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New Non-nucleoside Inhibitors of Hepatitis C Virus RNA-Dependent RNA Polymerase

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Abstract—Recombinant RNA-dependent RNA polymerase of hepatitis C virus was purified using a bacterial expression system (*Escherichia coli*). The system for enzyme activity detection was optimized. The maximum activity was achieved when the reaction was carried out at 30°C in the presence of 3 mM Mg^{2+} or 0.75 mM Mn^{2+}. Among α- and β-pyrogallaldehydes, effective inhibitors were found. It was shown that they acted at the primer elongation stage, and their binding to the protein is reversible.

Key words: hepatitis C virus, RNA-dependent RNA polymerase, non-nucleoside inhibitors

Hepatitis C virus (HCV) is a major human pathogen, which has infected an estimated 170 million people [1]. Among them, more than 80% develop chronic hepatitis that later often progresses into cirrhosis (>20%) and hepatocellular carcinoma (1-4% per year) [2]. In comparison, hepatitis B infection turns into chronic hepatitis in only 5% of cases.

At present, HCV therapy is based on the use of the interferon-α alone or in combination with ribavirin [1, 2]. It should be noted that these medicines are effective in only 40% of patients infected by HCV genotype. Besides, they cause a number of side effects. The search for new anti-HCV agents is hindered by the lack of laboratory infection cell systems for screening compounds [3]. The only model for antiviral activity determination is the artificial HCV RNA replicon system [4]. Hence, the isolation of the key HCV enzymes and construction of the system for *in vitro* searching of enzyme inhibitors is a problem of great importance.

HCV is a positive-stranded RNA virus with a genome of 9.6 kb that encodes a single polyprotein. The processing of the latter results in at least nine proteins [5], and they can be classified as structural (C-protein of nucleocapsid, E1 and E2 glycoproteins of virus capsule) and non-structural proteins. The latter are NS2-protein (the component of an NS2-3 autoproteinase) and NS3-protein, whose N-terminal domain is a chymotrypsin-like protease and the C-terminal one is an NTP-dependent helicase. NS4A- and NS4B-proteins are a cofactor of NS3-protease and a negative regulator of helicase, accordingly. NS5A is a phosphoprotein and is believed to be a component of the replication complex; moreover, it is involved in developing of interferon resistance. NS5B is an RNA-dependent RNA polymerase (RdRp), and is the main component of virus replication complex. This enzyme can direct *de novo* RNA synthesis [6, 7], and it takes part in the replication of both (+) and (–) strands of viral RNA [8]. Inhibition of RdRp affects replication and inhibits virus reproduction.

At present, the intense search for new inhibitors of HCV being carried out in many laboratories is based on the screening of low molecular weight compounds using *in vitro* recombinant protein assays [1]. The isolation of full-length NS5B is hindered by the presence of 21 hydrophobic amino acids on the C-terminal of the protein molecule that results in the formation of inclusion bodies [9]. The deletion of this domain allows the isolation of soluble form of the protein (NS5BΔ21) without affecting the enzymatic activity [9, 10].

The inhibitors of HCV RdRp described so far can be classified into three groups [1]. The first consists of modified nucleoside analogs such as 2′-O-methyladenosine

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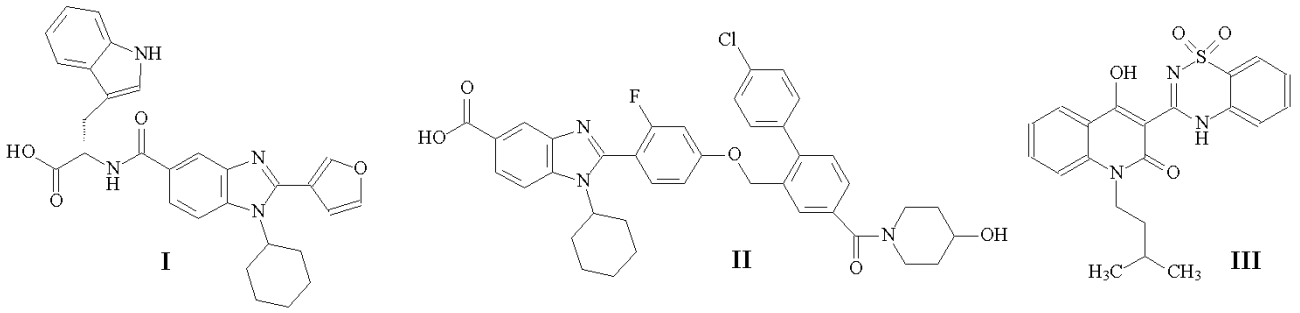


Scheme

and 2′-O-methylcytidine [11]. The compounds that belong to the second group are pyrophosphate mimics, and their action is based on chelating of the metal ions in the enzyme active site [1]. The compounds of the third group are non-nucleoside inhibitors. Among them, the derivatives of benzimidazole (I, II) and benzothiadiezine (III) have been found (see Scheme).

Subgenomic replicon study showed that submolecular concentrations of the compound III effectively decreased the level of viral RNA without apparent cytotoxicity [12]. These agents appear to bind directly to the polymerase before elongation complex formation, thus preventing viral RNA synthesis [13]. Nevertheless, both binding sites and a detailed mechanism of action of such compounds remain unknown. Hence, the synthesis of new non-nucleoside inhibitors of RdRp and elucidation of mechanism of their action are of great value.

Here we present the expression and purification of the HCV RNA-dependent RNA polymerase, optimization of the RdRp assay, and a new class of non-nucleoside inhibitors of the enzyme.

MATERIALS AND METHODS

Reagents and materials were purchased from the following sources: *E. coli* strain BL-21-CodonPlus® (DE3)-RIL from Stratagene (USA); vector pET-21d and Ni-NTA-agarose from Novagen (USA); poly(U)-Sepharose CL-6B from Amersham (UK); bacto-tryptone, yeast extract, and bacto-agar from Difco (USA); Tris and 2-mercaptoethanol from Merck (Germany); glycerol, dithiothreitol, imidazole, Triton X-100, Nonidet P-40, ammonium persulfate, phenylmethylsulfonyl fluoride (PMSF), and leupeptin from Sigma (USA); acrylamide and methylene-bis-acrylamide from Roth (Germany); EDTA and pepstatin from Serva (Germany); Tween-20 and Coomassie Brilliant Blue R-250 from Bio-Rad (USA); TEMED from Reanal (Hungary); and DE-81 filters were from Whatman (UK). Other reagents were from Reakhim (Russia). The enzymes were purchased from Promega (USA) and Sibenzyme (Russia). The synthesis of the non-nucleotide inhibitors will be the subject of the next publication.

Expression and isolation of HCV NS5BΔ21 protein. cDNA corresponding to nucleotide sequence of NS5B was the kind gift of Prof. R. Bartenschleger (Institute for Virology, Johannes-Gutenberg University Mainz, Germany). We used site-directed mutagenesis and polymerase chain reaction (PCR)-technique and isolated plasmid pET-21;5BΔ21 based on vector pET-21d for subsequent enzyme expression. The final recombinant construction was controlled by sequencing. *E. coli* strain BL-21-CodonPlus® (DE3)-RIL, containing additional copies of genes of rare tRNAs (*argU*, *ileY*, *leuW*), was chosen as the host. The transformation of the competent cells BL-21-CodonPlus® (DE3)-RIL was performed using the standard methods [14]. The cells were grown in LB medium containing 150 μg/ml ampicillin at 37°C with stirring (160 rpm) to an optical density of 0.6 at 550 nm. Lactose was added to the final concentration of 10 g/liter and the cells were grown for an additional 18 h at 22°C and then harvested by centrifugation (4000*g* for 30 min at 4°C). The cell pellet obtained from 1 liter of culture was resuspended in 40 ml of buffer A (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10 mM imidazole, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 1 mM 2-mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). The cells were sonicated on ice (8 × 30 sec with 1-min intervals) using a Cole-Parmer (USA) ultrasonic disintegrator at 44 kHz. Recombinant protein was isolated according to [12] with several modifications. The suspension was centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was loaded onto a Ni-NTA-agarose column (Novagen) and the column was washed with buffer A and then with the same buffer containing 50 mM imidazole. The recombinant protein was eluted using 200 mM imidazole in buffer A. The fractions containing NS5BΔ21 were diluted threefold with buffer B (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 5% (v/v) glycerol, 1% Nonidet P-40) and applied to a poly(U)-Sepharose CL-6B column. The column was washed with buffer B containing 400 mM KCl, and then the protein was eluted with the same buffer containing

800 mM KCl. The target fractions were stored in aliquots at −20°C in 30% glycerol.

RNA polymerase activity. In a standard 20-μl reaction volume, 0.3 μg of NS5B was incubated with 100 μg/ml poly(A), 25 μg/ml $(Up)_5U$, 1 μM UTP, and 1 μCi of [α-^{32}P]UTP (4000 Ci/μmol) in transcription buffer C (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 μg/ml BSA). The enzyme was preincubated with poly(A) and $(Up)_5U$ in the same buffer C for 20 min at 20°C, then UTP and [α-^{32}P]UTP were added and the samples were incubated for 30 min at 30°C. Afterwards the samples were applied onto DE-81 filters. The filters were washed four times with 0.5 M sodium phosphate buffer (pH 7.0), then once with ethanol and dried in air. The bound radioactivity was measured by the Cherenkov method.

Investigation of properties of the inhibitor. The IC_{50} was determined under the experimental conditions described above. The inhibitor concentrations varied from 0.3 μM to 1 mM. In a standard assay, the inhibitors were added to the reaction mixture together with UTP after preincubation. The compounds of 1,4-diaminopiperazine (DAP), N^4-(methyl)benzyl-N^1-aminopiperazine (MB), and aminomorpholine (MO) series (see table) were dissolved in dimethylsulfoxide (DMSO), and 1 μl was added to the reactions, the same volume of DMSO being added to the control samples. The compounds of series N^4-methyl-N^1-aminopiperazine (MM) and N^4-dimethyl-N^1-aminopiperazine (DM) were dissolved in water. For the investigation of the effect of the compounds on primer elongation, 20 ng/ml of heparin was added to the samples simultaneously with UTP and the inhibitors.

Investigation of reversibility of enzyme–inhibitor binding. NS5B (0.3 μg) and 10 μM of compound MM-345 were incubated in 2 μl of 10 mM Tris-HCl (pH 7.5) buffer containing 200 mM KCl and 0.5 mM 2-mercaptoethanol. After 10-min incubation, the mixture was diluted to 18 μl with transcription buffer containing necessary preincubation components (see above). After 10 min of preincubation, UTP and [α-^{32}P]UTP were added and the reactions were carried out as described above.

RESULTS AND DISCUSSION

NS5BΔ21 expression and purification. To obtain recombinant NS5B as a fusion protein containing a $(His)_6$ at the C-terminus of the molecule, we chose the pET-21d expression vector. Owing to a considerable quantity of rare codons within NS5B, we used *E. coli* BL-21-CodonPlus® (DE3)-RIL strain, whose genome contains additional copies of genes of several rare tRNAs. Protein expression was induced by adding lactose; this agent is an inexpensive and available analog of isopropyl-1-thio-β-D-galactopyranoside (IPTG) [15]. In some cases, the use of this reagent reduces inclusion body formation [16]. The expression was carried out at 22°C because more than 90% of the recombinant protein was found in soluble form under these conditions.

Previously described methods for NS5BΔ21 isolation include a number of requirements for the composition of the lysis buffer. For example, the concentration of NaCl or KCl must be at least 300 mM [10], and the glycerol concentration must be no less than 2.5% for complete protein extraction [17]. In our experiments, the isolation of the protein was performed in lysis buffer containing 500 mM NaCl, 10% glycerol, and 0.5% Triton X-100. It should be noted that in the absence of the detergent and lower glycerol concentration, the enzyme yield markedly decreased; moreover, a high level of contaminant proteins was observed during the affinity chromatography.

The presence of several protease inhibitors in the lysis buffer is the other essential requirement for the successful isolation of NS5BΔ21 protein. When the purification procedure was carried out in the absence of leupeptin, the SDS-PAGE analysis of fractions obtained after the Ni-NTA-agarose chromatography showed that the target NS5B underwent a proteolysis that resulted in a number of shortened proteins (50-62 kD), and the yield of full-length protein was rather low.

The purity of the protein obtained was >95% according to SDS gel electrophoresis data. To concentrate the enzyme, we performed chromatography on a poly(U)-Sepharose CL-6B column. After this procedure, the concentration of the protein was 0.5 mg/ml. The yield of the enzyme was 0.6-0.8 mg from 1 liter of cell culture.

Optimization of RdRp assay conditions. The RdRp activity was measured by incorporation of [α-^{32}P]UTP into the 3′-terminal of the oligonucleotide in a system of poly(A)-template and $(Up)_5U$-primer. Such assay has been previously described by several groups worldwide. However, a number of key parameters of the assay differ in various papers. They are: concentration of the metal ions, reaction temperature, and time of incubation, and, finally, the necessity and the time of preincubation procedure for the formation of the enzyme ternary complex. For example, Lohmann et al. [18] reported that the maximal enzyme activity with poly(C)–oligo(G) could be achieved in the presence of 12.5 mM Mg^{2+} or 7-9 mM Mn^{2+}, whereas the best results for poly(A)–oligo(U) system were obtained at 2.5-5 mM Mg^{2+} according to Yamashita et al. [9]. Besides, the latter paper [9] asserted the optimal reaction temperature to be 30°C; however, in a recent work of O'Farrell et al. [19] it was shown that the enzyme activity at 37°C was 3-5 times higher than at 30°C. It was also found that the HCV RdRp activity increased after the preincubation of the enzyme with template and primer in the absence of nucleoside triphosphate when compared to the assay without preincubation

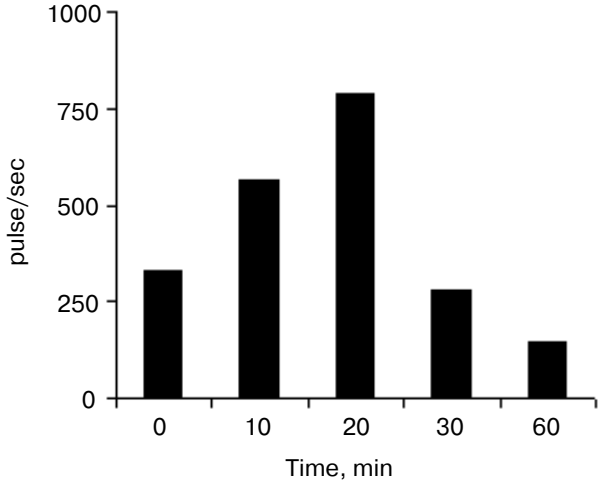


Fig. 1. Dependence of the enzyme activity on the time of preincubation with poly(A) template and $(Up)_5U$ primer.

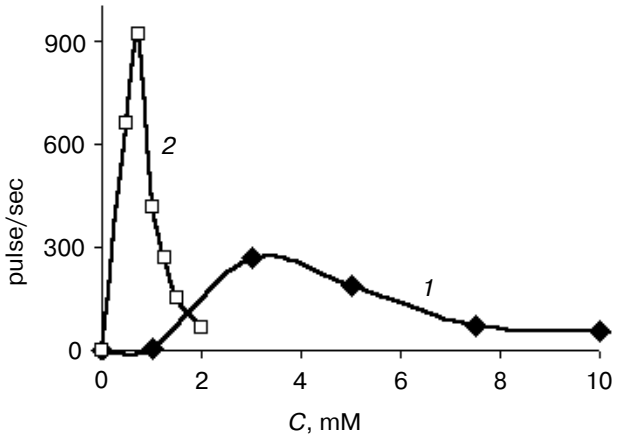


Fig. 2. Dependence of the enzyme activity on Mg^{2+} (*1*) and Mn^{2+} (*2*) concentration.

[20]. Proceeding from the aforesaid, several assay conditions had to be optimized.

The dependence of the enzyme activity upon the preincubation time with template and primer is presented at Fig. 1. One can see that the optimum preincubation time is 20 min; longer preincubation led to a drop in activity, apparently due to the inactivation of the enzyme being released from the complex with RNA template.

The determination of the optimum Mg^{2+} and Mn^{2+} concentrations was also performed. It was reported that in the polymerization reaction Mg^{2+} could be replaced by Mn^{2+} [18], but no other metal ions, such as Fe^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, and Zn^{2+} [19]. Moreover, Zn^{2+} being in concentration >100 mM inhibits the enzyme activity [18]. It was shown that the maximum activity of RdRp was achieved in the presence of 3 mM Mg^{2+} or 0.75 mM Mn^{2+} (Fig. 2). In spite of the fact that the RdRp activity in the presence of Mn^{2+} was several times higher than in the presence of Mg^{2+}, for further experiments Mg^{2+} was chosen because of a lesser influence of the changes in its concentration on the enzyme activity.

In agreement with the data of Yamashita et al. [9], we ascertained that the optimum reaction temperature was 30°C. Under these conditions, the linear dependence of product accumulation on the reaction time was maintained during 30-40 min (Fig. 3). Subsequent decrease in the reaction rate appears to be due to the destruction of the RNA/RdRp complex followed by inactivation of the enzyme [20]. Therefore, we attempted to stabilize the enzyme by the addition to the reaction mixture of BSA (50 μg/ml), glycerol (10%), or nonionic detergent Tween-20 (0.05%). As can be seen from Fig. 3, neither BSA nor Tween-20 affected the polymerase activity or the linear part of the kinetic plot. In contrast, the addition of glycerol led to a substantial decrease in the reaction rate together with the prolongation of the linear kinetic plot up to 2 h. A most likely explanation of a low reaction rate could be a high viscosity of the reaction mixture.

Thus, maximum enzyme activity was achieved when RdRp was preincubated with the template and the primer for 20 min at room temperature in the buffer containing 3 mM Mg^{2+} before the addition of nucleoside triphosphate followed by the reaction for 30 min at 30°C.

Pyrogallaldehydes and their hydrazones as inhibitors of HCV RdRp. Antibiotics that block the DNA/RNA-interacting enzymes often contain a hydroxylated aromatic moiety (i.e., rifamycin, amanitin, etc.). The polarity of the phenolic hydroxyl group makes them effective donors and/or acceptors of hydrogen bonds, thus giving

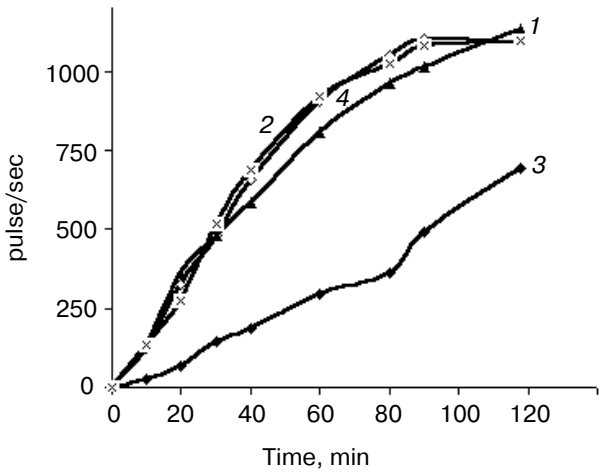
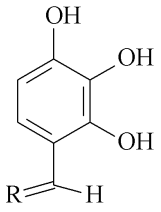
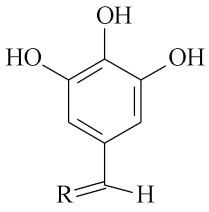


Fig. 3. Kinetic plot of accumulation of product of primer elongation reaction catalyzed by HCV RNA-polymerase in the presence of BSA (*2*), glycerol (*3*), Tween-20 (*4*), or their absence (*1*).

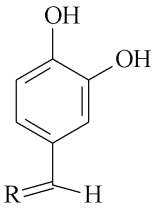
good possibilities for the interaction with different amino acid residues. For example, the O–H bond dissociation might lead to electrostatic interaction with positively charged arginine and lysine residues, which may contact with phosphate groups of DNA or RNA in the enzyme active site [21]. The detailed investigation of the inhibition of HIV integrase activity by polyatomic phenols has been reported earlier [22-24]. The inhibition of a number of bacterial DNA polymerases by β-C-nucleoside-5′-triphosphate bearing 3,4-dihydroxybenzene instead of nucleic base has also been described [25]:



series 234



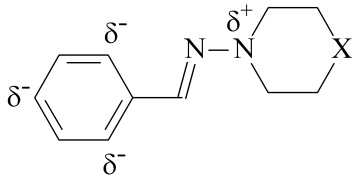
series 345



series 34

We found that isomeric 2,3,4- and 3,4,5-trihydroxybenzaldehydes can act as rather effective inhibitors of HCV RdRp. The IC_{50} values for α-isomers are two orders of magnitude lower than that for their β-isomers (see table). In spite of the fact that two OH-groups in positions 3 and 4 are common in both structures, the corresponding 3,4-dihydroxybenzaldehyde appears to be almost inactive. To explain such significant differences in the properties of the compounds under study, we assume that (–)-mesomeric effect of the formyl group can significantly influence the inhibitory properties of the compounds by decreasing pK_a values of the phenolic hydroxyl groups. In the case of α-pyrogallaldehyde there are two groups being affected, namely, 2-OH and 4-OH, whereas in the case of both β-pyrogallaldehyde and 3,4-dihydroxybenzaldehyde only the 4-OH group is affected.

To elucidate the effect of the residue, we synthesized three parallel series of hydrazones of α- and β-pyrogallaldehydes and 3,4-dihydroxybenzaldehyde with N-aminomorpholine, 1-amino-4-methylpiperazine, and derivatives of the latter (see table, substituents MO, MM, DM, and MB). In comparison with formyl group, a substituted hydrazine exerts donor effect upon the aromatic system according to the (+)-mesomeric mechanism:

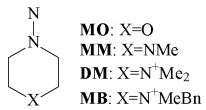
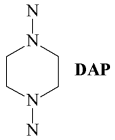


It is well known that donor substituents do not affect significantly pK_a values of phenolic hydroxyl groups; therefore, the differences in acidity of hydrazone hydroxyls are leveling out. As can be seen from the table, hydrazones of series 34 proved to be rather weak inhibitors of HCV RdRp. Compounds of series 345 demonstrated 100-fold higher activity, whereas the activity of the compounds of series 234 was 5-fold lower than that of the corresponding aldehydes. It should be noted that the fixed positive charge in piperazine moiety (X = N^+Me_2 and N^+MeBn) had no influence on the IC_{50} values of the inhibitors of DM and MB series, despite the proposed reduction of donor effect for these compounds.

As one can see from the table, the derivatives of β-pyrogallaldehyde containing the electron donor piperazine moiety as well α-pyrogallaldehyde are the most effective inhibitors of HCV RdRp. The inhibition can be characterized by a double bend, and the almost total suppression of the enzyme can be observed at concentrations of 20-30 μM (IC_{90}).

It is noteworthy that the observed change in the inhibition properties of hydrazones compared to the corresponding aldehydes appears not to be the result of the interaction of the enzyme with the piperazine fragment of the inhibitor. Thus, neither variation of charge (MO and DM) nor size (DM and MB) of substituents at aliphatic moiety affected the activity of compounds of both series. Moreover, as can be seen from the table, the IC_{50} values of symmetrically substituted hydrazones of 1,4-diaminopiperazine (DAP) in each series were twice lower than that of the corresponding mono-substituted analogs. This could be accounted for by the doubling of the aromatic component of the inhibitors. These data support the statement that the aromatic system and its internal electronic density play the crucial role in the enzyme–inhibitor interaction. Detailed study of the effect of the substituents of pyrogallic structures on their

Structures of the tested compounds

R	IC_{50}, μM		
	series 234	series 345	series 34
O	7	1000	>1000
 **MO**: X=O **MM**: X=NMe **DM**: X=N^+Me_2 **MB**: X=N^+MeBn	30-40	10	>400
 **DAP**	10-20	5	100-200

Note: The IC_{50} values were determined as described in the "Materials and Methods" section.

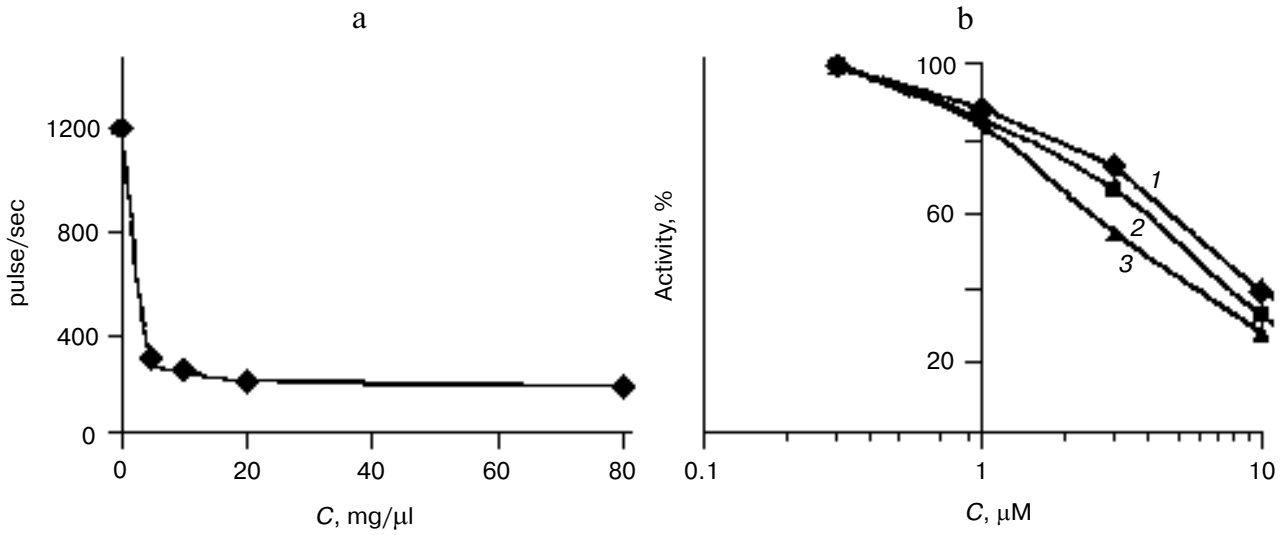


Fig. 4. Dependence of enzyme activity on the heparin (a) and DAP-345 concentration (b) when the inhibitor was added after preincubation together with heparin (*1*), in the absence of heparin (*2*), or before preincubation (*3*).

inhibitory activity for HCV RdRp and other related enzymes will be reported elsewhere.

Study of the inhibition mechanism. In the experiments described above, the inhibitors were added to the reaction sample together with nucleoside triphosphate after preincubation, i.e., after the formation of the ternary complex. Under these conditions, the observed effect may be due to affecting either transcription initiation or primer elongation. To elucidate the stage of enzymatic process we used heparin sulfate. According to Tomei et al. [20], the addition of heparin to the reaction mixture after the preincubation traps unbound enzyme molecules, thus preventing reinitiation of the RNA synthesis. This experiment can be used to study the solely elongation stage of the process. It was shown that heparin at concentrations higher than 10 ng/μl completely inhibited the synthesis reinitiation (Fig. 4a). It should be noted that in this case the activity of RdRp was 5-6-fold lower than that of the enzyme in the absence of heparin. The IC_{50} value for the symmetrical derivative of pyrogallaldehyde DAP-345 was the same both in the presence and absence of heparin (Fig. 4b). Hence, the compounds under study appear to affect the primer elongation reaction.

The possibility of the hampering of the enzyme–template–primer ternary complexes by the inhibitors was also taken into account. To study this possibility, DAP-345 was added into the reaction mixture either before or after preincubation of the polymerase with poly(A) and $(Up)_5U$. As can be seen in Fig. 4b, the efficacy of the inhibitor in these two assays was about the same. Thus, the inhibitor affects the primer elongation reaction only.

One of the possible mechanisms of the inhibitory effect of compound DM-345 at the stage of primer elongation might involve competition with UTP. However, the inhibition was found to be noncompetitive towards this substrate (Fig. 5). Hence, the inhibitor can bind to the enzyme both in the absence and in the presence of UTP, and the inhibitor does not hamper the UTP binding.

The next step was to study the reversibility of the binding of the inhibitor that causes the enzyme inactivation. For a number of structurally related polyphenolic compounds that are inhibitors of HIV integrase, the irreversible mechanism of action was proposed [24]. For the study of the nature of binding of the compounds to the RdRp, the following method was used. When the enzyme is incubated with the reversible inhibitor, being in concentrations close to its IC_{50} value, the dilution of the reaction mixture by tenfold is accompanied by a recovery of the enzyme activity to the control level. On the contrary, in the case of irreversible binding the inhibition is not

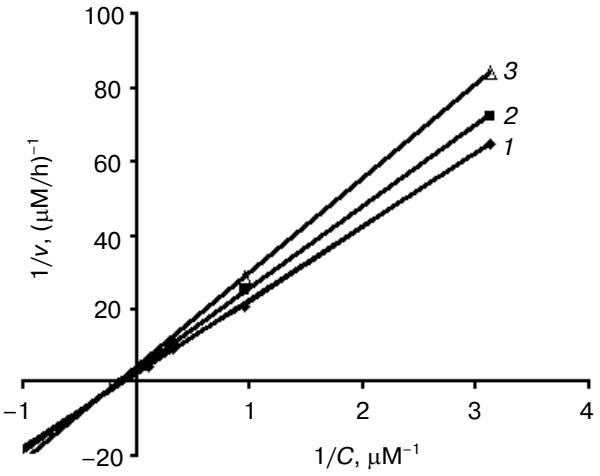


Fig. 5. Dependence of the enzyme activity on UTP concentration in the absence of inhibitor MM-345 (*1*) or in the presence of 5 (*2*) and 10 μM (*3*) of MM-345.

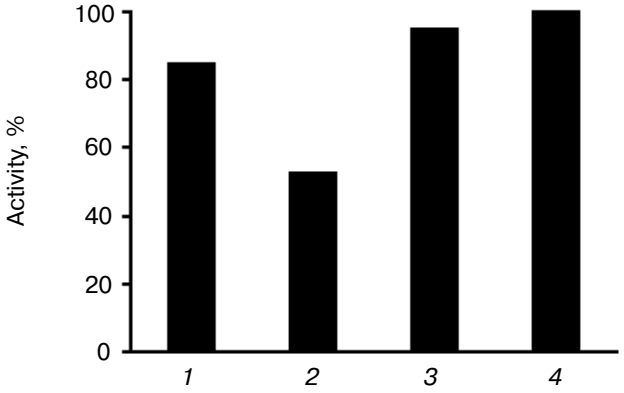


Fig. 6. Analysis of the reversibility of the enzyme–inhibitor binding. The inhibitor concentrations during the initial incubation and after the sample dilution were 10 and 1 μM, correspondingly (column *1*), 10 μM constant (column *2*), 1 μM constant (column *3*), and 0 μM constant (column *4*).

affected by subsequent dilution of the sample. For this experiment, we chose the water-soluble derivative of pyrogallaldehyde DM-345 (for details see "Materials and Methods"). The results are presented in Fig. 6. When the enzyme was first incubated with 10 μM of DM-345 followed by tenfold dilution, the enzyme activity was 85% of control value (Fig. 6). However, when the inhibitor concentration of 10 μM was kept during all stages of the experiment, 50% inhibition of the enzyme activity was observed. Therefore, the studied compounds are reversible inhibitors of HCV RdRp.

In conclusion, RNA-dependent RNA polymerase of HCV was isolated and the enzyme activity assay was optimized. Among the series of α- and β-pyrogallaldehydes and their derivatives, a number of effective enzyme inhibitors were found. The compounds affected the primer elongation reaction, and their binding to the enzyme was found to be reversible.

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