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RNA-Dependent RNA Polymerase of Hepatitis C Virus: Study on Inhibition by α,γ-Diketo Acid Derivatives

M. V. Kozlov*, K. M. Polyakov, S. E. Filippova, V. V. Evstifeev, G. S. Lyudva, and S. N. Kochetkov

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, 119991 Moscow, Russia; fax: (495) 135-1405; E-mail: kozlovmv@hotmail.com

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Abstract—It is supposed that α,γ-diketo acids (DKAs) inhibit the activity of hepatitis C virus RNA-dependent RNA polymerase (RdRP HCV) via chelation of catalytic magnesium ions in the active center of the enzyme. However, DKAs display noncompetitive mode of inhibition with respect to NTP substrate, which contradicts the proposed mechanism. We have examined the NTP substrate entry channel and the active site of RdRP HCV for their possible interaction with DKAs. The substitutions R48A, K51A, and R222A greatly facilitated RdRP inhibition by DKAs and simultaneously increased K_m values for UTP substrate. Interestingly, C223A was the only one of a number of substitutions that decreased K_m(UTP) but facilitated the inhibitory action of DKAs. The findings allowed us to model an enzyme–inhibitor complex. According to the proposed model, DKAs introduce an additional Mg^{2+} ion into the active site of the enzyme at a stage of phosphodiester bond formation, which results in displacement of the NTP substrate triphosphate moiety to a catalytically inactive binding mode. This mechanism, in contrast to the currently adopted one, explains the noncompetitive mode of inhibition.

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Key words: hepatitis C virus, RNA-dependent RNA polymerase, α,γ-diketo acid derivatives, inhibition mechanism, molecular modeling

The screening of a library of 200,000 compounds by a team of Italian researchers has shown that 2,4-dioxo-4-phenylbutyric acid (α,γ-diketo acid, DKA) (Fig. 1) functions as a selective and reversible inhibitor of RNA-dependent RNA polymerase of the hepatitis C virus (RdRP HCV, viral nonstructural protein NS5b) [1]. According to data of the same authors, inhibition was noncompetitive with respect to the NTP substrate and RNA template. Comparison of the effects of DKAs and a pyrophosphate analog, foscarnet, also showed that both compounds competed for the common binding center in the active center of RdRP [2]. Based on this fact, the authors suggested considering diketo acids as pyrophosphate analogs. According to their hypothesis, the enol-form dianion of DKAs forms a complex bond with Mg^{2+} or Mn^{2+} present in the enzyme active center, which, in turn, prevents the binding of NTP substrate and formation of phosphodiester bond. In accordance with this mechanism, the aromatic component of the inhibitor is responsible for specificity and strength of additional interactions with the binding center [3].

It should be noted that the competition between NTP substrate and DKAs for chelation of the same catalytic magnesium ions, assumed by the authors, does not correspond in any way to the noncompetitive mode of inhibition they revealed. It is obvious that the mechanism of action of diketo acids is more complex and needs further study. As has been asserted recently in respect to this issue, on transition from initiation to elongation DKAs change the mode of inhibition in relation to NTP substrate from noncompetitive to competitive [4]. Unfortunately, the authors give no detailed description of the experiments and their results, which impedes their critical analysis. Most likely, they indicate that the binding centers of NTP substrate and DKAs have common boundaries, which probably begin to overlap under structural rearrangements of the active center.

In spite of the great diversity of synthesized structural variants of DKAs, it is still unclear how DKAs are bound to the enzyme. The first assumptions in this

Abbreviations: DKAs, α,γ-diketo acids; RdRP HCV, hepatitis C virus RNA-dependent RNA polymerase.

* To whom correspondence should be addressed.

respect were suggested by Korean researchers in 2006 [5]. Using the crystal structure of an unusual RdRP complex with three UTP molecules [6] and remote structural similarity between DKAs and UTP, the authors proposed a model of interaction between inhibitor and enzyme. It included: (i) electrostatic interaction of diketo acid residue with catalytic Mn^{2+} ion; (ii) hydrogen bonds with Phe224 and Asp225; and (iii) a hydrogen bond with Leu159. As one can see, this model is in the channel of ideas suggested by the discoverers of DKAs [2].

In the previous work [7] we obtained the first indications that the inhibiting effect of DKAs can be realized on interaction with completely formed catalytic complex at the stage of phosphodiester bond formation. We have also suggested that Arg222 intensifies the interaction of enzyme and inhibitor due to formation of an additional hydrogen bond. In this report, we present further results on localization of the binding center of DKAs obtained by oligonucleotide-directed mutagenesis of the RdRP active center.

MATERIALS AND METHODS

Synthesis of DKA derivatives. DKA derivatives (Fig. 1) were synthesized in accordance with published procedures [1, 8]. The structures of the compounds were confirmed by ^{1}H-NMR spectra (DMSO-d6). Previously unpublished NMR spectral data on the compounds are given below: DKA-PrCO_2H – δ 7.81 (d, J = 6.9 Hz, 1H, CH), 7.60 (t, J = 7.2 Hz, 1H, CH), 7.26 (s, 1H, CH), 7.20 (d, J = 8.4 Hz, 1H, CH), 7.10 (t, J = 7.6 Hz, 1H, CH), 4.15 (t, J = 6.2 Hz, 2H, CH_2), 2.46 (t, J = 7.5 Hz, 2H, CH_2), 1.96-2.05 (m, 2H, CH_2); DKA-BnCO_2H – δ 7.94 (d, J = 7.8 Hz, 1H, CH), 7.64-7.70 (m, 2H, CH), 7.57-7.63 (m, 2H, CH), 7.43-7.52 (m, 2H, CH), 7.30 (d, J = 8.1 Hz, 1H, CH), 7.02 (s, 1H, CH), 5.53 (s, 2H, CH_2).

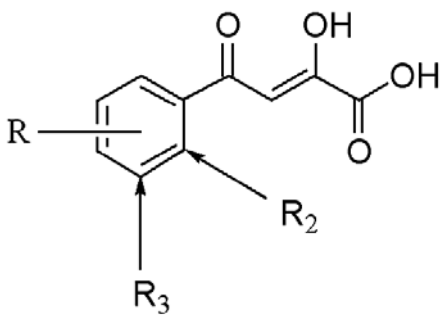
Expression and purification of RdRP. The source of RdRP shortened by 55 amino acid residues from the C-terminus was plasmid pET-21d-2c-5BΔ55 constructed previously in work [9]. Directed mutagenesis of the RdRP gene was carried out using the QuickChange kit (Stratagene, USA) according to the accompanying protocol. The sequences of forward (F) and reverse (R) primers for obtaining single amino acid substitutions (R48A, K51A, Q148A, K151A, R222A, C223A, and D352A) are given in Table 1. PCR included preheating for 30 sec at 95°C and then 16 cycles in the mode of 30 sec at 95°C, 1 min at 55°C, and 7 min 20 sec at 68°C. All substitutions in necessary positions were confirmed by sequencing.

The resultant mutant plasmids were used for transformation of *Escherichia coli* from the Rosetta line (DE3). After induction of the expression, the enzyme was purified on a column with Ni-NTA-agarose as described previously [9]. Target fractions were dialyzed at 4°C against buffer containing 20 mM Tris (pH 7.5), 30% glycerol, 350 mM NaCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol and stored at –20°C.

Determination of polymerase activity. Standard reaction mixture contained 0.3 μg RdRP, 100 μg/ml poly(rA), 25 μg/ml $(Up)_5U$, 10 μM UTP, and 1 μCi [α-^{32}P]UTP in 20 μl of buffer containing 20 mM Tris (pH 7.5), 20 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml BSA. The enzyme was preincubated with poly(rA) and $(Up)_5U$ for 15 min at 20°C, followed by addition of UTP and [α-^{32}P]UTP and further incubation for 30 min at 30°C. Then 10 μl of the reaction mixture was transferred to a DE-81 filter that was washed four times with 0.5 M PBS (pH 7.0) and once with ethyl alcohol and then dried in air. Radioactivity of the filters was measured according to Cherenkov.

The K_m(UTP) values were determined in the UTP concentration range of 1 to 500 μM and calculated using Origin 6.1 (OriginLab Corp., USA) in the Michaelis–Menten kinetic model. The K_m(UTP) measurement error was 20% with three parallel experiments.

Measurement of inhibiting effect of DKA. Polymerase reaction was performed in the presence of inhibitors in



DKA	R= H
DKA-Pr	R_2= -O-$(CH_2)_3$-H
DKA-PrCN	-O-$(CH_2)_3$-CN
DKA-PrCO_2H	-O-$(CH_2)_3$-CO_2H
DKA-Bn	R_3= -O-CH_2-Ph
DKA-BnCN	-O-CH_2-[(o-CN)Ph]
DKA-BnCO_2H	-O-CH_2-[(o-CO_2H)Ph]

Fig. 1. Molecular structure of DKA presented in enol form and list of compounds used in the work with indication of substituents.

Table 1. Structures of primers for directed mutagenesis of NS5bΔ55

Mutation	Forward (F) and reverse (R) primers
R48A	F 5' GCAGCGCAAGCCTG**GC**GCAGAAGAAGGTCACC 3' R 5' GGTGACCTTCTTCTGC**GC**CAGGCTTGCGCTGC 3'
K51A	F 5' GCCTGCGGCAGAAG**GC**GGTCACCTTTGACAGACTG 3' R 5' CAGTCTGTCAAAGGTGACC**GC**CTTCTGCCGCAGGC 3'
Q148A	F 5' GAGGTTTTCTGCGTC**GCG**CCAGAGAAGGGGGGC 3' R 5' CGGCCCCCCTTCTCTGG**CGC**GACGCAG 3'
K151A	F 5' CTGCGTCCAACCAGAG**GCT**GGGGGCCGC 3' R 5' GCTTGCGGCCCCC**AGC**CTCTGGCGC 3'
R222A	F 5' GCTTCTCATATGACACC**GCA**TGCTTTGACTCAACAGTCACTG 3' R 5' TCAGTGACTGTTGAGTCAAAGCA**TGC**GGTGTCATATGAGAAGCCC 3'
C223A	F 5'GGCTTCTCATATGACACCCG**GGC**CTTTGACTCAACAGTCACTG 3' R 5' CAGTGACTGTTGAGTCAAAG**GCC**CGGGTGTCATATGAGAAGCC 3'
D352A	F 5' CCCCCTGGGG**C**CCCGCCCCAACCAGAATAC 3' R 5' GTATTCTGGTTGGGGCGGG**G**CCCCAGGGGG 3'

the concentration range of 0.1 to 300 μM; for this purpose, DKA derivatives were first dissolved in DMSO, then mixed with an aqueous solution of UTP/[α-^{32}P]UTP and added to the reaction mixture after preincubation of the enzyme with the template and the primer as described. The IC_{50} measurement error was 10-20% with four parallel experiments. The inhibition mode was determined by Dixon's method at inhibitor concentrations of 1 to 300 μM and three UTP concentrations (0.5, 5, and 50 μM).

Molecular modeling and docking were performed in an Indigo 2 workstation (Silicon Graphics) using Turbo Frodo [10] and Molscript [11].

RESULTS AND DISCUSSION

In the previous work, we proposed a mechanism of RdRP inhibition by DKA derivatives according to which the diketo acid residue of the inhibitor chelates two Mg^{2+} ions of the catalytic complex via three oxygen atoms at the stage of phosphoryl group transfer [7]. In the proposed model, the cyano group of DKA-PrCN oxypropyl substituent (see color insert; Fig. 2) forms a hydrogen bond with Arg222 of the enzyme active center, which is in agreement with the experimentally observed intensification of inhibition as compared with DKA or DKA-Pr. For proof or disproof of the presumed position of the inhibitor, it was decided to study the effect of R222A substitution on the inhibiting effect of DKA-PrCN. In this context, it also seemed interesting to obtain Ala substitutions for other amino acids of the active center located in close proximity to Arg222 with the purpose of testing their effects on the strength of inhibiting action of various DKA derivatives.

Effect of RdRP mutations on polymerase activity. Amino acid residues Arg48, Lys51, Gln148, Lys151, Arg222, Cys223, and Asp352 were selected for mutagenesis based on analysis of crystal structure of the RdRP and UTP complex, where the triphosphate residue of the substrate, in the authors' opinion, occurs in the catalytically active mode [12]. As can be seen from Fig. 2, the mutations in the aggregate almost completely surround the NTP substrate entry channel and the triphosphate residue of the nucleotide in the catalytic center of the enzyme. The functional significance of residues Arg48, Lys151, and Arg222 for polymerase activity has been characterized previously. In particular, it has been shown that the mutant form of RdRP R48A in the presence of Mn^{2+} uses GTP as *de novo* initiating substrate with the K_m(GTP) value of 25.9 μM, which is 7.8 times higher than the wild type value. In the presence of Mg^{2+}, RdRP R48A, in contrast to the wild type polymerase, was unable to initiate RNA synthesis on the heterotemplate from dinucleotide primer GU. With the longer primer GUAUA, the activity was restored but the yield of RNA products was six times less compared with the wild type [13]. These results were a direct indication that R48A mutation disturbed K_m for

the initiating substrate. The K151A and R222A substitutions insignificantly influenced the polymerase activity of RdRP in the reaction of reverse copying of heterotemplate, the 3′-end sequence of which served as an internal primer. However, under conditions of a test system containing poly(rA) template and pUpU primer, the properties of the two mutant polymerases were drastically different. It was shown that RdRP K151A could elongate dinucleotide primer with 1.8-fold higher V_{max} value than in the initial enzyme; at the same time, the K_m(UTP) value was 300 μM, i.e. about 3-fold higher compared with the wild type. In the same system, the R222A substitution caused the complete loss of enzyme activity [14]. In the authors' opinion, this pointed to a more significant role of Arg222 in the delivery of NTP substrate to the active center of RdRP as compared with Lys151. There are no data in the literature on the mutagenesis of Lys51, Gln148, Cys223, and Asp352.

The yields of wild type RdRP and the mutant proteins were similar (Table 2). Them had different stability during storage and therefore were used within a few weeks but not later than one month after the stage of dialysis. Since the ratio of catalytically active and inactive polymerases in enzyme preparations was not determined, the activity values given in Table 2 are rather relative. It should be noted that the measurements were performed at UTP substrate concentrations much lower than the K_m values and, in this context, the observed defects in the activity could be associated both with disturbance of catalysis and with disturbance of substrate binding. Nevertheless, all of the proteins had polymerase activity under conditions of a test system containing the poly(rA) template and $(Up)_5U$ primer, including the mutant form R222A unable to elongate the dinucleotide primer pUpU on the same template [14]. Length extension of the primer to six nucleotides leveled the effect of this mutation on polymerase activity and restored the latter to the initial level. Nevertheless, the K_m(UTP) value was 2.5-fold higher than the wild type value: 160 μM. The K151A substitution proved to be insignificant both for the activity and for the K_m(UTP) value. Thus, the defects in RdRP due to this mutation and observed in the system with the primer pUpU [14] were compensated on application of the $(Up)_5U$ primer.

The substitution of Ala for positively charged Arg48 or Lys51 gave practically identical results, i.e. threefold decrease in polymerase activity and approximately 5-fold increase in K_m(UTP) values. The results for RdRP R48A correlate well with the literature [13]. It should be noted that the similar effect of R48A and K51A substitutions on enzyme activity suggests similar functional significance of these two amino acid residues. This conclusion is also confirmed by crystallographic data showing that Arg48 in the active center of RdRP forms a hydrogen bond with the γ-phosphate group of the NTP substrate, while Lys51 has electrostatic interaction with the same group [12]. The C223A substitution reduced the K_m(UTP) value 2.6 times, which is probably explained by diminution of steric obstacles for UTP entry to the catalytic center and(or) removal of the partial negative charge of the sulfhydryl group of cysteine. In comparison with the wild type, the changes in the properties of RdRP Q148A and D352A were minimal.

Selection of DKA derivatives. The phenyl ring of DKAs, with allowance for symmetry, has three different positions for modification. As is known from the literature, bulky substituents in *para*-position abruptly worsen the inhibiting properties of diketo acids, whereas bulky substituents in *ortho*- or *meta*-positions have different influence depending on their structure and the presence of functional groups (Cl, Br, CN, CO_2H, etc.) [1, 8]. In this work we have used *ortho*-oxypropyl and *meta*-oxybenzyl substituted DKAs with identical functional groups in the substituents (Fig. 2). Among Ala-substituted RdRP, we hoped to find an enzyme variant discriminating the *ortho*- and *meta*-series of DKAs that would indicate possible contact with the substituent.

Inhibition of polymerase activity by DKA derivatives. The initial experiments on inhibition of polymerase activity of RdRP WT by a set of diketo acid derivatives revealed the exceeding of IC_{50} values by 5-30 times as compared with the literature data (Table 3). An analogous 10-fold divergence of results for some DKAs had been observed still earlier, but its reason was not ascertained [15]. Nevertheless, the DKA-PrCN and DKA-BnCN derivatives under the experimental conditions showed an abrupt increase of inhibition intensity compared with

Table 2. Characteristics of the isolated mutant forms of RdRP

Characteristic	RdRP							
	WT	R48A	K51A	Q148A	K151A	R222A	C223A	D352A
Yield, mg/liter culture	4-6	4-6	4-6	1.5-2.5	1.5-2.5	1.5-2.5	1.5-2.5	4-6
Relative activity*	1.00	0.35	0.31	1.29	0.94	1.24	0.81	0.91
K_m(UTP), μM	67	340	350	85	64	160	26	86

* Values normalized to wild type RdRP activity.

Table 3. IC_{50} values (μM) for DKA derivatives tested on WT and Ala-substituted RdRP under conditions of polymerase reaction

Inhibitor	RdRP							
	WT	D352A	Q148A	K151A	R48A	K51A	C223A	R222A
DKA	30/5.6*	40	34	18	14	12.2	5.8	0.9
DKA-Bn	102/8.0*	88	69	84	25.5	11.6	8.8	1.4
DKA-Pr	42/3.9*	30	25	24	6.7	7	4.3	2
DKA-$BnCO_2H$	11.4/1.8*	30	21	32	29.5	4.4	1.7	0.74
DKA-$PrCO_2H$	22.7/2.8*	33	18	14	4.8	6.8	3.4	0.94
DKA-BnCN	3.5/0.23*	8.1	3.4	1.9	1.4	1.3	0.35	0.4
DKA-PrCN	10.5/0.38*	3	6.4	1.8	0.5	0.6	0.18	0.27

* Values taken from [8].

their structural analogs DKA-Pr and DKA-Bn (4 and 29 times, respectively), which is in good agreement with the identically calculated values from the above-cited work [1] (11 and 35, respectively).

Further testing of Ala-substituted RdRP revealed that all mutations could be divided into three groups in accordance with their effect on inhibition: (1) R222A and C223A intensified it many fold; (2) Q148A, K151A, and D352A were neutral; and (3) R48A and K51A demonstrated an intermediate effect (Table 3).

The order of mutant polymerases in Table 3 corresponds to the sequential increase in efficiency of their inhibition by an unsubstituted DKA molecule as a compound suitable for comparison with other derivatives. The presented results show that the gradual decrease in IC_{50} values of DKA in the series of K151A→C223 is finished by an abrupt 6.5-fold drop for R222A. The profile of inhibition by a series of *ortho*-oxypropyl DKA derivatives was different and characterized by a threefold drop of IC_{50} values for R48A and then gradual decrease to the minimal values. One of the reasons for the observed effect of this substitution could be Arg48 moving away from the spatially close *ortho*-oxypropyl substitute for the inhibitor. Each of the *meta*-oxybenzyl DKAs had an individual inhibition profile, which was probably due to conformational differences between the molecules. However, for all inhibitors of this series the order of mutations had a "point of decline" of IC_{50} values of 3-5-fold (DKA-Bn/R48A, DKA-$BnCO_2H$/K51A, and DKA-BnCN/C223A).

Determination of IC_{50} values showed that none of the mutations impaired in principle the inhibiting properties of any compound. Thus, formation of an additional hydrogen bond between Arg222 and the cyano group of DKA-PrCN was not confirmed. This fact by no means contradicted our previous suggestion that DKAs interact with the catalytic complex at the stage of phosphoryl group transfer, but cast doubt on our previous results on molecular modeling and docking [7]. At the same time, it was probable that the C223A and R222A mutations, maximally intensifying the effects of all DKAs, changed in principle the inhibition mechanism as it is. However, the mode of inhibition both for DKA in case of initial RdRP WT and for DKA-PrCN in case of RdRP R222A remained noncompetitive with respect to NTP substrate (Fig. 3). It should be mentioned that the twofold decrease in mutant enzyme activity relative to the wild type differs from the data presented in Table 2 and is explained by the fact that the corresponding preparation of RdRP R222A was used at a later time from the beginning of its isolation.

It is interesting that the R48A, K51A, and R222A substitutions, being compactly located in the same region of the active center (Fig. 2), simultaneously increased K_m(UTP) values and many fold decreased IC_{50} values for DKA derivatives. In this connection, it was necessary to explain (i) why under the noncompetitive inhibition mode the same amino acids affected both the binding of substrate and the effect of inhibitor and (ii) why all Ala substitutions in this region including C223A intensified the inhibition.

The answer to the first question is rather simple: since Arg48, Lys51, and, with appropriate conformation, Arg222 interact with the γ-phosphate of NTP substrate within the catalytic complex [12], their substitution by Ala destabilizes the position of the triphosphate residue, which in turn intensifies the inhibiting effect of diketo acids at the stage of phosphodiester bond formation. The answer to the second question, in our view, is that all of the above Ala substitutions clear additional space close to the catalytic center, which slightly opens the binding site of diketo acids and facilitates conformational adjustment of the catalytic complex and the inhibitor to each other. This explanation is favored by the fact that we have not found in this region a single substitution that would reduce the inhibition. It should be noted that both explanations of the observed effects in case of R48A, K51A,

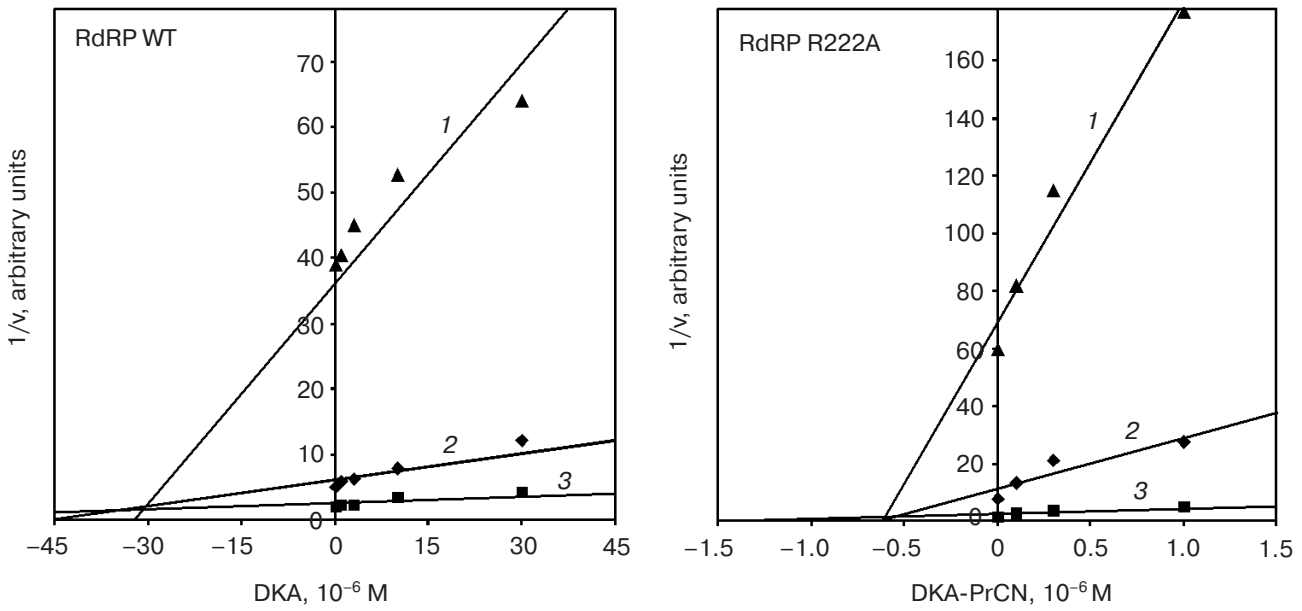


Fig. 3. Determination of inhibition mode by Dixon's method. Fragments of graphs in the region of crossing the abscissa axis are presented. UTP concentrations are designated as: 0.5 (*1*), 5 (*2*), and 50 μM (*3*).

and R222A substitutions are mutually complementary hypotheses. Indeed, intensification of the inhibition of activity of these mutant enzymes might be induced both by the weakening of NTP substrate binding and by intensification of inhibitor binding.

Docking of inhibitor. The baseline crystal structure of RdRP in complex with UTP (PDB ID 1NB7) was chosen for the docking of inhibitor in the enzyme active center [12]. In this structure, the triphosphate residue of the substrate was apparently in the catalytically active mode, because α-phosphate was located in the optimal position for the attack of the 3′-OH group of the growing nucleotide chain. Two catalytic Mn^{2+} ions are additionally coordinated by α- and β-phosphates, while γ-phosphate is directed from the catalytic center. It forms a hydrogen bond with Arg48 and interacts electrostatically with Lys51 and Lys151 (Fig. 2), which is in agreement with the literature data on the functional significance of these amino acid residues for RdRP activity [13, 14] and with our results (Table 2). The optimal position of the inhibitor was to satisfy several conditions simultaneously: to explain the very fact of inhibition and its noncompetitive character and to correlate with the results of mutational analysis and the relative force of the inhibiting effect of DKA derivatives. To meet these conditions, it was necessary to formulate the basic principle of the mechanism of inhibition.

We had to reject immediately the currently accepted hypothesis of inhibitor binding to the catalytic magnesium ion. The consequence of such binding would be lower availability of catalytic Mg^{2+} for NTP substrate and(or) disturbance of the catalytic complex geometry, which would inevitably result in competitive (before NTP binding) or uncompetitive (after NTP binding) inhibition mechanism and contradict the available literature and our own data.

Spectroscopic methods have shown that a DKA molecule is present in aqueous solutions of Mg^{2+} salts as an enol-form dianion due to the formation of a chelate with the metal in stoichiometric ratio of 1 : 1 [16]. The RdRP polymerase reaction requires Mg^{2+} concentration of the order of 5 mM. Thus, it is highly probable that DKA introduces an additional Mg^{2+} ion into the active center of the enzyme. Noncompetitive inhibition implies that substrate and inhibitor are bound simultaneously and independently and that the binding sites are not overlapped. We have supposed that on the interaction of inhibitor with the active center of RdRP the Mg^{2+} ion chelated by diketo acid involves the γ-phosphate of NTP substrate into its coordination sphere. As a result, there is a shift of the whole triphosphate residue relative to the catalytic mode of binding, which in turn makes impossible the formation of a phosphodiester bond. It should be noted that minimal chemical modifications of the diketo acid residue preventing the formation of enol-form dianion result in the complete loss of inhibiting effect [1].

The above assumption allowed us to begin work on inhibitor docking with positioning of the diketo acid-chelated Mg^{2+} ion in close proximity to the γ-phosphate of the NTP substrate. In accordance with the structure of the RdRP complex with UTP [12], amino acid residues Arg48, Lys51, Lys151, and Cys223 form the local environment for γ-phosphate (Fig. 2). It is easy to notice that Ala substitutions C223A, K51A, and R48A influenced the

strength of inhibition by all DKA derivatives (Table 3). Since the chelating diketo acid residue is their common structural element, it gave us a strong reason to position it into the free space between γ-phosphate and the above amino acid residues as shown in Fig. 4 (see color insert).

With the mentioned position of the inhibitor, the diketo acid chelate is open on the side of the Mg^{2+} ion for the attack by the γ-phosphate of the NTP substrate and on the opposite side for the hydrophobic interactions with R48 and K51. Below the plane of chelate, there is Cys223, the sulfhydryl group of which causes steric difficulties on the binding of the diketo acid residue. However, these difficulties can be partially eliminated by rotation about the C_α–C_β bond. Probably, just elimination of the sulfhydryl group of Cys223 as a result of C223A substitution is the reason of many fold increase in inhibition efficiency for all of the DKA derivatives (Table 3). In our model, Arg222 does not interact directly with any part of DKA molecule, and such a strong effect of R222A substitution on the inhibition of polymerase activity (Table 3) can be explained mainly by removal of steric obstacles in the way of the inhibitor to its binding center.

Orientation of the DKA phenyl ring directly bound to the diketo acid residue is obviously possible in two directions only: either to the catalytic center or to the NTP substrate entry channel (Fig. 4). The first direction was confirmed by the data on inhibition of mutant RdRP forms by the *ortho*-series of DKA derivatives, according to which Arg48 was spatially close to the *ortho*-oxypropyl substitute (see above). In the course of docking, we revealed that the adjacent *meta*-position of the phenyl ring could be directed towards the main protein chain in the region of Arg158. The distance between Arg158 and the DKA phenyl ring was 4.5 Å, implying their hydrophobic interaction (Figs. 4 and 5; see color insert). The literature shows that *para*-substituents of the DKA phenyl ring (Me, Et, and t-Bu) increase IC_{50} values (5, 47, and >50 μM, respectively) [1]. In our model, it could be due to limitation of the free space for *para*-substituents by Ser282 and by the main protein chain in the region of Ala159 (Fig. 5).

In comparison with the *ortho*-oxypropyl DKA derivatives, the docking of *meta*-oxybenzyl derivatives is much easier due to their lesser conformational flexibility and greater volume of the substituent. In Fig. 5, the DKA-BnCN molecule is shown in the internally unstrained conformation, which provides the optimal position of the 2-cyanobenzene residue in the enzyme cavity. This cavity is formed mainly by hydrophilic residues Tyr4, Asp225, Ser226, Cys279, Ser282, and, partially, hydrophobic residues Val161 and Ala281 and the main protein chain. The hydroxyl group of Ser226 stabilizes the position of inhibitor due to formation of a hydrogen bond with the cyano group of the benzene ring, which can explain 30-fold increase in the affinity of DKA-BnCN to the enzyme compared with DKA-Bn (Table 3). Besides, the hydrophilic nature of the cavity makes the position of hydrophilic benzyl residue of DKA-Bn energetically disadvantageous, reducing its affinity to the enzyme compared with an unsubstituted DKA molecule (Table 3).

It is considered that in the productive mode of binding to the catalytic center the α-phosphate of the NTP substrate forms two hydrogen bonds with the guanidine group of Arg158, while the 2′-OH group of ribose forms a hydrogen bond with the carboxyl group of Asp225 [6, 13]. The position of the inhibitor presented in Fig. 5 is not critical for these interactions, which is in agreement with the noncompetitive inhibition mechanism. Moreover, the presence of the NTP substrate probably stabilizes the position of the inhibitor due to the electrostatic interaction of γ-phosphate with the Mg^{2+} ion component of diketo acid chelate. It is known that the transfer of a phosphoryl group to the 3′-end of the growing polynucleotide chain is one of the two rate-limiting stages of the elongation cycle of viral RNA-dependent RNA polymerases [17, 18]. It is just the slow stages of the polymerase reaction where the inhibitor must act, and its activity is determined, in addition, by the rate of diffusion to the enzyme active center.

Thus, summarizing all the above, we believe that DKA derivatives block the polymerase activity of RdRp HCV at the stage of phosphodiester bond formation. The molecular mechanism of inhibition is based on the interaction of the inhibitor with the nucleotide mediated by the Mg^{2+} ion, resulting in a shift of the triphosphate residue of the NTP substrate relative to the catalytic binding mode. Such mechanism of inhibition of bacterial multisubunit RNA polymerases has been proposed previously for tagetitoxin – a natural phytotoxin produced by *Pseudomonas syringae* pv. *tagetis* [19, 20].

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