase activities

Recently, we have reported that the unwinding activity of WNV NTPase/helicase is significantly activated by *N*(7)-chloroethylguanine and *N*(9)-chloroethylguanine (850% and 220% of the control at concentrations of 200 μM and 250 μM, respectively). This effect was not associated with enhanced consumption of ATP. The ATPase activity of the enzyme remained unchanged up to the concentrations of the compounds in the high milimolar range. On the other hand, the chemically related O^6-benzyl-*N*(7)-chloroethylguanine activated the ATPase activity of the NTPase/helicase without affecting its unwinding activity (Borowski *et al.,* 2001a). Similar modulating effects were observed with the further investigated HCV and JEV NTPase/helicases (unpublished data). The mechanism of the modulating effect remains unclear. The well-documented nucleotide binding studies of Porter

Table 2. Comparison of inhibitory potential of anthracyclines towards helicase activity of HCV, WNV, JEV, and DENV NTPase/helicases

	HCV IC_{50} (μM)	WNV IC_{50} (μM)	JEV IC_{50} (μM)	DENV IC_{50} (μM)
Mitoxantrone	6.7	19	48	96
Doxorubicin	5	26	17	29
Daunomycin	57	120	100	150
Nogalamycin	0.1	>650	3.8	>650

The helicase reaction was performed under standard conditions described previously (Borowski *et al.,* 2001a) in the presence of increasing amounts of the compounds. The inhibition parameter was expressed as the concentration of the compound at which the half-maximal inhibition was observed (IC_{50}).

Although the compounds in question are widely used in the clinic as immunosuppressant drugs, their cytotoxicity and weak penetration in to the cell limit their application as potential antivirals. On the other hand, the high inhibiting potential and selectivity of the compounds may make them attractive antiviral drugs. Nevertheless, a search for less toxic derivatives is necessary.

(1998), together with our kinetic data (Borowski *et al.,* 2000) suggest strongly the existence of a second nucleotide binding site within the NTPase/helicase of *Flaviviridae*. One could speculate that the second binding site that could be occupied by a nucleotide, nucleoside and even nucleotide base, probably

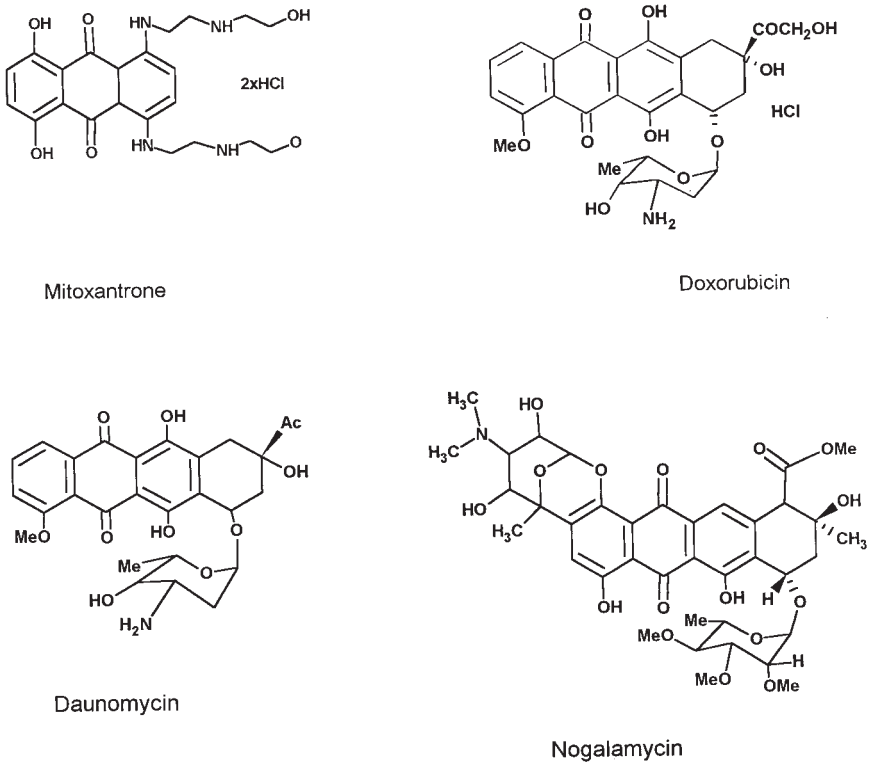

Figure 6. Structures of anthracyclines — mitoxantrone, doxorubicin, daunomycin and nogalamycin.

fulfils a regulatory function with respect to the NTPase and/or helicase activities of the enzyme. In agreement with the hypothesis are our ATP-binding studies performed with the isolated NTP-binding domain (domain 1) of the HCV NTPase/helicase demonstrating that the investigated chloroethylguanine derivatives do not influence ATP binding to the polypeptide.

CONCLUDING REMARKS

In view of the importance of modulation of RNA structures in diverse metabolic processes, RNA NTPase/helicases are probably of key importance in the life cycle of viruses whose genomes are composed of RNA. Thus, the compounds that reduce or modulate the activity of the enzymes could act as inhibitors of virus replication. Corroborating this hypothesis are the results of our *in vitro* and *in vivo* studies. An imidazo[4,5-d]pyridazine nucleoside analogue, 1-(2′-*O*-methyl-β-D-ribofuranosyl)imidazo[4,5-d]pyridazine-4,7(5*H*,6*H*)-dione, that caused an inhibition of the helicase activity of WNV NTPase/helicase (IC_{50} = 30 μM) displayed a similar inhibitory potency with respect to WNV replication in tissue cultures (Borowski *et al.,* 2002; and unpublished results). Paradoxically, the activators of the helicase activity *N*(7)-chloroethylguanine or *N*(9)-chloroethylguanine, when applied in cell culture also caused a strong inhibition of WNV replication. The mechanism of the antiviral effect remains, however, to be elucidated.

This observation is not without precedence. Recently, using high throughput screening, two independent groups identified the same class of aminothiazole derivatives, which inhibited the helicase activity of the herpes simplex virus (HSV) UL5/8/52 helicase/primase complex (Spector *et al.*, 1998; Crute *et al.*, 2002). 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, numer-

ous molecular details of the mechanism of this class of enzymes, particularly how the enzyme recognizes (or not) the nucleotide base, the mechanism by which NTP hydrolysis is coupled to the unwinding reaction or which residues (motifs) are involved in RNA binding, remain unsolved.

REFERENCES

Banerjee R, Dasgupta A. (2001) Specific interaction of hepatitis C virus protease/helicase NS3 with the 3′-terminal sequences of viral positive- and negative-strand RNA. *J Virol.*; **75:** 1708–21.

Bartenschlager R. (1997) Candidate targets for hepatitis C virus-specific antiviral therapy. *Intervirology.*; **40:** 378–93.

Boehmer PE, Lehman IR. (1993) Herpes simplex virus type 1 ICP8: helix-destabilizing properties. *J Virol.*; **67:** 711–5.

Borowski P, Heiland M, Oehlmann K, Becker B, Kornetzky L, Feucht H-H, Laufs R. (1996) Non-structural protein 3 of hepatitis C virus inhibits phosphorylation mediated by cAMP-dependent protein kinase. *Eur J Biochem.*; **237:** 611–8.

Borowski P, Heiland M, Feucht HH, Laufs R. (1999a) Characterisation of non-structural protein 3 of hepatitis C virus as modulator of protein phosphorylation mediated by PKA and PKC: evidences for action on the level of substrate and enzyme. *Arch Virol.*; **144:** 687–701.

Borowski P, Kühl R, Mueller O, Hwang L-H, Schulze zur Wiesch J, Schmitz H. (1999b) Biochemical properties of a minimal functional domain with ATP-binding activity of the NTPase/helicase of hepatitis C virus. *Eur J Biochem.*; **266:** 715–23.

Borowski P, Kühl R, Laufs R, Schulze zur Wiesch J, Heiland M. (1999c) Identification and characterization of a histone binding site of the non-structural protein 3 of hepatitis C virus. *J Clin Virol.*; **13:** 61–9.

Borowski P, Mueller O, Niebuhr A, Kalitzky M, Hwang L-H, Schmitz H, Siwecka MA, Kulikowski T. (2000) ATP-binding domain of NTPase/helicase as a target for hepatitis C antiviral therapy. *Acta Biochim Polon.*; **47:** 173–80.

Borowski P, Niebuhr A, Mueller O, Bretner M, Felczak K, Kulikowski T, Schmitz H. (2001a) Purification and characterization of West Nile virus NTPase/helicase, evidence for dissociation of the NTPase and helicase activities of the enzyme. *J Virol.*; **75:** 3220–9.

Borowski P, Lang M, Niebuhr A, Haag A, Schmitz H, Schulze zur Wiesch J, Choe J, Siwecka MA, Kulikowski T. (2001b) Inhibition of the helicase activity of HCV NTPase/helicase by 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-tiphosphate (ribavirin-TP). *Acta Biochim Polon.*; **48:** 739–44.

Borowski P, Lang M, Haag A, Schmitz H, Choe J, Chen HM, Hosmane RS. (2002) Characterization of imidazo[4,5-d]pyridazine nucleosides as modulators of unwinding reaction mediated by West Nile virus nucleoside triphosphatase/helicase: evidence for activity on the level of substrate and/or enzyme. *Antimicrob Agents Chemother.*; **46:** 1231–9.

Chambers TJ, Hahn CS, Galler R, Rice MC. (1990) Flavivirus genome organization expression and replication. *Annu Rev Microbiol.*; **44:** 649–88.

Chao K, Lohman TM. (1991) DNA-induced dimerization of the *Escherichia coli* Rep helicase. *J Mol Biol.*; **221:** 1165–81.

Chase JW, Wiliams KR. (1986) Single-stranded DNA binding proteins required for DNA replication. *Annu Rev Biochem.*; **55:** 103–36.

Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med.*; **3:** 866–71.

Cho HS, Ha NC, Kang LW, Chung KM, Back SM, Jang SK, Oh BH. (1998) Crystal structure of RNA helicase from genotype 1b hepatitis C virus, A feasible mechanism of un-

winding duplex RNA. *J Biol Chem.*; **273:** 15045–52.

Crute JJ, Grygon CA, Hargrave KD, Simoneau B, Faucher AM, Bolger G, Kibler P, Liuzzi M, Cordingley MG. (2002) Herpes simplex virus helicase-primase inhibitors are active in animal models of human disease. *Nat Med.*; **8:** 386–91.

Diana GD, Bailey TR. (1996) *Compounds composition and methods for treatment of hepatitis C.* U.S. Patent No. 5,633,388.

Diana GD, Bailey TR, Nitz TJ. (1998) *Compounds composition and methods for treatment of hepatitis C.* U.S. Patent No. 6,127,384.

Fuller-Pace FV. (1994) RNA helicases: modulators of RNA structure. *Trends Cell Biol.*; **4:** 271–4.

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

Ganapathi R, Kamath N, Constantinou A, Grabowski D, Ford J, Anderson A. (1991) Effect of the calmodulin inhibitor trifluoperazine on phosphorylation of P-glycoprotein and topoisomerase II: relationship to modulation of subcellular distribution DNA damage and cytotoxicity of doxorubicin in multidrug resistant L1210 mouse leukemia cells. *Biochem Pharmacology.*; **41:** 21–6.

Georgaki A, Strack B, Podust V, Hubscher U. (1992) DNA unwinding activity of of replication protein A. *FEBS Lett.*; **308:** 240–5.

Gorbalenya AE, Koonin EV. (1989) Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Res.*; **17:** 8413–40.

Gorbalenya AE, Koonin EV. (1993) Helicases: amino acid sequence comparisons and structure-function relationships. *Curr Opin Struct Biol.*; **3:** 419–29.

Gorbalenya AE, Koonin EV, Donschenko AP, Blinov VM. (1989) Two related superfamilies of putative helicases involved in replication recombination repair and expression of DNA and RNA genomes. *Nucleic Acids Res.*; **17:** 4713–30.

Gu B, Liu C, Lin-Goerke J, Malley DR, Gutshall LL, Feltenberger CA, Del Vecchio AM. (2000) The RNA helicase and nucleotide triphosphatase activities of the bovine viral diarrhea virus NS3 protein are essential for viral replication. *J Virol.*; **74:** 1794–800.

Gwack Y, Kim DW, Han JH, Choe J. (1996) Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochem Biophys Res Commun.*; **225:** 654–9.

Gwack Y, Yoo H, Song I, Choe J, Han JH. (1999) RNA stimulated ATPase and RNA helicase activities and RNA binding domain of hepatitis G virus nonstructural protein 3. *J Virol.*; **73:** 2909–15.

Halstead SB. (1992) The XXth century dengue pandemic: need for survieillance and research. *World Health StatQ* **45**: 292–8.

Hay B, Jan LY, Jan YN. (1988) A protein component of *Drosophila* polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. *Cell.*; **55:** 577–87.

Hesson T, Mannarino A, Cable M. (2000) Probing the relationship between RNA-stimulated ATPase and helicase activities of HCV NS3 using 2′-O-methyl RNA substrates. *Biochemistry.*; **39:** 2619–25.

Hsu CC, Hwang LH, Huang YW, Chi WK, Chu YD, Chen DS. (1998) An ELISA for RNA helicase activity: application as an assay of the NS3 helicase of hepatitis C virus. *Biochem Biophys Res Commun.*; **253:** 594–9.

Ishido S, Fujita T, Hotta H. (1998) Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. *Biochem Biophys Res Commun.*; **244:** 35–40.

Kadare G, Haenni A. (1997) Virus-encoded RNA helicases. *J Virol.*; **71:** 2583–90.

Kim DW, Gwack Y, Han JH, Choe J. (1997) Towards defining a minimal functional domain for NTPase and RNA helicase activities of the hepatitis C virus NS3 protein. *Virus Res.*; **49:** 17–25.

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

Koch JO, Bartenschlager R. (1999) Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3 NS4A and NS4B. *J Virol*.; **73:** 138–46.

Korolev S, Hsieh J, Gauss GH, Lohman TM, Waksman G. (1997) Major domain swiveling revealed by the crystal structures of complexes of *E. coli* Rep helicase bound to single-stranded DNA and ADP. *Cell*.; **90:** 635–47.

Kuo M-D, Chin C, Hsu S-L, Shiao J-Y, Wang T-M, Lin J-H. (1996) Characterization of the NTPase activity of Japanese encephalitis virus NS3 protein. *J Gen Virol*.; **77:** 2077–84.

Kwong AD, Risano C. (1998)) Development of a hepatitis C virus RNA helicase high troughput assay. In *Antiviral methods and protocols*. Kinchington D, Schinazi RF. eds. Humana Press Inc, Totowa NJ.

Kyono K, Miyashiro M, Tagushi I. (1998) Detection of hepatitis C virus helicase activity using the scintillation proximity assay system. *Anal Biochem*.; **257:** 120–6.

Lain S, Riechmann L, Martin MT, Garcia JA. (1989) Homologous potyvirus and flavivirus proteins belonging to a superfamily of helicase-like proteins. *Gene*.; **82:** 357–62.

Levin MK, Patel SS. (1999) The helicase from hepatitis C virus is active as an oligomer. *J Biol Chem*.; **274:** 31839–46.

Leyssen P, De Clercq E, Neyts J. (2000) Perspectives for the treatment of infections with *Flaviviridae*. *Clin Microbiol Rev*.; **13:** 67–82.

Li H, Clum S, You S, Ebner KE, Padmanabhan R. (1999) The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. *J Virol*.; **73:** 3108–16.

Lüking A, Stahl U, Schmidt U. (1998) The protein family of RNA helicases. *Crit Rev Biochem Mol Biol*.; **33**: 259–96.

Matson SW, Kaiser-Rogers KA. (1990) DNA helicases. *Annu Rev Biochem*.; **59:** 289–329.

Matusan AE, Pryor MJ, Davidson AD, Wright PJ. (2001) Mutagenesis of the dengue virus type 2 NS3 protein within and outside helicase motifs: effects on enzyme activity and virus replication. *J Virol*.; **75:** 9633–43.

Meyers G, Thiel HJ. (1996) Molecular characterization of pestiviruses. *Adv Virus Res*.; **7:** 53–118.

Miller H, Purcell R. (1990) Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc Natl Acad Sci U S A*.; **87:** 2057–61.

Monath TP. (1987) Yellow fever: a medically neglected disease. Report on a seminar. *Rev Infect Dis*. **9:** 165–75.

Monath TP, Heinz FX. (1996) Flaviviruses. In *Fields virology*. Fields BN, Knipe DM, Howley PM. eds, 3rd edn, vol 1, pp 961–1034. Lippincott-Raven Publishers, Philadelphia.

Moore KJM, Lohman TM. (1994) Kinetic mechanism of adenine nucleotide binding to and hydrolysis by the *Escherichia coli* Rep monomer. 1. Use of fluorescent nucleotide analogues. *Biochemistry*.; **33:** 14550–64.

Morgenstern KA, Landro JA, Hsiao K, Lin C, Young G, Su M-S, Thomson JA. (1997) Polynucleotide modulation of the protease nucleoside triphosphatase and helicase activities of hepatitis C virus NS3–NS4A complex. *J Virol*.; **71:** 3767–75.

Muerhoff AS, Leary TP, Simons JN, Pilot-Matias TJ, Dawson GJ, Erker JC, Chalmers ML, Schlauder GG, Desai SM, Mushahwar IK. (1995) Genomic organization of GB viruses A and B: two new members of the *Flaviviridae* associated with GB agent hepatitis. *J Virol*.; **69:** 5621–30.

Neddermann P, Clementi A, De Francesco R. (1999) Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3

protease NS4A NS4B and NS5A encoded on the same polyprotein. *J Virol.*; **73:** 9984–91.

Nettelton PE, Entrican G. (1995) Ruminant pestiviruses. *Br Vet J.*; **151:** 615–42.

Notarnicola SM, Park K, Griffith JD, Richardson CC. (1995) A domain of the gene 4 helicase/primase of bacteriophage T7 required for the formation of an active hexamer. *J Biol Chem.*; **270:** 20215–24.

Phoon CW, Ng YP, Ting AE, Yeo SL, Sim MM. (2001) Biological evaluation of hepatitis C virus helicase inhibitors. *Bioorg Med Chem Lett.*; **11:** 1647–50.

Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. (1998) Binding of hepatitis C virus to CD81. *Science.*; **282:** 938–41.

Porter D. (1998) Inhibition of the hepatitis C virus helicase associated ATPase activity by the combination of ADP NaF $MgCl_2$ and poly(rU). *J Biol Chem.*; **273:** 7390–6.

Porter DJ, Preugschat F. (2000) Strand-separating activity of hepatitis C virus helicase in the absence of ATP. *Biochemistry.*; **39:** 5166–73.

Preugschat F, Averett D, Clarke B, Porter D. (1996) A steady-state and presteady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. *J Biol Chem.*; **271:** 24449–57.

Pryor MJ, Gualano RC, Lin AD, Davidson AD, Wright PJ. (1998) Growth restriction of dengue virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins. *J Gen Virol.*; **79:** 2631–9.

Rice MC. (1996) *Flaviviridae*: the viruses and their replication. In *Fields virology.* Fields BN, Knipe DM, Howley PM. eds, 3rd edn, vol. 1, pp 931–60. Lippincott-Raven Publishers, Philadelphia.

Scheffner M, Knippers R, Stahl H. (1989) RNA unwinding activity of SV40 large T antigen. *Cell.*; **57:** 955–63.

Spector FC, Liang L, Giordano H, Sivaraja M, Petersen MP. (1998) Inhibition of herpes simplex virus replication by a 2-amino thiazole via interactions with the helicase component of the UL5-UL8-UL52 complex. *J Virol.*; **72:** 6979–87.

Subramanya HS, Bird LE, Brannigan JA, Wigley DB. (1996) Crystal structure of a DExx box DNA helicase. *Nature.*; **384:** 379–83.

Suzich J, Tamura J, Palmer-Hill F, Warrener P, Grakoui A, Rice MC, Feinstone S, Collett M. (1993) Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *J Virol.*; **67:** 6152–8.

Tai C-L, Chi W-K, Chen D-S, Hwang L-H. (1996) The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J Virol.*; **70:** 8477–84.

Tamura JK, Warrener P, Collett MS. (1993) RNA-stimulated NTPase activity associated with the p80 protein of the pestivirus bovine viral diarrhea virus. *Virology.*; **193:** 1–10.

Utama A, Shimizu H, Morikawa S, Hasebe F, Morita K, Idarash AI, Hatsu M, Takamizawa K, Miyamura T. (2000) Identification and characterization of the RNA helicase activity of Japanese encephalitis virus NS3 protein. *FEBS Lett.*; **465:** 74–8.

Walker JE, Saraste M, Runswick MJ, Gay NJ. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase myosin kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.*; **1:** 945–51.

Warrener P, Collett MS. (1995) Pestivirus NS3 (p80) protein possesses RNA helicase activity. *J Virol.*; **69:** 1720–6.

Wassarman DA, Steitz JA. (1991) RNA splicing, alive with DEAD proteins. *Nature.*; **349:** 463–4.

Westaway EG. (1987) Flavivirus replication strategy. *Adv Virus Res.*; **33:** 45–90.

Westaway EG, Brinton MA, Gaidamovich Ya, Horzinek MC, Igarashi A, Kääriäinen L, Lvov DK, Porterfield JS, Russell PK, Trent DW.

(1985) *Flaviviridae*. *Intervirology*.; **24:** 183–92.

Wu Q, Bounaud PY, Kuduk S, Yang CP, Ojima I, Horwitz S, Orr G. (1998) Identification of the domains of photoincorporation of the 3′- and 7-benzophenone analogues of taxol in the carboxyl-terminal half of murine mdr1b P glycoprotein. *Biochemistry*.; **37:** 11272–9.

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

Zhang S, Maacke H, Grosse F. (1995) Molecular cloning of the gene encoding nuclear DNA helicase II, A bovine homologue of human RNA helicase A and *Drosophila* Mle protein. *J Biol Chem*.; **270:** 16422–7.

Zhang N, Chen H-M, Koch V, Schmitz H, Liao C-L, Bretner M, Bhadti VS, Fattom A, Naso R, Hosmane RS, Borowski P. (2002) Ring-expanded ("fat") nucleoside and nucleotide analogues exhibit potent *in vitro* activity against *Flaviviridae* NTPases/helicases including those of the West Nile virus (WNV) hepatitis C virus (HCV) and japanese encephalitis virus (JEV). Submitted to *J Med. Chem*.