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Interaction of HIV-1 Reverse Transcriptase with Modified Oligonucleotide Primers Containing 2′-O-β-D-Ribofuranosyladenosine

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Abstract—Modified synthetic oligodeoxyribonucleotides containing 2′-O-β-D-ribofuranosyladenosine were used as primers in the RNA-dependent DNA synthesis catalyzed by HIV-1 reverse transcriptase. The degree of elongation of the primers depends on the position of the additional ribose unit, its presence in the specific position of the primer (−4) (and only in it) completely preventing elongation. Computer-modeled binding of the modified primers to the active site of reverse transcriptase demonstrated that steric hindrances arising from the interaction of the additional ribose residue with the reverse transcriptase region 262-270 interacting with the minor groove of the DNA substrate prevents elongation in the above mentioned case.

Key words: HIV-1, reverse transcriptase, 2′-O-β-D-ribofuranosyladenosine, oligonucleotides, inhibitory analysis, computer modeling

Human immunodeficiency virus type I (HIV-1) is a causative agent of acquired immunodeficiency syndrome (AIDS), one of the most dangerous and still incurable diseases. Reverse transcriptase (RT) is a key enzyme of the life cycle of this virus; it synthesizes the double-stranded proviral DNA, using viral RNA as the template. A DNA copy then incorporates in the host cell genome. Since RT is the only DNA polymerase functioning in the cytoplasm of HIV-infected cells, it is the main target for compounds blocking virus replication [1]. Many highly specific nucleoside (acting as terminators of DNA strand growth) and non-nucleoside (non-competitive) RT inhibitors have been described [2, 3]. However, none of such drugs is able to completely suppress the infection. The appearance of mutant virus forms resistant to a given drug is one of the main reasons for this. That is why the search for principally new HIV RT inhibitors is quite urgent [3, 4].

Modified oligodeoxyribonucleotides (ON) are also potential RT inhibitors: they mainly inhibit primers bearing terminating nucleotide (without 3′-hydroxy group) on their 3′-end [5-8]. However, these inhibitors are not widely used because the above mentioned mutant RT forms exhibit increased ability for hydrolytic cleavage of the terminating nucleotide at the 3′-end [8-10]. Earlier we suggested the use of a novel type of ON primers bearing a modified nucleoside 2′-O-β-D-ribofuranosyladenosine, not at the end but within the nucleotide strand, as primers in the reserve transcription reaction [11].

We recently developed convenient methods for obtaining 2′-O-β-D-ribofuranosylnucleosides [12] and their protected derivatives for ON synthesis [13-15]. Such modified nucleosides are known in nature, particularly, 2′-O-β-D-ribofuranosyladenosine (and the corresponding guanosine derivative) were found in the T-domain stem of yeast initiatory tRNA [16]. It is important that the substance can be used for post-synthetic modification of ON, i.e., oxidation of the additional ribofuranose residue with sodium periodate, giving dialdehyde derivatives. Earlier it was shown that such ON with reactive groups can be used for affinity modification of lysine residues in the ON-binding sites of proteins [13, 14]. In this work we studied the ability of ON containing 2′-O-β-D-ribofura-

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nosyladenosine to participate as substrates (primers) or inhibitors in the reaction catalyzed by HIV RT.

MATERIALS AND METHODS

Synthesis of modified nucleosides and oligonucleotides. For oligonucleotide synthesis, 2′-O-β-D-ribofuranosyladenosine and its corresponding derivatives were synthesized as described by us earlier [12-15]. Oligonucleotides were synthesized in micromolar quantities using commercial 2-cyanoethylphosphoramidites and standard synthetic procedures. To increase the yields, incorporation of a modified unit was performed during longer time of synthesis of the phosphodiester bond (80 sec) and at higher concentrations (0.15 M) of modified synthons. The ON were deblocked, removed from the solid carrier, and purified as described in [13-15]. The structure of ON **1a-6a** was proved by mass-spectrometry. 5′-^{32}P-terminal labeling of oligonucleotides **1a-6a** was performed using T4 polynucleotide kinase [17]; as a result, corresponding phosphorylated primers **1b-6b** were obtained:

1a 5′-GAC-GTT-GTA-AAA-CG-3′ (control),

2a 5′-G**X**C-GTT-GTA-AAA-CG-3′,

3a 5′-GAC-GTT-GT**X**-AAA-CG-3′,

4a 5′-GAC-GTT-GTA-**X**AA-CG-3′,

5a 5′-GAC-GTT-GTA-A**X**A-CG-3′,

6a 5′-GAC-GTT-GTA-AA**X**-CG-3′,

where **X** = 2′-O-β-D-ribofuranosyladenosine.

Isolation of HIV RT and estimation of its activity. Recombinant HIV-1 RT was purified from *E. coli* producer strain as described in [18]. 151-mer RNA fragment obtained by T7 transcription of *Pvu*II-linearized plasmid pTZR7G [19] was used as a template. ON 1-6 were annealed with the template according to the standard protocol [17] and used as primers in the RT-catalyzed reaction. For analysis of the primer elongation reaction catalyzed by RT, two systems of activity assay were used.

a) The substrate mixture (10 μl) contained 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.05% Nonidet NP-40, 3% glycerol, 200 U/ml RNAsine, 20 μM each dNTP, 0.05 μg RT, and variable concentrations of template with annealed 5′-^{32}P-phosphorylated primers **1b-6b**.

b) To determine kinetic parameters (K_m and relative V_{max}) of primer elongation by one nucleotide, the same substrate mixture contained $2 \cdot 10^6$ cpm [α-^{32}P]dATP and variable concentrations (1-10 μM) of unlabeled duplex of template with primers **1a-6a**. To determine K_i for **2a-6a**, the reaction was performed at available concentrations of template/primer **1b** and several fixed concentrations of template/primer **2a-6a**.

The samples were incubated for 45 min at 37°C and analyzed by polyacrylamide gel electrophoresis according to [17]. Then the gels were autoradiographed for 12 h at −70°C. For quantitative determination of primer elongation and determination of kinetic parameters of the reaction, the autoradiographs were scanned using a Fast Scan 300A densitometer from Molecular Dynamics (USA) and programs from Image Quant.

Preparation of oxidized modified primers and study of their interaction with RT. Primers **1a-6a** were phosphorylated with polynucleotide kinase [17], oxidized with sodium periodate according to [14], annealed with the template according to the standard protocol [17], and used in kinetic experiments. In experiments on affinity modification, the oxidized duplexes thus obtained were incubated with RT (30 min, 37°C) and treated with 100-fold excess sodium borohydride (1 h in the dark, 25°C). Then the samples were diluted with buffer for protein electrophoresis (without boiling) [17], incubated for 18 h at 25°C, and analyzed by electrophoresis with subsequent autoradiography as described earlier.

Binding of modified primers to the RT active site was modeled using the 3D-structure of the RT complex with oligodeoxyribonucleotide duplex of the template (25 nucleotides) and primer (21 nucleotide) and dNTP analog, ddTTP, [20] (Brookhaven Protein Data Bank, ID 1RTD) as the initial model. The WebLabViewerPro 3.7 program from Molecular Simulations Inc. was used for modeling. Additional ribosyl residues were incorporated in the primer in positions corresponding to the modified oligonucleotides **1a-6a**. Position and conformation of the additional ribose residue were modeled in accord with the structure of the decamer RNA duplex containing 2′-O-β-D-ribofuranosyladenosine determined by NMR [21] (C3′-*endo*-conformation, exposition into the minor groove of the duplex, torsion angle O2′-C1″-C2″-O2″ is −134°). To evaluate steric hindrances between additional ribose and protein, close interatomic contacts less than 70% of the sum of covalent radii were used.

RESULTS AND DISCUSSION

RT catalyzes DNA synthesis using HIV RNA as a template. The presence of ON primer bearing a free 3′-hydroxyl group is necessary for the elongation reaction to proceed. It was interesting to study the substrate properties of the modified primers bearing a bulky ribofuranose residue at the 2′-position in the reactions of DNA synthesis catalyzed by HIV RT using RNA as a template. Earlier we obtained several ON having such modification and their thermal stability was evaluated. Their melting

points demonstrated that modified ON form stable duplexes with DNA (ΔT_{melt} = 0°C) [15]. Insertion of 2′-O-β-D-ribofuranosylnucleosides to ON slightly destabilizes formation of duplexes with DNA (ΔT_{melt} = −2-3°C) [15]. To determine the effect of this modification on the structure of self-complementary decaribonucleotide 5′-r(GCGXAUUCGC)-3′ (X = 2′-O-β-D-ribofuranosyladenosine) in solution, high-resolution NMR spectroscopy and molecular dynamics were used [21]. Insertion of a bulky substituent at the 2′-position does not noticeably affect the thermal stability of the duplex. RNA in the composition of the duplex was found to correspond to A-helix, and an additional ribose residue is exposed into the minor groove of the ON duplex [21]. This residue adopts C3′-*endo*-conformation, and the additional O-glycoside bond is characterized by torsion angle O2′-C1″-C2″-O2″ equal to −134° (Fig. 1).

Modified oligonucleotides **1-6** synthesized in this study were annealed with the template according to the standard protocol [17], forming RNA–DNA duplexes of the following type:

3′-r[(N_{64}-CUG-CAA-CAU-UUU-GCU-GCC-N_{69})-5′]

5′-d[GAC-GTT-GTA-AAA-CG]-3′,

where N = A, G, C, U.

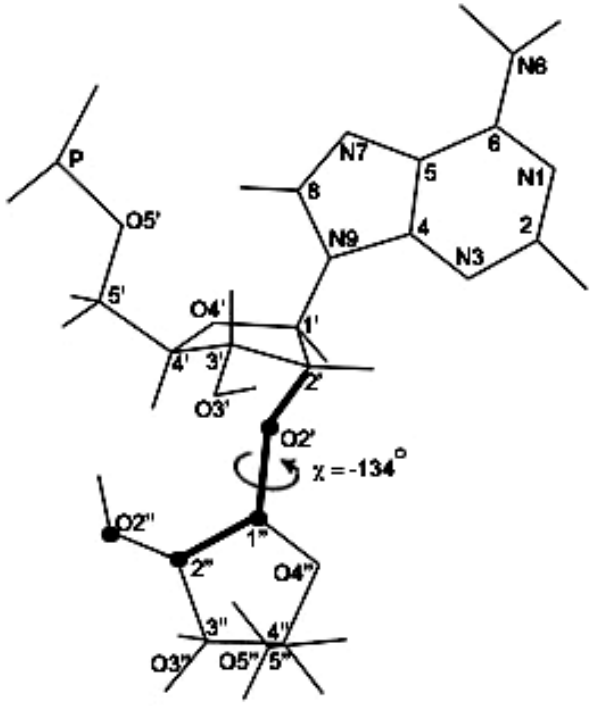

Fig. 1. Structure of 2′-O-β-D-ribofuranosyladenosine residue in RNA according to [21].

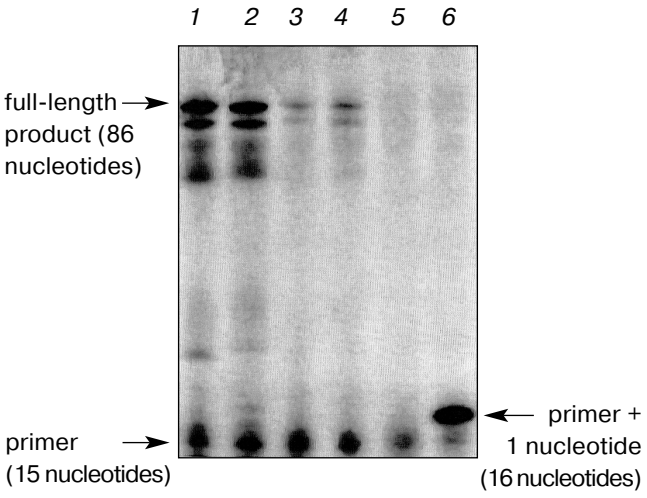

Fig. 2. Elongation of 5′-^{32}P-labeled modified primers catalyzed by RT: *1*) **1b** (control); *2*) **2b**; *3*) **3b**; *4*) **4b**; *5*) **5b**; *6*) **6b**.

These duplexes were used as template/primer in the reverse transcription reaction.

Figure 2 and Table 1 present the results of electrophoretic separation of the products of synthesis of the full-length product of reverse transcription with primers **1b-6b**. As shown, primer **2b** with modification at the −13 position from the 3′-end (the reactive center) was elongated as efficiently as unmodified oligonucleotide **1b**. As the modified unit was shifted to the 3′-end of the primer, elongation of the latter became less efficient, although direct correlation was not observed: primer **4b** modified at −5 position was elongated more efficiently than **3b** (modification at −6 position). Primer **5b** with modification at the −4 position was essentially not elongated by RT. However, primer **6b** having a modified unit closer to the 3′-end (−3) was efficiently elongated, but only by one nucleotide. We suppose that the origin of the reaction termination after elongation by one unit and subsequent isomerization of ES complex is identity of the latter to a complex formed with primer **5b**. Thus, in the case of **6b**, incoming dATP formally acts as terminator of the reaction.

Experiments with the duplexes in which the ribofuranose residue of the primer was oxidized by sodium periodate did not demonstrate any difference from the "usual" primers **1b-6b**.

So, for efficient elongation of the primer, not only relative distance of the modified nucleotide from the 3′-end but also specific position of the latter in the nucleotide strand determined by the topography of the binding site of the primer in the active center of the enzyme seem to be important.

The complete scheme of the reaction catalyzed by DNA polymerases is known to be rather complicated, since the reaction includes five substrates (four dNTP and

Table 1. Kinetic parameters of full-length product synthesis in the reverse transcription reaction using primers **1-5**

Primer	Product	Yield, %	K_m, μM	Relative V_{max}, %	V_{max}/K_m
1	DNA	100	0.45 ± 0.11	100	222
2	DNA	90	0.62 ± 0.13	100	161
3	DNA	10	1.47 ± 0.24	86	58
4	DNA	20	0.19 ± 0.05	18	93
5	No elongation, K_i = 1.05 ± 0.33				
6	Primer elongation by one nucleotide (see Table 2)				

template/primer). That is why for comparison of structurally similar substrates, kinetic analysis is usually performed in the approach of the single-substrate Michaelis–Menten equation (varying concentrations of one substrate at fixed saturating concentrations of the others). Kinetic parameters of reaction of synthesis of the full-length product thus obtained are presented in Table 1.

As seen, the K_m value increases as the modified nucleotide is shifted to the 3′-end of the primer; consequently, efficiency of the enzyme binding with such substrate decreases. For the modified primers, V_{max} is several times lower than for unmodified primer **1**, whereas the values for **2** and **4** practically coincide. It should be noted that primer **5**, which is not elongated during the reaction, efficiently inhibits elongation of primer **1**.

Correct comparison of kinetic parameters of all primers from Table 1 is impossible because a full-length product is not formed with primer **6**. That is why K_m and V_{max} were determined for the elongation reaction by one nucleotide. The products of elongation reaction of primers **1-4** and **6** by one nucleotide were analyzed by electrophoresis in polyacrylamide gel with subsequent densitometry (Fig. 3). Kinetic parameters of elongation of primers by one nucleotide are presented in Table 2.

Table 2. Kinetic parameters of elongation of primers by one nucleotide

Primer	K_m, μM	Relative V_{max}, %	V_{max}/K_m
1	0.33 ± 0.05	100	303
2	2.62 ± 0.40	49	19
3	4.80 ± 0.78	10	2
4	6.01 ± 1.19	55	9
6	0.56 ± 0.07	100	178

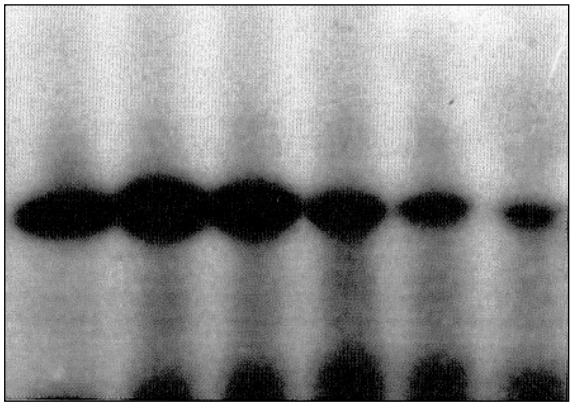

Fig. 3. Rate of reaction of primer elongation by one nucleotide versus template/primer **2** duplex concentration. Lanes: *1*) 3; *2*) 1.6; *3*) 1; *4*) 0.5; *5*) 0.32; *6*) 0.25 μM.

Analyzing the data of Table 2, it can be noted that the values of K_m and V_{max} for primers **1-3** and **6** vary negligibly. In case of primer **4b**, both parameters decrease (modification at the –5 position). The data suggest that K_m decreases due to tighter binding to the enzyme (e.g., due to formation of a new hydrogen bond(s) between the additional ribofuranose group and the protein), and V_{max} decreases due to formation of the "improper" enzyme–substrate complex. Superposition of these two effects results in the value of V_{max}/K_m parameter comparable with that for other primers; this parameter is known to characterize efficiency of enzymatic reaction. It is interesting that kinetic parameters of primer elongation by one nucleotide for **6** are close to those for unmodified primer **1**.

We tried to perform affinity modification of RT using primers containing reactive aldehyde groups according to an earlier developed procedure [13, 14]. Oxidized nucleotide derivatives are known to react with lysine residues of proteins, forming unstable dihydroxymorpholine derivatives that can be reduced by borohydrides to the stable morpholine derivatives [22]. Covalent adducts of

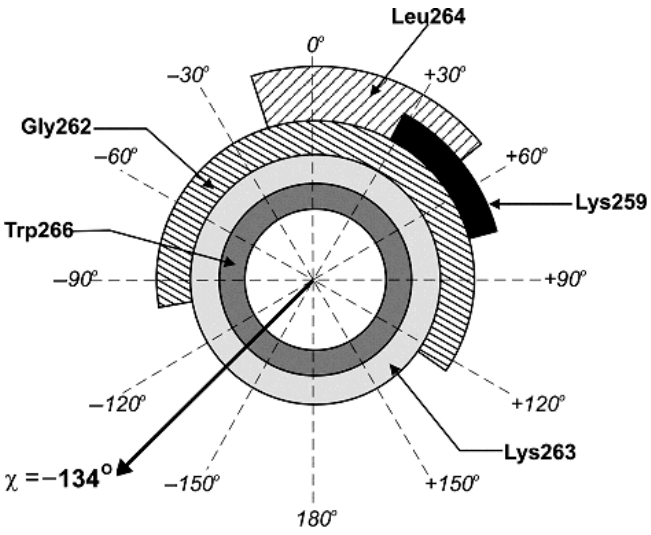

Fig. 4. Range of torsion angle χ values (°) in which forbidden contacts between additional ribose residue in 2′-O-β-D-ribofuranosyladenosine of primers and amino acid residues of α-helix H of RT appear.

protein and ON can be detected by electrophoresis. However, we did not detect formation of such adducts with any of the primers examined.

To explain different properties of the modified primers in the enzymatic reaction, we modified binding of the latter in the RT active center based on the 3D-structure of its complex with the template and primer [20]. The structure of RT complex with the substrates was used as a model (Brookhaven Protein Data Bank, ID 1RTD). The position and conformation of additional ribose residue were modeled in accord with the structure of the decamer RNA duplex containing 2′-O-β-D-ribofuranosyladenosine determined by NMR [21]. Using structural characteristics of the RNA duplex for modeling [21], we relied on the fact that in the RT structure the template–primer duplex near the active center is close to a classical A-form [20, 23, 24]. According to NMR data [21], the additional ribose residue is fixed in the minor groove of the duplex and adopts C3′-*endo*-conformation. The position of this residue is characterized by torsion angle O2′-C1″-C2″-O2″ (χ) = –134°. Insertion of additional ribose residues into nucleotides 19 and 20 of RT/template/primer complex [20] topologically corresponding to modifications in primers **5** and **6** is demonstrated in Figs. 3 and 4. As can be seen, for primer **5** at χ = –134° there exist significant forbidden steric contacts between the additional ribose residue in 2′-O-β-D-ribofuranosyladenosine and side chains of Lys263 and Trp266 amino acid residues. On change in χ value (rotation around O-glycoside bond) interactions with Lys259, Gly262, and Leu264 residues also appear (Table 3 and Fig. 4). It should be noted that these amino acid residues are incorporated into α-helix of H enzyme (helix H); according to X-ray data, this helix is the only structural element of RT contacting with the minor groove of DNA duplex in the enzyme active center [23]. For primers **6**

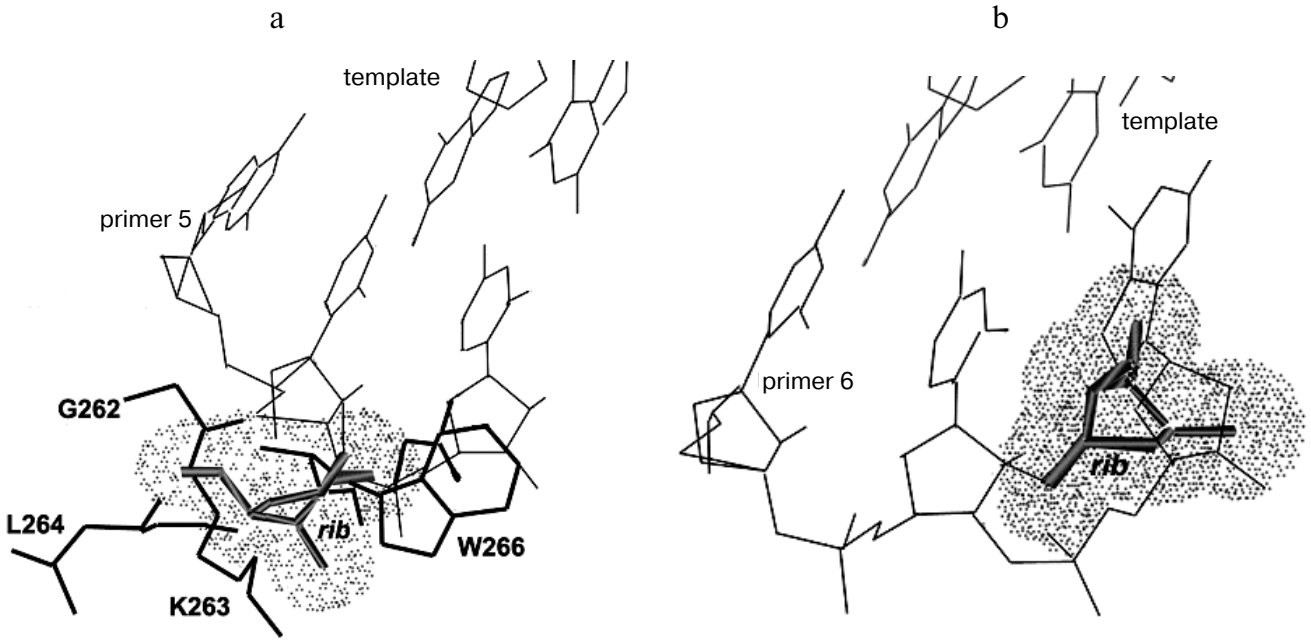

Fig. 5. Modeling of binding of the modified primers **5** (a) and **6** (b) in the RT active center.

Table 3. Forbidden interatomic contacts between the additional ribose residue in 2′-O-β-D-ribofuranosyladenosine of primers and amino acid residues of α-helix H of RT

Amino acid residue of helix H	Range of angle χ values (°) in which forbidden contacts appear		
	primers **1–4**	primer **5**	primer **6**
Asn255	—	—	—
Asp256	—	—	—
Lys259	—	(+24.9) – (+-67)	—
Leu260	—	—	—
Val261	—	—	—
Gly262	—	(–111) – (+128)	—
Lys263	—	**(–180) – (+180)**	—
Leu264	—	(–18.7) – (+41.8)	—
Asp265	—	—	—
Trp266	—	**(–180) – (+180)**	—
Ala267	—	—	—
Ser268	—	—	—
Gln269	—	—	—
Ile270	—	—	—

and **2-4** no such interactions are detected at any values of torsion angle χ.

Thus, primer **6** in the bound state retains significant conformation freedom; this allows formation of a proper enzyme–substrate complex, reaction with incoming substrate (dNTP), and elongation by one nucleotide. As a result, structure **6** becomes equal to the structure of primer **5**, and further elongation is impossible due to arising steric hindrances discussed above. The results of modeling of primers **5** and **6** binding in the RT active center are presented in Fig. 5 (a and b).

So, the presence of the additional 2′-ribofuranose residue in –4 position of DNA primer prevents its elongation by HIV-1 RT due to steric hindrances on interaction with amino acid residues of helix H of RT. The primer characterized in this study is an RT inhibitor of a new type. In this case, inhibition is based on termination of the elongating DNA strand. However, not a usual modified dNTP incapable of further strand elongation (a classical example is azidothymidine-5′-triphosphate) but a natural dNTP plays the role of terminator, and termination is performed due to the presence of an additional group in the primer and translocation of the latter in the active center because of elongation. We suppose that such primers with bulky additional groups could be used for specific inhibition of various polymerases.

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REFERENCES

1. Kaufmann, G. R., and Cooper, D. A. (2000) *Curr. Opin. Microbiol.*, **3**, 508-514.
2. Balint, G. A. (2001) *Pharmacol. Ther.*, **89**, 17-27.
3. De Clercq, E. (2002) *Med. Res. Rev.*, **22**, 531-565.
4. De Clercq, E. (2002) *Biochim. Biophys. Acta*, **1587**, 258-275.
5. Heidenreich, O., Kruhoeffer, M., Grosse, F., and Eckstein, F. (1990) *Eur. J. Biochem.*, **192**, 621-625.
6. Austermann, S., Kruhoffer, M., and Grosse, F. (1992) *Biochem. Pharmacol.*, **43**, 2581-2589.
7. Canard, B., Sarfati, S. R., and Richardson, C. C. (1998) *J. Biol. Chem.*, **273**, 14596-14604.
8. Sarafianos, S. G., Clark, A. D., Jr., Das, K., Tuske, S., Birktoft, J. J., Ilankumaran, P., Ramesha, A. R., Sayer, J. M., Jerina, D. M., Boyer, P. L., Hughes, S. H., and Arnold, E. (2002) *EMBO J.*, **21**, 6614-6624**.**
9. Meyer, P. R., Matsuura, S. E., Schinazi, R. F., So, A. G., and Scott, W. A. (2000) *Antimicrob. Agents Chemother.*, **44**, 3465-3472.
10. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001) *J. Virol.*, **75**, 4832-4842.
11. Andreeva, O. I., Golubeva, A. S., Kochetkov, S. N., van Aerschot, A., Herdewijn, P., Efimtseva, E. V., Ermolinsky, B. S., and Mikhailov, S. N. (2002) *Bioorg. Med. Chem. Lett.*, **12**, 681-684.
12. Mikhailov, S. N., Efimtseva, E. V., Gurskaya, G. V., Zavodnik, V. E., De Bruyn, A., Rozenski, J., and Herdewijn, P. (1997) *J. Carbohydr. Chem.*, **16**, 75-92.
13. Tunitskaya, V. L., Rusakova, E. E., Memelova, L. V., Kochetkov, S. N., van Aerschot, A., Herdewijn, P., Efimtseva, E. V., Ermolinsky, B. S., and Mikhailov, S. N. (1999) *FEBS Lett.*, **442**, 20-24.
14. Gritsenko, O. M., Mikhailov, S. N., Efimtseva, E. V., van Aerschot, A., Herdewijn, P., and Gromova, E. S. (2000) *Nucleosides, Nucleotides and Nucleic Acids*, **19**, 1805-1820.
15. Efimtseva, E. V., Bobkov, G. V., Mikhailov, S. N., van Aerschot, A., Schepers, G., Busson, R., Rozenski, J., and Herdewijn, P. (2001) *Helvetica Chim. Acta*, **84**, 2387-2397.
16. Basavappa, R., and Sigler, P. B. (1991) *EMBO J.*, **10**, 3105-3111.
17. Maniatis, T., Frich, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor University Press, Cold Spring Harbor, N. Y.
18. Pokholok, D. K., Gudima, S. O., Esipov, D. S., Dobrynin, V. N., Rechinsky, V. O., and Kochetkov, S. N. (1993) *FEBS Lett.*, **325**, 237-241.
19. Gudima, S. O., Kostyuk, D. A., Grishchenko, O. I., Tunitskaya, V. L., Memelova, L. V., and Kochetkov, S. N. (1998) *FEBS Lett.*, **439**, 302-306.

20. Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998) *Science*, **282**, 1669-1675.
21. Luyten, I., Esnouf, R. M., Mikhailov, S. N., Efimtseva, E. V., Michiels, P., Heus, H. A., Hilbers, C. W., and Herdewijn, P. (2000) *Helvetica Chim. Acta*, **83**, 1278-1289.
22. Ermolinsky, B. S., and Mikhailov, S. N. (2000) *Bioorg. Khim.*, **26**, 483-504.
23. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 6320-6324.
24. Ding, J., Das, K., Hsiou, Y., Sarafianos, S. G., Clark, A. D., Jr., Jacobo-Molina, A., Tantillo, C., Hughes, S. H., and Arnold, E. (1998) *J. Mol. Biol.*, **284**, 1095-1111.