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Viral NS3 helicase activity is inhibited by peptides reproducing the Arg-rich conserved motif of the enzyme (motif VI)

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ABSTRACT

The NTPase/helicase of *Flaviviridae* viruses is one of the essential components of their replication complex. The enzyme is defined by the presence of seven highly conserved amino acid motifs. Random screening of numerous hepatitis C virus (HCV) derived peptides, revealed a basic amino acid stretch corresponding to motif VI of the HCV NTPase/helicase (amino acids 1487–1500 of the HCV polyprotein). This peptide inhibited the unwinding activity of the enzyme with an IC_{50} = 0.2 μM. Peptides corresponding to motif VI of HCV, West Nile virus (WNV) and Japanese encephalitis virus (JEV) were synthesized and tested as inhibitors of NTPase and unwinding reactions mediated by the viral enzymes. Peptides distinguished in regard to their length and structure. Between the peptides tested HCV(1487–1500) reproducing the sequence of motif VI was the most potent inhibitor of helicase activities of investigated enzymes. Other respective peptides were rather modest inhibitors. The examined peptides inhibited the *Flaviviridae* helicases in the following order of potency: HCV(1487–1500) > WNV(1959–1572) > JEV(1962–1975). Interestingly, the susceptibility of the helicase activity to the inhibition by the peptides was similar and in the row: HCV > WNV > JEV.

The inhibition results from binding and blockade of the active site of the enzyme lyes beyond the NTP-binding and hydrolyzing site. The kinetic analyses indicated that the binding of the peptides do not interfere with the NTPase activity of the enzymes. The peptide may serve as effective and selective tool to reduce the virus propagation.

1. Introduction

Viruses of the *Flaviviridae* family are small, enveloped, spherical particles of 40–50 nm in diameter with single-stranded, positive sense RNA genomes. The viruses could be classified into three genera: hepacivirus, flavivirus, and pestivirus [1,2]. Hepaciviruses and flaviviruses are known to be the cause of severe encephalitic, hemorrhagic, hepatic, and febrile illnesses in humans [3,4]. The viral genome of the genera hepacivirus and flavivirus encodes a polyprotein of

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3000–4000 amino acids. The NH_2-terminus of the polyprotein of both genera is processed into three structural proteins. The COOH-terminal part of the polyprotein of hepaciviruses is cleaved into six mature nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and the polyprotein of flaviviruses is processed into seven NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [3,5–7]. Among these proteins the NS3 appears to be a promising target for antiviral agents. It possesses numerous enzymatic activities including serine protease, RNA stimulated nucleoside triphosphatase (NTPase), and RNA helicase activities. The catalytic domain of the chymotrypsin like NS3 protease has been mapped to the NH_2-terminus region of the NS3, whereas the NTPase and helicase activities are associated with the COOH-terminus of NS3 [5,8].

Some mutational and inhibitory studies demonstrated that the intact enzymatic properties of NS3 are necessary for the virus propagation [9–11]. In this context, the development of effective inhibitors of the enzymes is an important part of the antiviral strategy. In contrast to numerous and well-characterized protease inhibitors there are only few works presenting effective NTPase/helicase inhibitors [9,12,13].

NTPase/helicases are capable of unwinding duplex RNA and DNA structures by disrupting the hydrogen bonds that keep the two strands together. The solved crystal structure of the HCV NTPase/helicase demonstrated that the enzyme consists of three equally-sized domains (1, 2 and 3) separated by deep clefts and connected by flexible amino acid stretches so-called hinge regions [14,15]. NTPase/helicases are NTP binding and consuming enzymes. The interaction with the nucleotide is mediated by a well-characterized binding pocket, detected due to the presence of highly conserved Walker motifs A and B [16,17]. Both motifs are short amino acid sequences participating in binding and hydrolysis of β- and γ-phosphate groups of NTP. Another important amino acid motif – present on the surface of the NTPase/helicase domain of NS3 – is the Arg-rich segment localized between amino acids 1487 and 1500 of the HCV polyprotein [17–19]. This conserved motif is characteristic for a broad range of RNA and DNA specific NTPase/helicases [14]. Nevertheless, the function of this amino acid stretch remains controverse up to date [17,20,21]. There are crystallographic studies demonstrating a key role of the motif in the binding of RNA (or DNA) substrate(s) [17]. On the other hand, there are modelling and crystallographic studies strongly suggesting that this segment participates in the binding of NTP to the NTPase/helicase and/or in stabilization of this binding [17,19,20,22].

According to the mentioned crystallographic and mechanistic studies, two alternative mechanisms of the unwinding reaction have been postulated [17,22]. Both models predict binding and hydrolysis of NTP as well as cooperation of the domains of the enzyme in course of the process. The energy released is used for the ''march'' of the enzyme along the DNA or RNA structures, and the unwinding reaction results from capturing single strand (ss) regions which arise due to thermal fluctuations at the fork. Alternatively, the energy could be transferred to the fork and used for disruption of the hydrogen bonds that keep the strands together [15,17,19]. Consistent with the proposed models the following mechanisms of inhibition of the helicase activity could be considered: (a) inhibition of the NTPase activity by interference with NTP binding, (b) inhibition of the NTPase activity by an allosteric mechanism, and (c) inhibition of the coupling of NTP hydrolysis to unwinding reaction. Additional possibilities include (d) interference in the interaction of helicase with its RNA or DNA substrate *via* competitive blockade of the substrate-binding site or by (e) inhibition of the unwinding by steric blockade of translocation of the enzyme along the polynucleotide chain [23]. Nevertheless, there are further possibilities by which the helicase or NTPase activities could be inhibited. One of this, postulated here, is the blockade of the intramolecular interaction of the NTPase/helicase domains that might inhibit the activities of the viral enzyme.

2. Materials and methods

2.1. Reagents

Peptides used were synthesized and purified by Dr. Kullmann and Dr. Buck at the Center for Molecular Neurobiology (University of Hamburg, Germany) and, alternatively, at the Louisiana State University Health Sciences Center (New Orleans, USA). All peptides are dissolved in water. DNA oligonucleotides were synthesized by Dr. M. Schreiber (Bernhard-Nocht-Institute, Hamburg, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) and *N*-succinimidyl-[2,3-^{3}H]propionate were obtained from Amersham or Du Pont (Bad Homburg, Germany), respectively. All other chemicals were purchased from Sigma unless otherwise stated.

2.2. Methods

2.2.1. Cell culture and infection

Vero E6 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Scotland) containing 10% fetal calf serum (Sigma) and supplemented with 80 μg/ml gentamicin (Merck), 100 mM sodiumpyruvate and 200 mM glutamine (Gibco). Cells in the log phase of growth were infected with WNV (ATCC strain VR-82) as described previously [24]. The cell culture medium harvested five days after infection served further as start material for purification of the WNV NTPase/helicase.

2.2.2. Purification of WNV NTPase/helicase

This procedure was established in our laboratory and is applicated with small modifications [24]. The cell culture medium (200 ml aliquots) was concentrated in the presence of 5 U/ml aprotinin, 1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 1 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone, 10 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 5 mM EGTA and 0.5% Triton X-100 to a volume of 10 ml. The concentrate was subjected to chromatography on 10 ml Reactive Red 120 agarose (Sigma) equilibrated with buffer containing 20 mM Tris–HCl pH 7.5, 10% glycerol, 0.05% Triton X-100 (TGT), 1 mM EDTA and 1 mM β-mercaptoethanol (TGT buffer) for 4 h at 4 °C. The matrix was washed with the buffer; the bound protein was eluted with 1 M KCl in the same buffer, and concentrated by ultrafiltration on a 30-kDa membrane to a final volume of 2 ml. The resulting concentrate

was subjected to gel exclusion chromatography on a HiLoad Superdex-200 column. Fractions expressing ATPase and helicase activities were chromatographed again on Reactive Red 120 agarose (5 ml). The salt-eluted protein was precipitated with polyethylene glycol (30% w/w), collected by centrifugation, solubilized with TGT buffer, and applied to experiments.

2.2.3. *Sources and purification of the HCV, and JEV NTPase/helicases*

The NTPase/helicase domain of HCV NS3 containing a His tag at the COOH-terminus was expressed in *E. coli* and purified as previously described [25] with some modifications. The bacteria were collected by centrifugation and disrupted by sonication in lysis buffer (100 mM Tris–HCl pH 7.5, 20% glycerol, 0.1% Triton X-100, 200 mM NaCl, 1 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 10 mM imidazole). The insoluble material was pelleted at 26,000 × *g* and the supernatant was mixed with 3 ml nickel charged resin (Qiagen) equilibrated with TGT buffer for 12 h. The matrix was transferred into the column and washed with TGT buffer supplemented with 200 mM NaCl and 20 mM imidazole. The protein bound was eluted with 0.5 M imidazole in the same buffer. The purity of the obtained HCV protein was around 65–70%. The eluted fraction was concentrated by ultrafiltration on a 30-kDa membrane and fractionated over a HiLoad Superdex-200 column equilibrated with TGT buffer. Fractions containing the most ATPase and helicase activities were pooled and served to investigate the properties of the NTPase/helicase.

The JEV NTPase/helicase was expressed in *E. coli* [26] and purified according to the protocol established for the HCV enzyme. Final preparations of the enzymes contain homogenous HCV and JEV NTPase/helicases as demonstrated by Coomassie Blue stained SDS/polyacrylamide gel (data not shown).

2.2.4. *ATPase and helicase assays*

The ATPase activity of the WNV, HCV and JEV NTPase/helicases was determined under conditions described previously [27] with slight modifications. Briefly, the assay was performed with 0.5 pmol of HCV, 2 pmol of WNV, and 4 pmol of JEV NTPase/helicases. The amounts of the enzymes showed the same relative unwinding activity determined at ATP concentrations equal to the K_m values (see below). The enzymes were incubated in a reaction mixture (final volume 25 μl) that contained 20 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol, 0.01% Triton X-100, 0.1 mg/ml BSA and [γ-^{32}P]ATP (25 nCi). The ATP concentration was adjusted to K_m values estimated in the NTPase reaction for each enzyme (105 μM for HCV, 9.5 μM for WNV, and 235 μM for JEV NTPase/helicases). The reaction was proceeded for 30 min at 30 °C and terminated by heating at 95 °C for 5 min. Afterwards, 1 μl of each sample was spotted onto a thin layer plate (POLYGRAM® CEL 300 PEI, Macherey-Nagel) and subjected to separation in 0.4 M KH_2PO_4 pH 3.4 buffer. After chromatography the plate was dried and exposed to a Kodak X-ray film at −70 °C. Parts of the plates corresponding to ''hot spots'' of the non-hydrolyzed [γ-^{32}P]ATP and to the released $^{32}P_i$ were cut out and Cerenkov radiation was measured. As a positive control of the *Flaviviridae* NTPase/helicase mediated NTP hydrolysis, an established NTPase – Apyrase (isolated from *Solanum tuberosum*, from Sigma) – was applied.

The helicase activity unless otherwise specified, was proceeded as described for NTPase assay. The reaction mixture contained 4.7 pM DNA substrate and the amounts of enzymes indicated in the legends. The ATP concentrations were adjusted as specified above. The reaction was stopped by addition of 5 μl of termination buffer (100 μM Tris–HCl pH 7.5, 20 mM EDTA, 0.5% SDS, 0.1% Triton X-100, 25% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol). Samples were subjected to electrophoresis on a 15% Tris–borate–EDTA (TBE) polyacrylamide gel containing 0.1% SDS [24]. Gels were dried and exposed to Kodak X-ray films at −70 °C. Subsequently, parts of the gels corresponding to the released strand and to not unwound substrate were cut out and ^{32}P radioactivity was measured. The inhibitory effect was estimated as a function of increasing concentrations of the peptides.

2.2.5. *Effect of preincubation of HCV(1487–1500) with enzyme on unwinding and hydrolysis efficacy*

The HCV enzyme was preincubated with HCV(1487–1500) at 30 °C in 20 μl of TGT buffer containing 0.1 mg/ml BSA for various periods of time and various concentrations of peptide. The hydrolysis and unwinding reactions were initiated by addition of $MgCl_2$, ATP and DNA substrate at concentrations used in the standard ATPase or helicase assays in TGT buffer. In additional tests the effect of preincubation on NTPase reaction the hydrolysis was started by addition of $MgCl_2$ and ATP at concentrations used in the standard ATPase assay in TGT buffer. In control experiments, DNA substrate or ATP were preincubated alone under the same conditions. Thereafter, the reaction mixture was added and reactions were started.

2.2.6. *Synthesis of the substrate for helicase reaction*

The substrate for helicase assays was obtained by annealing two partially complementary DNA oligonucleotides synthesized with sequences corresponding to the deoxynucleotide version of the RNA strands described previously [8]. The release strand (26-mer) with a sequence: 5′-CAA ACU CUC UCU CUC UCA ACA AAA AA-3′ was 5′-end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (MBI, Fermentas) as recommended by the manufacturer. For the annealing reaction the labeled oligonucleotide was combined at a molar ratio of 1:10 with the template strand (40-mer) of the following sequence: 5′-AGA GAG AGA GGU UGA GAG AGA GAG AGU UUG AGA GAG AGA G-3′, denatured for 5 min at 96 °C and slowly renatured. The duplex DNA was electrophoresed on a 15% native TBE polyacrylamide gel, visualized by autoradiography and extracted as described previously [24].

2.2.7. *Immobilization and renaturation of the HCV NTPase/helicase*

Aliquots of HCV NTPase/helicase were mixed with 20 μl of sample buffer, boiled, and subjected to SDS/PAGE. After being run on SDS/polyacrylamide gels, proteins were electroblotted onto nitrocellulose filters. Membranes were dried and incubated for 5 h with 1 mg/ml BSA in 20 mM Tris–HCl pH 7.5, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100, 1 mM 2 mercaptoethanol, 150 mM NaCl, 0.3% Tween 20, and 2 mM $MgCl_2$ (renatura-

tion buffer) for at least 24 h at room temperature. The nitrocellulose sheets with the immobilized protein were used for binding assays (see below).

2.2.8. Binding assays

The binding of HCV(1487–1500) to HCV NTPase/helicase was investigated by two alternative methods: by an overlay method and by an equilibrium size exclusion gel chromatography. In the overlay assay the protein was subjected to SDS/PAGE, and afterwards electroblotted onto a nitrocellulose filter. After renaturation (see above) the buffer was replaced by the same buffer supplemented with ^{3}H radiolabeled HCV(1487–1500) adjusted to a final concentration of 0.1 nM. After incubation for 30 min at room temperature, the filter was washed five times with 3 mg/ml BSA in TGT buffer, dried and exposed to a Kodak X-ray film. As control protein casein was applied.

In the binding assay basing on the gel filtration technique [^{3}H]HCV(1487–1500) was incubated in the presence (or absence) of 50 μg of HCV NTPase/helicase for 30 min. The binding was conducted in TGT buffer containing 100 mM KCl and applied to HiLoad Superdex 200 column equilibrated with the same buffer. Fractions of 3 ml were collected and the radioactivity was counted.

2.2.9. ^{3}H labeling of HCV(1487–1500)

The labeling of HCV(1487–1500) was performed with *N*-succinimidyl-[2,3-^{3}H]propionate according to the procedure described by Muller [28] with following modifications. The reaction mixture was applied to a HA-Ultrogel column (approx. 5 mg peptide/1 ml matrix), and the column was washed with 10 mM ammonium acetate pH 7.8. Bound peptide was eluted with 0.5 M ammonium acetate pH 9.5, the fraction was freeze-dried for 24 h, solved with the carrier (DMSO) and used in further assays.

2.2.10. Other assays

The protein concentration in all preparations of the NTPase/helicases was determined in SDS/polyacrylamide gel as described previously [29]. Kinetic parameters were determined by non-linear-regression analysis using ENZFITTER (BioSoft) and SIGMA PLOT (Jandel Corp.).

3. Results

3.1. Sources and purity of the enzymes

The HCV and JEV NTPase/helicases used in the studies were obtained from bacteria expressing respective proteins. The WNV enzyme was isolated from medium collected from WNV infected cell culture. Proteins were highly purified, up to a homogeneous preparation, as documented in SDS/PAGE followed by Coomassie Blue staining [30]. Properties of the NTPase/helicases were extensively studied, and characterized in numerous previous works [13,31]. To make it possible to compare the inhibitory parameters of investigated peptides it was necessary to employ the same reaction conditions and substrates described previously [13,20,31].

3.2. Selection of the amino acid sequences of the peptides used in this study

A broad range of peptides reproducing motif VI of HCV NTPase/helicase and of related WNV as well as JEV was tested as inhibitors of the unwinding reaction. First, peptides (5-, 8-, 11-, 14-, and 17-tide) reproducing shorter or enlarged version of HCV-1b motif VI and a respective 17-tide, basing on amino acid sequence of HCV-1a polyprotein, were synthesized. Both 17-tides, named HCVB(1484–1500) and HCVA(1484–1500), reproducing respective sequences of HCV NTPase/helicase localized between amino acids 1484 and 1500 of HCV polyprotein. The position of the 17-tide within the HCV-1b NTPase/helicase domain is schematically presented in Fig. 1. The amino acid sequences of the peptides used in this work and their terminology are stated in Fig. 2. The specificity of the inhibition was tested by employing a peptide with amino acid composition of HCV(1487–1500) but with random sequence. The peptide is termed [rd]HCV(1487–1500).

Further, analogous sets of peptides reproducing the motif VI of WNV and JEV NTPase/helicases were synthesized and investigated as potential inhibitors of respective NTPase/helicases. The synthesized amino acid stretches of motif VI of the investigated enzymes are localized between 1956 and 1972 of WNV as well as between 1959 and 1975 of JEV polyproteins. Fig. 2 presents the alignment of the amino acid sequences of the 17-tides synthesized, and the amino acid sequences of the shortened variants of the peptides used in this study.

3.3. Helicase activity of HCV NTPase/helicase

The helicase activity of the HCV enzyme was determined *vs* DNA substrate. The enzyme was assayed under optimal conditions established in previous works [13,31]. ATP was applied at concentration of 105 μM, that corresponds to the K_m value measured in the ATPase reaction. The helicase activity

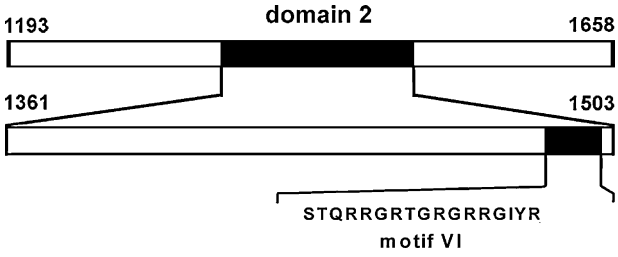

Fig. 1 – Schematic presentation of the localization of the extended version of motif VI of HCV-1b NTPase/helicase. This amino acid sequence served as a basis for the synthesis of the 17-tide HCVB(1484–1500), its shortened derivatives and an analogous 17-tide from HCV-1a, HCVA(1484–1500), used in this study. The top bar represents the NTPase/helicase domains (1, 2 and 3) of the HCV NS3 protein. The bar below represents the extended domain 2 of the enzyme with indicated position of the motif VI. In the case of WNV and JEV analogous sequences are localized between amino acids 1956 and 1972 of WNV polyprotein and 1959 and 1975 of JEV polyprotein. One single code was used.

Peptide	Sequence
HCV(1496-1500)	RGIYR
HCV(1493-1500)	RGRRGIYR
HCV(1490-1500)	RTGRGRRGIYR
HCV(1487-1500)	RRGRTGRGRRGIYR
HCVA(1484-1500)	RTQRRGRTGRGKPGIYR
HCVB(1484-1500)	STQRRGRTGRGRRGIYR
[rd]HCV(1487-1500)	
WNV(1968-1972)	SQVGD
WNV(1965-1972)	RNPSQVGD
WNV(1962-1972)	RIGRNPSQVGD
WNV(1959-1972)	RRGRIGRNPSQVGD
WNV(1956-1972)	AAQRRGRIGRNPSQVGD
JEV(1971-1975)	NQVGD
JEV(1968-1975)	RNPNQVGD
JEV(1965-1975)	RVGRNPNQVGD
JEV(1962-1975)	RRGRVGRNPNQVGD
JEV(1959-1975)	AAQRRGRVGRNPNQVGD

Fig. 2 – Overview of the sequences of HCV, WNV and JEV NTPase/helicase motifs VI derived peptides and their deleted derivatives used in the experiments presented above. The sequences correspond to the amino acids stretches (and/or their shortened variants) localized between the residues 1484 and 1500 of HCV polyprotein, 1956 and 1972 of WNV polyprotein and 1959 and 1975 of JEV polyprotein. The shortened derivatives are NH_2 terminally deleted. One single code was used.

Table 1 – Inhibition of HCV helicase activity by HCV, WNV and JEV peptides

Peptide	IC_{50} (μM)
HCVA(1484–1500)	27.1 ± 2.4
HCVB(1484–1500)	24.3 ± 1.8
HCV(1487–1500)	0.2 ± 0.01
HCV(1490–1500)	34.6 ± 3.2
HCV(1493–1500)	313 ± 11.3
HCV(1496–1500)	>500
[rd] HCV(1487–1500)	386 ± 13.4
WNV(1968–1972)	>500
WNV(1965–1972)	>500
WNV(1962–1972)	417 ± 17.6
WNV(1959–1972)	169 ± 6.8
WNV(1956–1972)	358 ± 13.2
JEV(1971–1975)	>500
JEV(1968–1975)	>500
JEV(1965–1975)	374 ± 12.8
JEV(1962–1975)	196 ± 9.4
JEV(1959–1975)	442 ± 17.7

The table summarizes the results of experiments graphically presented in Fig. 3A–C. The helicase activity was determined under standard conditions as described in Section 2 in the presence of ATP adjusted to the K_m values of 105 μM and 4.7 pM of DNA substrate (concentration of nucleotide base). The substrate and the released strand were separated on TBE polyacrylamide gel and visualized by exposure of the dried gel to X-ray film. The parts of the gels corresponding to the released strand were excised, and the ^{32}P radioactivity was quantified as described in Section 2. The helicase activity of the enzyme measured in the absence of the compounds was referred to as 100%. The term IC_{50} is defined as the concentration of peptides required for 50% inhibition of enzyme activity. The results shown are representative of three independent experiments.

of the enzyme was measured as a function of increasing concentrations of the peptides. Fig. 3A presents the inhibition curves obtained with increasing concentrations of the HCV peptides and Table 1 summarizes the results of the tests expressed as IC_{50} values. The comparison of the data emphasizes that the 14-tide HCV(1487–1500) is the most potent inhibitor of the HCV NTPase/helicase unwinding activity. Shortening or lengthening the peptide reduces significantly the inhibitory potential of the derivatives. Similar, randomizing of the sequence of HCV(1487–1500) impaired significantly the inhibition of the enzyme. Although the sequences of HCVA(1484–1500) and HCVB(1484–1500) differ in some amino acids, the obtained inhibition curves and IC_{50} values estimated for both 17-tides did not show detectable discrepancies.

In further part of the study the mentioned WNV and JEV derived sets of peptides were investigated on their inhibitory effect towards the HCV enzyme. The obtained inhibition

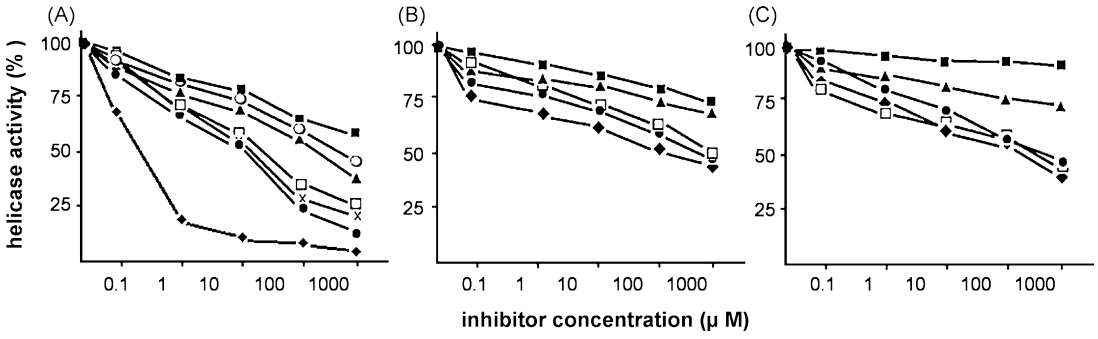

Fig. 3 – Inhibition of the helicase activity of HCV NTPase/helicase mediated by peptides derived from amino acid sequence of motif VI of HCV (A), WNV (B) and JEV (C) enzymes. The peptides were simultaneously added to the reaction mixture with all other ingredients. After the unwinding reaction the helicase activity was determined as a function of increasing concentrations of the peptides. The symbols used in Fig. A are: HCVA(1484–1500) (×), HCVB(1484–1500) (●), [rd]HCV(1487–1500) (○), HCV(1487–1500) (◆), HCV(1490–1500) (□), HCV(1493–1500) (▲), and HCV(1496–1500) (■). The symbols used in figure (B) are: WNV(1956–1972) (●), WNV(1959–1972) (◆), WNV(1962–1972) (□), WNV(1965–1972) (▲), and WNV(1968–1972) (■). The symbols used in figure (C) are: JEV(1959–1975) (●), JEV(1962–1975) (◆), JEV(1965–1975) (□), JEV(1968–1975) (▲), and JEV(1971–1975) (■). All results presented are representatives of three independent experiments.

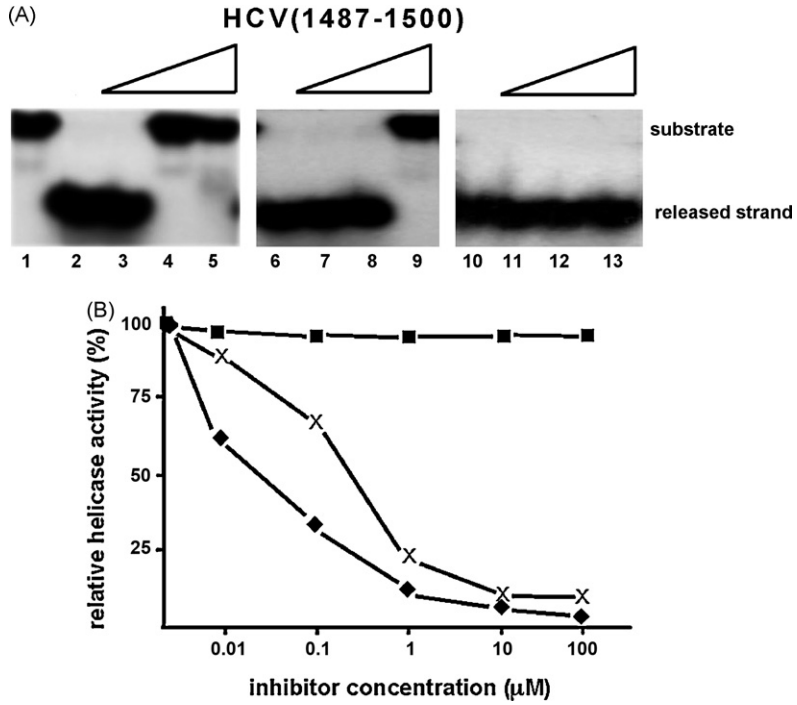

Fig. 4 – The effect of various concentrations of HCV NTPase/helicase on the inhibitory potential of HCV(1487–1500) demonstrated as autoradiography (A) and extended graphical analysis (B). (A) Presents the autoradiography of the helicase assay obtained with different concentrations of the enzyme and with the peptide inhibitor. The enzyme was applied at following concentrations: 0.15 pmol (lanes 2–5), 0.5 pmol (lanes 6–9), and 1.5 pmol (lanes 10–13). Lane 1 presents the native substrate. The peptide was applied at concentrations: 5 nM (lanes 3, 7, and 11), 50 nM (lanes 4, 8, and 12), and 500 nM (lanes 5, 9, and 13). In lanes 2, 6, and 10 no peptide was added. (B) Presents more detailed graphical analysis of this effect. Amounts of the NTPase/helicase: 0.15 pmol (◆), 0.5 pmol (×), and 1.5 pmol (■). Concentrations of HCV(1487–1500) are presented in the figure. All results presented are representatives of three independent experiments.

curves are shown in Fig. 3B as well as Fig. 3C, respectively, and the results are summarized in Table 1. It should be noted that HCV(1487–1500) is a nanomolar inhibitor whereas the respective 14-tides WNV(1959–1972) and JEV(1962–1975) were only modest inhibitors of the enzyme.

In our previous study we showed that the preincubation of the HCV NTPase/helicase with 5,6-dichloro-1-(β-D-ribofuranosyl)benzotriazole (DRBT) enhanced the inhibitory potential of the compound [30]. Although the peptides are chemically not related to DRBT, the effect of preincubation on the unwinding activity was tested. However, the incubation of the HCV NTPase/helicase or DNA substrate with HCV(1487–1500) and other peptides before starting the reaction, did not affect their inhibitory potential. Similarly, no detectable influence of the IC_{50} values was measured when the DNA substrate was applied at different concentrations varying in the range of 1.6–14.7 pM. To complete the characterization of the peptide mediated inhibition, the reaction was proceeded at different amounts of the enzyme and the peptide was added at increasing concentrations. It is namely possible that the peptide competes with DNA for the substrate-binding site of the enzyme. Indeed, as presented in Fig. 4A and B enhancing of the enzyme amount in the reaction mixture reduced and even abolished the peptide mediated inhibition.

3.4. *The responses of the helicase activities of WNV and JEV enzymes to peptide inhibitors*

Similar to HCV NTPase/helicase the activities of WNV and JEV enzymes were determined with DNA substrate as a function of increasing concentration of the peptides. The ATP was adjusted to concentrations corresponding to their respective K_m values determined in ATPase reactions of the respective enzymes: 9.5 μM for WNV and 235 μM for JEV.

First, the helicase activity of both enzymes was analyzed for possible inhibition by HCV derived peptides. Fig. 5A and B present the inhibitory curves of the helicase activity of WNV and JEV enzymes, respectively, obtained with increasing concentrations of the peptides HCVB(1484–1500), HCVA(1484–1500), HCV(1487–1500), HCV(1490–1500), HCV(1493–1500) and HCV(1496–1500). In the case of both enzymes (similar to HCV NTPase/helicase) the most potent inhibitor was HCV(1487–1500). Table 2 summarizes the inhibitory potential of investigated peptides – expressed as IC_{50} values.

Next, inhibition of WNV and JEV helicase activities were tested with the mentioned set of WNV peptides: WNV(1956–1972), WNV(1959–1972), WNV(1962–1972), WNV(1965–1972) and WNV(1968–1972). The obtained inhibitory curves resulting from the examination of the WNV enzyme with WNV peptides are shown in Fig. 5C and the results from inhibition of the JEV

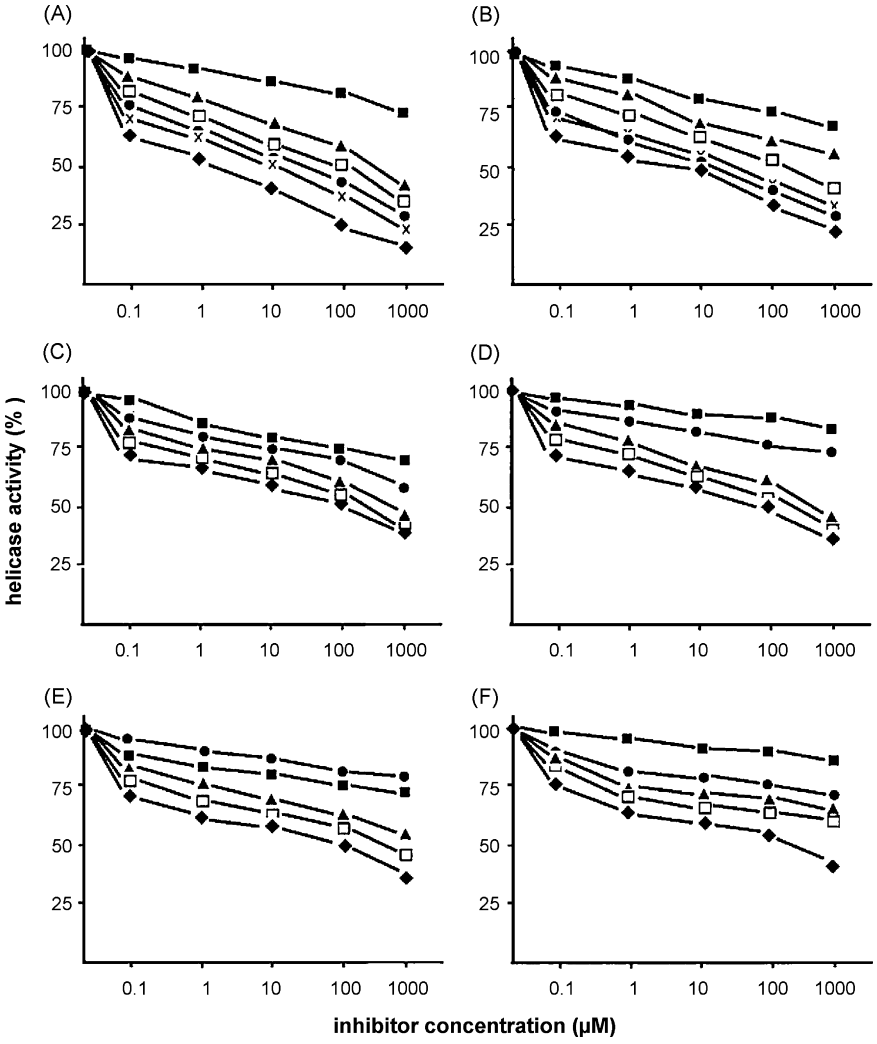

Fig. 5 – Inhibition of the helicase activity of WNV (A, C, E) and JEV (B, D, F) NTPase/helicases mediated by peptides derived from amino acid sequence of motif VI of HCV (A, B), WNV (C, D) and JEV (E, F) enzymes. The peptides were simultaneously added to the reaction mixture with all other ingredients. After the unwinding reaction the helicase activity was determined as a function of increasing concentrations of the peptides. The symbols used in The symbols used in (A, B) HCVA(1484–1500) (×), HCVB(1484–1500) (●), HCV(1487–1500) (◆), HCV(1490–1500) (□), HCV(1493–1500) (▲), and HCV(1496–1500) (■). The symbols used in (C, D) are: WNV(1956–1972) (●), WNV(1959–1972) (◆), WNV(1962–1972) (□), WNV(1965–1972) (▲), and WNV(1968–1972) (■). The symbols used in (E, F) are: JEV(1959–1975) (●), JEV(1962–1975) (◆), JEV(1965–1975) (□), JEV(1968–1975) (▲), and JEV(1971–1975) (■). All results presented are representatives of three independent experiments.

helicase activity by the same peptides in Fig. 5D. All IC_{50} values are presented in Table 3. Analogous inhibitory studies were performed with JEV derived peptides: JEV(1959–1975), JEV(1962–1975), JEV(1965–1975), JEV(1968–1975) and JEV(1971–1975). Curves obtained with WNV and JEV NTPase/helicases are shown in Fig. 5E and F, respectively, and IC_{50} values are listed in Table 4. Also in the case of WNV and JEV enzymes the shortened peptides WNV(1959–1972) and JEV(1962–1975) were the most potent derivatives. Nevertheless, WNV(1959–1972) and JEV(1962–1975) were significantly weaker inhibitors of both enzymes when compared with HCV(1487–1500). Further investigations demonstrated that WNV and JEV enzymes, like the HCV NTPase/helicase, interact with the inhibiting peptide. The extend of the inhibition exerted by the peptides depended strictly on the concentration of enzyme. At higher amounts of the enzymes in the reaction mixture the inhibition was weaker or even abolished (data not shown).

3.5. *ATPase activity of HCV NTPase/helicase*

As mentioned above, all investigated NTPase/helicases were assayed at ATP concentrations corresponding to their K_m values estimated in the ATPase reaction. Since the Arg-rich motif participates in the binding and/or ATP hydrolysis [20], it could be not excluded that the peptides interfere with the ATPase reaction mediated by the enzymes. Attention was then directed to the effects of the various peptides on the ATPase activity of the HCV enzyme, monitored by the release of $^{32}P_i$ from [γ-^{32}P]ATP. To examine the possibility a range of reaction parameters was changed. First, the NTPase activity of the HCV

Table 2 – Inhibition of WNV and JEV helicase activities by HCV peptides

Peptide	IC_{50} (μM)	
	WNV	JEV
HCVA(1484–1500)	37.1 ± 3.9	49.8 ± 4.3
HCVB(1484–1500)	33.6 ± 3.5	47.2 ± 4.7
HCV(1487–1500)	2.7 ± 0.3	21.1 ± 2.2
HCV(1490–1500)	106 ± 6.1	322 ± 9.7
HCV(1493–1500)	397 ± 10.6	>500
HCV(1496–1500)	>500	>500

The table summarizes the results of experiments graphically presented in Fig. 5A and B. The helicase activity was determined under standard conditions as described in the legend to Table 1 in the presence of ATP adjusted to the K_m values of 9.5 μM and 235 μM for WNV and JEV helicase, respectively. The results shown are representative of three independent experiments.

Table 3 – Inhibition of WNV and JEV helicase activities by WNV peptides

Peptide	IC_{50} (μM)	
	WNV	JEV
WNV(1968–1972)	>500	>500
WNV(1965–1972)	383 ± 12.3	437 ± 13.4
WNV(1962–1972)	285 ± 8.1	334 ± 12.1
WNV(1959–1972)	156 ± 6.9	191 ± 7.3
WNV(1956–1972)	>500	>500

The table summarizes the results of experiments graphically presented in Fig. 5C and D. The helicase activity was determined under standard conditions as described in the legend to Table 2. The results shown are representative of three independent experiments.

enzyme was studied with HCV(1487–1500) applied at a concentration corresponding to the IC_{50} value estimated in the helicase reaction. The ATP concentration was changed over the range of 0.1–1000 μM. Next, the enzyme was preincubated with different concentrations of ATP (0.1, 10, and 1000 μM) for various time periods (0, 30 and 60 min) and the reaction was started by addition of DNA substrate and peptide. In further experiments, the concentration of HCV(1487–1500) was altered over the range of 1–300 μM at a constant ATP concentration. Nevertheless, all modifications of the reaction conditions did not influence the relative turnover of ATP hydrolysis (data not shown). Thus, it may be concluded that the peptides do not interact with the ATP binding site.

Table 4 – Inhibition of WNV and JEV helicase activities by JEV peptides

Peptide	IC_{50} (μM)	
	WNV	JEV
JEV(1971–1975)	>500	>500
JEV(1968–1975)	>500	>500
JEV(1965–1975)	448 ± 14.3	>500
JEV(1962–1975)	215 ± 7.2	253 ± 6.5
JEV(1959–1975)	>500	>500

The table summarizes the results of experiments graphically presented in Fig. 5E and F. The helicase activity was determined under standard conditions as described in the legend to Table 1. The results shown are representative of three independent experiments.

3.6. *Interaction of the HCV NTPase/helicase with HCV(1487–1500)*

The data presented above supply indications that the peptides interact directly with the enzymes. To corroborate this finding two independent binding assays demonstrating protein–peptide binding were performed.

In the first one, the protein was immobilized onto a nitrocellulose membrane, renatured and overlaid with ^{3}H-labeled HCV(1487–1500) as described in Section 2. The autoradiography of dried nitrocellulose sheets revealed unambiguously a binding of [^{3}H]HCV(1487–1500) to HCV NTPase/helicase as shown in Fig. 6A. To exclude any possible artificial effects resulting from denaturation and/or renaturation of the protein a second assay was performed. The native HCV NTPase/helicase obtained from HiLoad Superdex-200 column was mixed, incubated with [^{3}H]HCV(1487–1500) and then subjected again to size gel chromatography on the same HiLoad column. As shown in Fig. 6B the activity migrated in three peaks: the first minor peak eluted at void volume (fractions 3–5) that might reflect multimerized status of the enzyme; the second (fractions 9 and 10) corresponds to protein with molecular mass of 50–60 kDa resulting from the binding of [^{3}H]HCV(1487–1500) to enzyme, and the third eluted at exclusion volume (fractions 17 and 18) resulting from unbound [^{3}H]HCV(1487–1500).

4. Discussion

NTPase/helicases of HCV, WNV and JEV were obtained and purified from *E. coli* expressing the proteins, or from native source. The enzymes were targets for peptide inhibitors derived from motif VI of these enzymes. The viruses possess a similarly structured NS3 protein with highly conserved amino acid motifs of the NTPase/helicase domain. Some differences in the amino acid composition of motif VI derived peptides might suggest some discrepancies regarding selectivity, specificity and inhibitory potential of the peptides. Indeed, testing of HCV, WNV and JEV derived peptides revealed numerous interesting features of the relatively similar derivatives: (I) In the case of each NTPase/helicase the 14-tide variant of the peptides was the most potent inhibitor of the enzymes. The inhibitory potential of the peptides corresponds strongly to their length: 14-tide > 11-tide > 8-tide > 5-tide. Interestingly, the longer 17-tide was also a significantly weaker inhibitor than the 14-tide. (II) A further general tendency was observed: the helicase activity of all investigated NTPase/helicases was inhibited by the 14-tides in the following order: HCV(1487–1500) > WNV(1959–1972) > JEV(1962–1975). (III) The peptides inhibited the NTPase/helicase by a direct interaction with the enzyme highly probably by a competitive mechanism. (IV) The susceptibility of the helicases for the inhibition by the peptides decreased in the following order: HCV > WNV > JEV. The basis of the

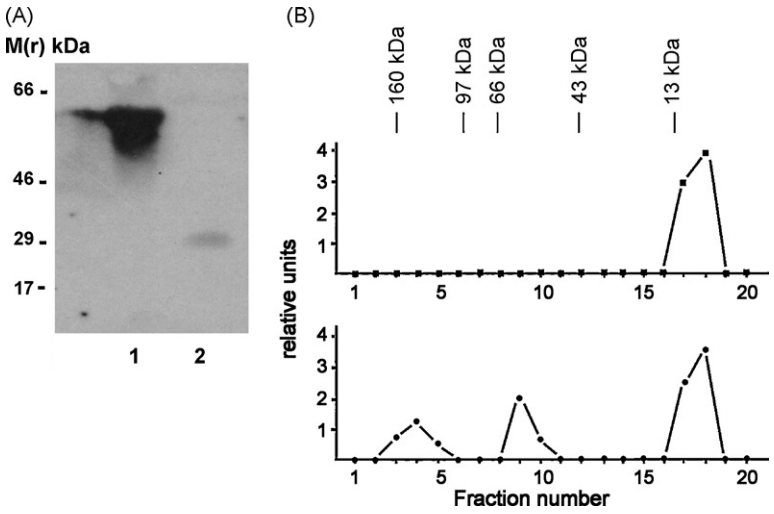

Fig. 6 – The binding of HCV(1487–1500) to HCV NTPase/helicase. The protein–peptide binding was investigated by (A) an overlay method and (B) an equilibrium size exclusion chromatography. (A) 5 μg of HCV NTPase/helicase (lane 1) or 5 μg casein (as control, lane 2) were immobilized on nitrocellulose, renatured and overlaid with [3H]HCV(1487–1500) (0.1 nM). The nitrocellulose was washed, dried and exposed to Kodak X-ray film as described in Section 2. (B) The migration of [3H]HCV(1487–1500) in the gel filtration chromatography in the absence or presence of 50 μg of HCV NTPase/helicase. Details are described in Section 2. The upper panel demonstrates the migration of [3H]HCV(1487–1500) alone and the lower panel after incubation of the peptide with protein. The column was calibrated with following marker proteins: IgG (160 kDa), phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (43 kDa), and cytochrome c (14 kDa).

specificity remains unclear. Probably it results from differences in the structures of the active site of the enzymes. Furthermore, the sequence of HCV 14-tide contains more arginines which led to its highly hydrophobic character than in the case of sequences of WNV or JEV. In comparison of all results it is to see that peptides containing aa RRGR are better inhibitors than those without. Additional, as described in a further study [32] aa 1498–1500 play a crucial role in binding of the peptide to domain 1. These two facts combined explain the higher IC_{50} value of the random peptide.

On the basis of these and further data it could be speculated about the possible mechanism of action of the peptide. The crystallographic analysis of the HCV NS3 NTPase/helicase performed by Kim and co-workers predicted a direct contact between motifs II and VI of the enzyme [19,21]. This process is regulated by the binding of the polynucleotide substrate and is, in consequence, responsible for the status of the enzyme (''open *vs* closed'') [19,22]. Thus, it is conceivable that the addition of HCV(1487–1500), a surrogate of motif VI, could block the function of the ''gatekeepers'' that is fulfilled by motifs II and VI. In accordance with this supposition are observations that the modus of the inhibition of the helicase by the peptide is rather competitive (with an estimated K_i of 90 nM). On the other hand, it is known that motif II participates directly in binding and hydrolysis of NTP [19,20,22]. Therefore, additionally soluble motif VI should interfere with the NTPase reaction. Nevertheless, no changes of the kinetic parameters of the ATPase reaction were observed. Our second study describes the binding sites of the peptide [32]. As shown in a model the peptide is twisted around domain 1, and therefore, fills the cleft between domains 1 and 2 as well as domains 1 and 3. It was previously reported that the HCV NTPase/helicase, similarly to that of *Bacillus stearothermophilus*, possesses an overwhelming negatively charged surface [17,33]. Arg 1487, Arg1490 and Arg1493 are esposed to the solvent and critical for interaction with the substrate. Furthermore the negative electrostatic surface appears capable of binding nucleic acids [17]. This was also confirmed by us [32]. Other mechanisms of action of the peptide – although less possible – could be taken into account. Apparently, at the tested concentrations of DNA substrate no change of the inhibitory parameters was observed. However, it could be not certainly excluded that the concentration range of the DNA substrate was too narrow to draw any conclusion regarding the mode of inhibition. As previously shown with the WNV NTPase/helicase and HCV enzyme strong substrate/product inhibition of the unwinding reaction was observed when the DNA substrate concentration exceeded 15 pM ([24,30], and data not shown).

The inhibition of all helicases was strongly dependent on the structure of the peptide. The scrambled [rd]HCV(1487–1500) has the same charge like HCV(1487–1500) but acts only modestly as inhibitor of the helicase activity. Similar evidences were obtained with the respective 17-tides. Extending the HCV(1487–1500) peptide by three NH_2-terminal amino acid residues reduced significantly the inhibitory potential of the peptides. Probably, the additional amino acids changed the structure of the peptide and in consequence hindered an effective binding of the peptide to the enzyme. An analogous situation was observed in intact holoenzyme. Mutagenesis studies performed with numerous NTPase/helicases indicated that for their intact biochemical functions correct folding of motif VI is necessary. The structure of the Arg-rich motif guarantees the exposition of Arg residues to the solvent and

respective burial of other amino acids within the protein [17,19,20]. Thus, it is conceivable that the structure of HCV(1487–1500) mimics the conformation of the motif VI embedded in domain 2 [17].

In this context, previous findings are very interesting demonstrating the effective inhibition of protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA) by here described HCV(1487–1500) and its shortened derivatives [34,35]. Some features of the inhibition are common for protein kinases and HCV NTPase/helicase. For example in both cases the 14-tide was the most potent inhibitor and this effect decreased in the order: 11-tide > 8-tide > 5-tide. Further, the peptide was a competitive inhibitor in regard to macromolecular substrates. Surprisingly, the protein kinases possess very similar amino acid motifs, localized on their regulatory domains. Peptides reproducing the motifs are also potent competitive inhibitors of PKC and PKA respectively [33,34]. These observations make the mechanisms of inhibition of the unrelated enzyme groups somewhat similar. In this context it could be carefully suggested that the amino acid stretch HCV(1487–1500) fulfills the function of an ''autoregulatory'' domain of HCV NTPase/helicase. Thus, the development of a novel class of peptide inhibitors reproducing the structure of the ''autoregulatory'' motif could lead to effective antivirals. Additionally, further amino acid substitutions combined with chemical modifications could lead to high effective and selective compounds. Such attempts were performed with PKC and other protein kinases [36].

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