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3′-Mercapto-2′,3′-dideoxynucleotides are high effective terminators of DNA synthesis catalyzed by HIV reverse transcriptase

Alexander A. Yuzhakov, Zurab G. Chidgeavadze, Robert Sh. Beabealashvilli

Cardiology Research Centre, Academy of Medical Sciences of Russia, Moscow 121552, Russia

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Four 3′-mercapto-2′,3′-dideoxynucleoside 5′-triphosphates (A, G, C and T) were tested as DNA chain terminator substrates for calf thymus α-DNA polymerase, *E. coli* DNA polymerase I Klenow fragment, terminal deoxynucleotidyl transferase and reverse transcriptases of AMV, HIV and MLV viruses. It was shown that the analogues selectively and irreversibly terminated DNA chain elongation by AMV and HIV reverse transcriptases and the terminal transferase. Other DNA polymerases tested did not use the nucleotide analogues as chain terminator substrate.

DNA polymerase; Reverse transcriptase; Virus; HIV; Nucleotide; Analogue; Chain termination

1. INTRODUCTION

Selective inhibitors of DNA polymerase of different origins are important tools for investigating the mode of the enzyme action. We continue the study of analogues modified at 3′-position of sugar moiety of nucleotides [1,2]. Earlier we reported about termination properties of 3′-mercapto-2′,3′-dideoxythymidine 5′-triphosphate towards AMV reverse transcriptase and antiviral activity of the corresponding nucleoside on lymphoblastoid T-cell line with HIV-1 virus [2]. Unfortunately, we did not possess pure HIV reverse transcriptase at that time. Now in this report we present new data on the 3′-mercapto-derivatives of dNTP.

2. MATERIALS AND METHODS

TTP(3′SH) was synthesized as described earlier [3], dCTP(3′SH), dGTP(3′SH) and dATP(3′SH) were synthesized from 3′-mercaptothymidine by the chemical *trans*-glycosyltation reaction [4]. The molar content of free SH-group in NTP(3′SH) was measured according to [5] and accounted for more than 90%.

Recombinant DNA polymerase I Klenow fragment with specific activity of 20,000 units/mg, AMV reverse transcriptase with activity of 12 units/μl and HIV reverse transcriptase with activity of 2 units/μl were kindly provided by Dr. L. Savochikina, Dr. A. Shevelev and Dr. T. Rozovskaya (Cardiology Research Centre, Russia). All the enzymes were nearly homogeneous. Concentration of stock solution of MLV reverse transcriptase (Gibco-BRL, USA) was 100 units/μl. DNA polymerase α and Bollum terminal transferase from calf thymus were purified according to modified methods of Grosse et al. [6] and Bollum [7]. Specific activity of the enzymes were 5000 units/mg and 20,000 units/mg, respectively.

For AMV, MLV and HIV reverse transcriptases one unit of activity corresponds to incorporation of 1 nmol of dAMP into an acid-precipitable product with oligo(dT)poly(rA) as template-primer in 10 min at 37°C under specified condition. For other DNA polymerases one incorporation of 1 nmol of ^{32}P-labeled nucleotide with M13 standard primer and M13 DNA template 30 min for DNA polymerase I and 1 h for DNA polymerase α and terminal transferase.

dGTTTTCCCAGTCACGAC primer was provided by Farminvestprom (Russia). It was 5′-^{32}P-labeled with T4 polynucleotide kinase according to [8]. The oligonucleotide was purified by Nensorb-20 (DuPont, USA) and used for the synthesis with M13 mp10 DNA as a template. M13 mp10 DNA was taken from the laboratory stock. dNTP (Sigma, USA) were used without special purification. Electrophoresis in 20% polyacrylamide gel was made as in [9]. 3′-Azido-3′-dideoxythymidine 5′-triphosphate (TTP(3′N$_3$) was kindly given by Dr. Azhayev (Institute of Bioorganic Chemistry, Academy of Sciences of Russia).

DNA synthesis was carried out in two steps: main reaction with limited set of nucleoside triphosphates and run-away step under high concentration of all dNTPs. Run-away reaction was made with terminal transferase (for the transferase main reaction) or with α- or Klenow DNA polymerase for the rest of the enzymes.

DNA synthesis with α-DNA polymerase, Klenow fragment of DNA polymerase I *E. coli*, AMV and HIV reverse transcriptases was performed in 20 μl total volume in a mixture containing 50 mM of Tris-HCl (pH 8.0), 10 mM of $MgCl_2$, 10 mM of 2-mercaptoethanol and 10 μM of each of dATP, dGTP and TTP for dCTP(3′SH), dGTP and TTP – for ATP(3′SH), dGTP – for TTP(3′SH), 0.2 μg of M13 mp10 DNA and molar equivalent of 5′[^{32}P]primer. Reactions were initiated by the addition of 80 units of the AMV reverse transcriptase, 2 units of the Klenow enzyme, 4 units of alpha DNA polymerase, or 8 units of HIV reverse transcriptase at 37°C. DNA synthesis with MLV reverse transcriptase was performed under the similar reaction

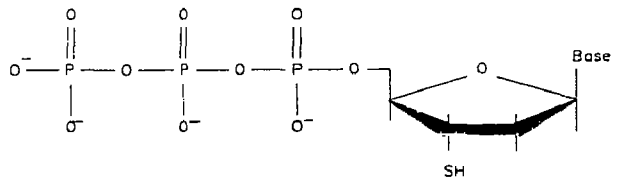

Fig. 1. 3′-Mercapto-2′,3′-dideoxynucleoside 5′-triphosphates, where base is adenine, cytosine, guanine or thymine.

Correspondence address: A.A. Yuzhakov, Cardiology Research Centre, Academy of Medical Sciences of Russia, Moscow 121552, Russia.

conditions, but the reaction media contained 50 mM of Tris-HCl (pH 8.3), 75 mM of KCl, 3 mM of $MgCl_2$ and 10 mM of DTT. Reaction was initiated by the addition of 100 units of MLV reverse transcriptase at 37°C. Main reaction as well as run-away reaction for Klenow enzyme was made for 10 min, while for DNA polymerase α, AMV reverse transcriptase and Bollum enzyme the main reaction was for 60 min and run-away reaction was for 30 min. Time of both main and run-away reactions for MLV and HIN reverse transcriptase were 15 min. Concentrations of nucleotide analogues are shown in legends to figures.

Nucleotide sequence of the product is:

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          10        20        30
          *         *         *
5'-GTTTTCCCAGTCACGACGTTGTAAAACGACGGCC
(primer is underlined)
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3. RESULTS

We had tested dNTP(3'SH) with calf thymus α DNA polymerase and *E. coli* polymerase I Klenow fragment. Both of the enzymes did not use any of the dNTP(3'SH) (Fig. 2).

AMV reverse transcriptase is able to incorporate dNMP(3'SH) at the 3'-end of the product. Fig. 2 exhibits the data produced for dAMP(3'SH) (tracks 12, 13). The incorporation is irreversible under experimental conditions used; DNA chain is terminated (track 12; Fig. 2). It is clear from the absence of 23A-line elimination (track 13; Fig. 2), while 22T-line is fully eliminated under the run-away reaction. These data for each of dNTP(3'SH) conform our earlier results for TTP(3'SH) [2].

MLV reverse transcriptase used dNTP(3'SH) very unwillingly. It is evident from Fig. 2, tracks 16 and 17.

HIV reverse transcriptase incorporates very efficiently all of dNTP(3'SH) and terminates DNA chain elongation. It is obvious from Fig. 2, tracks 20, 21: 22T-line is transformated into 23A-line without remnant, and the enzyme meets no free 3'-OH end of DNA chain for the run-away reaction. We studied HIV reverse transcriptase DNA chain termination efficiency upon the variation of TTP(3'SH) or TTP(3'N_3) concentration with the background of 1 μM of natural TTP concentration (Fig. 3). It is seen that TTP(3'SH) is at least as efficient as TTP(3'N_3).

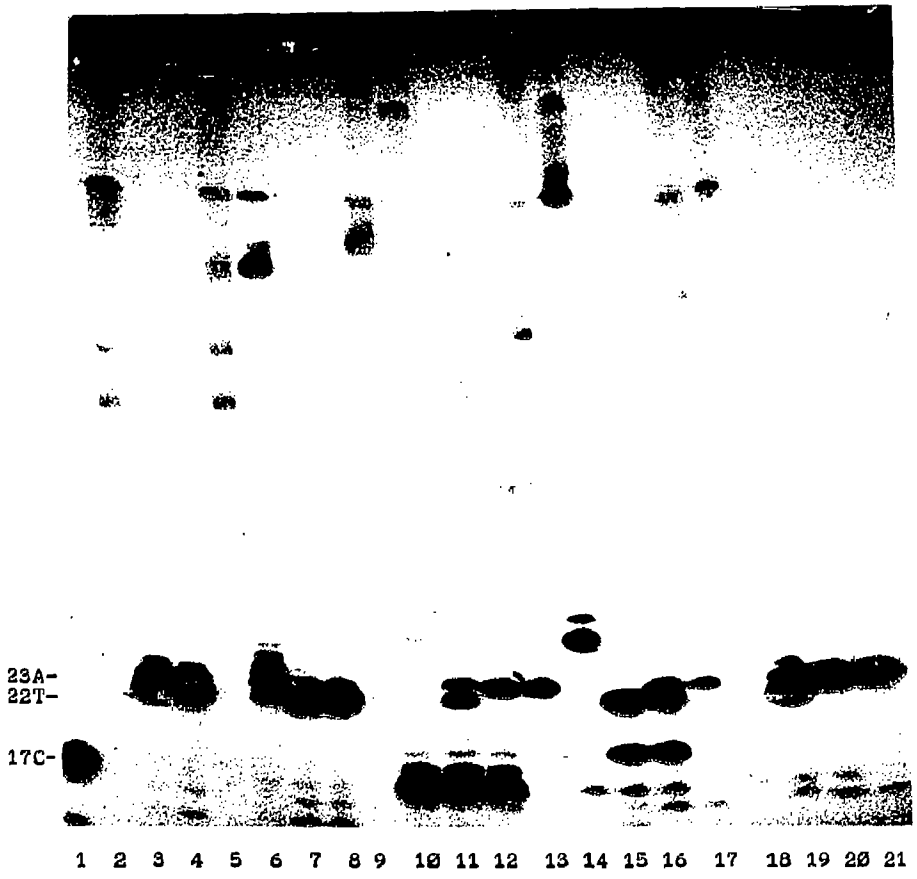

Fig. 2. Electrophoretogram of DNA synthesized in the presence of dATP(3'SH) of Klenow enzyme (tracks 2–5), α- DNA polymerase (tracks 6–9); AMV (tracks 10–13), MLV (tracks 14–17), HIV (tracks 18–21) reverse transcriptases. Track 1 = 5'-[^{32}P] primer. Tracks 2,6,10,14,18 = synthesis with 250 μM of each of the four natural dNTPs. Tracks 3,7,11,15,19 = synthesis with 10 μM of each of dGTP and TTP. Tracks 4,8,12,16,20 = synthesis with 10 μM of each of dGTP, TTP and 200 μM dATP(3'SH). Tracks 5,9,13,17,21 = run-away experiment with 2 units of Klenow enzyme and 250 μM of each of the four natural dNTPs added after completion of reactions shown in tracks 4,8,12,16,20.

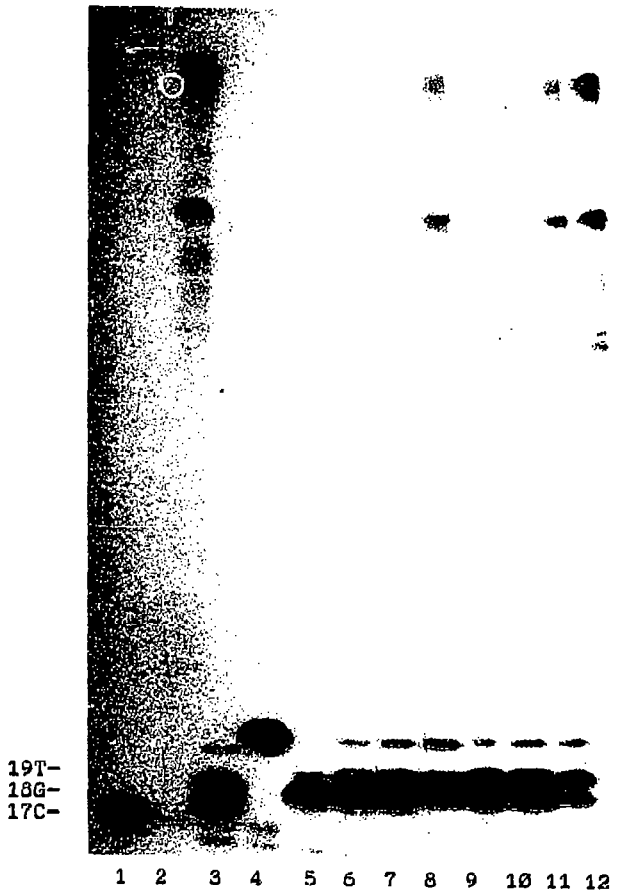

Fig. 3. Termination of DNA chain with variable concentration of TTP(3′SH) and TTP(3′N_3) for HIV reverse transcriptase. Track 1 = 5′-[^{32}P]primer; track 2 = 250 μM of each of the four natural dNTP; track 3 = 10 μM of dGTP and 1 μM of TTP; tracks 5–8 and 9–12 = run-away reactions with 250 μM of each of dNTPs added after completion of main reactions conducted with 1 μM of TTP and 10 μM of dGTP and TTP(3′SH) (tracks 5–8) or TTP(3′N_3) (tracks 9–12) at 200 μM (track 5,9), 20 μM (track 6,10), 10 μM (track 7,11), 1 μM (track 8,12). Both main and run-away reactions were for 15 min.

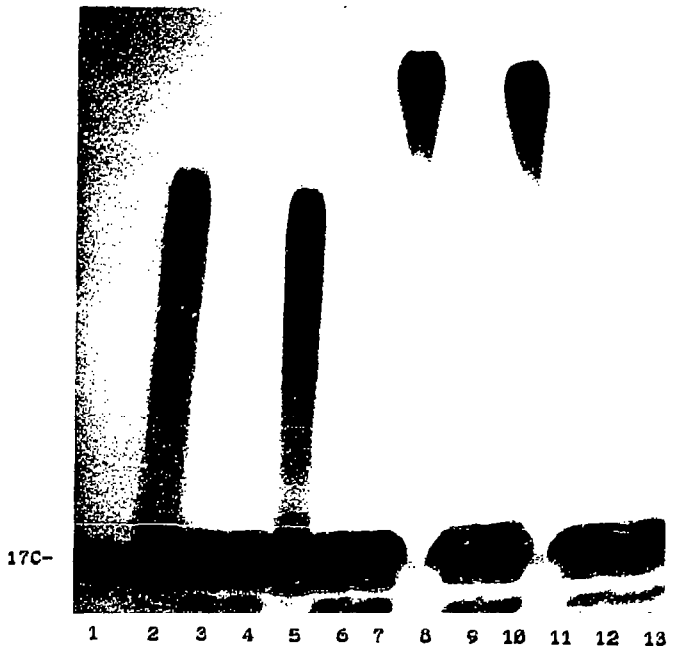

Fig. 4. DNA synthesized by deoxyribonucleotidyl transferase. Track 1 = 5′-[^{32}P]primer; tracks 2,5,8,11 = synthesis with 200 μM of natural dGTP (track 2), dATP (track 5), dCTP (track 8), TTP (track 11). Tracks 3,6,9,12 = synthesis with 200 μM dNTP(3′SH): dGTP(3′SH) (track 3), dATP(3′SH) (track 6), dCTP(3′SH) (track 9), TTP(3′SH) (track 12). Tracks 4,7,10,13 = run-away reactions with 200 μM of natural dNTP, after completion of reactions shown in tracks 3,6,9,12.

Terminal transferase is able to incorporate dNTP(3′SH) at the 3′-end of the products (Fig. 4, tracks 1, 3, 6, 9, 12) like reverse transcriptase. The incorporation are irreversible under experimental conditions (tracks 4, 7, 10, 13).

We did not observe any difference in substrate properties between dATP(3′SH), dGTP(3′SH), dCTP(3′SH) or TTP(3′SH) for all the enzyme studied. The sequencing gel (Fig. 5) illustrates it for HIV reverse transcriptase.

We had investigated 3′-end of DNA synthesis product with Terminal transferase or HIV reverse transcriptase in the presence of dNTP(3′SH) in reaction with

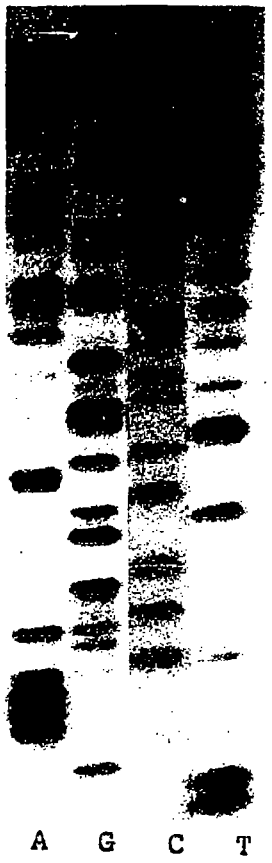

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Fig. 5. Reactions of sequence with HIV reverse transcriptase in the presence of 10 μM of dATP(3′SH) (track A); dGTP(3′SH) (track G); dCTP(3′SH) (track C); TTP(3′SH) (track T) and each of 50 μM dNTP.

Ellman reagent. According to our HPLC analysis data there is 3'-SH group of last nucleotide on 3'-end of synthesized DNA [4].

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