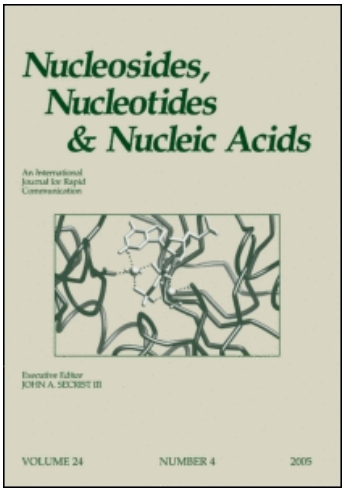


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Chain Termination With Sugar- Modified Nucleotide Analogs in the DNA Synthesis by DNA Polymerase γ

Shunji Izuta[a]; Shonen Yoshida[a]
[a] Laboratory of Cancer Cell Biology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya, Japan

To cite this Article Izuta, Shunji and Yoshida, Shonen(1996) 'Chain Termination With Sugar- Modified Nucleotide Analogs in the DNA Synthesis by DNA Polymerase γ', Nucleosides, Nucleotides and Nucleic Acids, 15: 1, 683 — 692
To link to this Article: DOI: 10.1080/07328319608002415
URL: http://dx.doi.org/10.1080/07328319608002415

NUCLEOSIDES & NUCLEOTIDES, 15(1-3), 683-692 (1996)

CHAIN TERMINATION WITH SUGAR-MODIFIED NUCLEOTIDE ANALOGS IN THE DNA SYNTHESIS BY DNA POLYMERASE γ [1]

Shunji Izuta* and Shonen Yoshida

Laboratory of Cancer Cell Biology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan.

ABSTRACT: We examined the effects of sugar-modified nucleotide analogs on the reaction with DNA polymerase γ and HIV reverse transcriptase. The 3'-modified derivative, 3'-amino-3'-deoxythymidine 5'-triphosphate inhibited the reaction of both enzymes. However, where it did not cause chain termination in the DNA polymerase γ reaction, it did cause termination in HIV reverse transcriptase reaction. On the other hand, the triphosphate derivative of oxetanocin G which has a 3, 4-dihydroxymethyloxetane at the sugar moiety instead of ribofuranose showed the chain termination in both polymerase reactions, however, termination sites with DNA polymerase γ reaction were different from those with HIV reverse transcriptase or with DNA polymerase α. These results, combined with our previous data, indicate that the action of several sugar-modified nucleotide analogs on DNA polymerase γ could be very different from those on other eukaryotic DNA polymerases including HIV reverse transcriptase.

Many nucleic acid related compounds have been developed and examined for their anti-cancer or anti-viral effects (1). Among them, the effective analogs have been further studied with respect to their target of action. DNA polymerases have been shown to be targets for inhibition by nucleoside analogs which could be metabolized to their triphosphate derivatives, and some of the sugar-modified nucleotide analogs cause chain termination in DNA synthesis. For example, the 5'-triphosphate of 1-β-D-arabinofuranosylcytosine (araC)[2], a potent anti-cancer drug, is known to inhibit eukaryotic DNA polymerases, especially α-polymerase, and also DNA primase associated with DNA polymerase α (2-4), and the 5'-triphosphate of araC is reported to be incorporated into

1. This paper is dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.
2. Abbreviations used are: araC, 1-β-D-arabinofuranosylcytosine; HIV, human immunodeficiency virus; OXT-GTP, oxetanocin G triphosphate.

DNA by DNA polymerase α or β and causes chain termination (5, 6). On the other hand, we have recently reported (7) that the triphosphate derivative of a novel nucleoside analog, 9-[(2*R*, 3*R*, 4*S*)-3, 4-bis(hydroxymethyl)-2-oxetanyl] guanine (oxetanocin G) which exhibits potent anti-cancer and anti-viral activities (8-11) could be incorporated into DNA by DNA polymerase α, herpes simplex virus type-II DNA polymerase or human immunodeficiency virus (HIV)-coded reverse transcriptase and thereby caused the chain termination at the unique site. Although this type of reseach has been performed with several prokaryotic and eukaryotic DNA polymerases or viral-coded DNA synthesizing enzymes, little is known about DNA polymerase γ because of the difficulty of purification of this enzyme.

DNA polymerase γ is a mitochondrial enzyme and is responsible for mitochondrial DNA replication (12). The content of this enzyme is estimated to be less than 1% of total DNA polymerase activities in the cells or tissues. Previously, we successfully purified DNA polymerase γ from bovine liver by means of sequential column chromatography (13). Using this preparation, we have reported that 3'-deoxythymidine 5'-triphosphate caused chain termination on the DNA synthesis with bovine DNA polymerase γ but 3'-azido-3'-deoxythymidine 5'-triphosphate does not (13, 14). However, in the case of HIV reverse transcriptase, both compounds terminate chain elongation. These observations suggest that some sugar-modified nucleotide analogs could show a different inhibition mechanism against DNA polymerase γ among several DNA polymerases. To know the distinctive effects of 3'-substituent groups on dTTP analogs, we extended our work to the chain termination effects of another 3'-substituted dTTP analog, 3'-amino-3'-deoxythymidine 5'-triphosphate which is a strong inhibitor for DNA polymerase β (15). This compound contains a hydrophilic group at the 3'-position which is comparable to the previous two analogs. Furthermore, we examined the effect of the triphosphate derivative of oxetanocin G (OXT-GTP), which is an inhibitor of several DNA polymerases as described above (7), for an another type of sugar-modified nucleotide analog. Here we report that DNA polymerase γ is affected by the sugar-modified analogs in a manner which differs from other polymerases.

MATERIALS AND METHODS

Enzymes. DNA polymerase γ was purified from bovine liver as described previously (13). HIV-1 reverse transcriptase was purchased from Seikagaku Co. (Tokyo, Japan).

Nucleotide analogs. 3'-Amino-3'-deoxythymidine 5'-triphosphate (16) was kindly gifted from Dr. Mineo Saneyoshi (The Nishi-Tokyo University, Japan). The triphosphate

derivative of oxetanocin G was provided by Nippon Kayaku Co. (Tokyo, Japan) (7). The chemical structures are shown in TABLE 1.

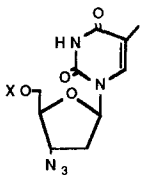
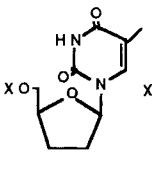
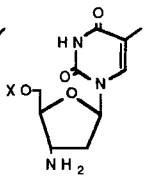
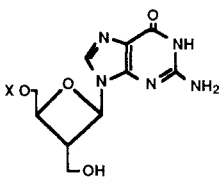
Assay of DNA polymerase γ activity. DNA polymerase γ and HIV reverse transcriptase activities were measured according to the reported methods (7, 13) with activated calf thymus DNA.

Analysis of the products synthesized on singly primed M13mp18 single-stranded DNA. M13mp18 single-stranded DNA (Pharmacia, Sweden) was annealed to a universal primer (17-mer) and then used as a template-primer. The mixtures (25 μl) comprising 40 mM potassium phosphate, pH 7.5 (for DNA polymerase γ) or 40 mM Tris-HCl, pH 7.5 (for HIV reverse transcriptase), 8 mM $MgCl_2$, 8 μg/ml singly primed M13mp18 single-stranded DNA, 5 μM each of dGTP, dCTP and dTTP, 2 μM [α-^{32}P]dATP (370 kBq), 4 mM dithiothreitol, 100 mM KCl, and various concentrations of nucleotide analog as indicated. 0.5 Units of DNA polymerase γ or HIV reverse transcriptase were added to the mixture and incubated for 1 h at 37°C. After the reaction, the mixture was treated with 1 mg/ml (final concentration) proteinase K in the presence of 0.2% sodium dodecylsulfate, and ^{32}P-labeled product DNA was precipitated with ethanol and subjected to 8% sequencing gel. The radioactive bands were detected by autoradiography with X-ray film (Fuji Photo Film Co., Tokyo, Japan).

RESULTS

Chain termination by 3'-amino-3'-deoxythymidine 5'-triphosphate. We first analyzed the inhibitory action of 3'-amino-3'-deoxythymidine 5'-triphosphate. The *Ki* value of 3'-amino-3'-deoxythymidine 5'-triphosphate against DNA polymerase γ was 10 μM with activated calf thymus DNA as a template-primer and the inhibition mode was competitive with respect to the incorporation of dTTP. We then examined whether the 3'-amino-3'-deoxythymidine 5'-triphosphate causes the chain termination in the DNA synthesis with DNA polymerase γ by measuring the size of products synthesized on the singly primed M13mp18 single-stranded DNA in the presence of analog with a sequencing gel. If the analog is incorporated and causes the chain termination during DNA synthesis, short oligonucleotide fragments will be observed at the sites of adenines on the template. If the analog is not incorporated and merely competes with substrate on the enzyme, the total amount of product will be reduced without yielding the short terminated fragments (FIG. 1). Results are shown in FIG. 2. In the absence of inhibitor, no short fragments were detected except for the natural arrest bands (FIG. 2, lane 1, arrow). Even if the reaction

TABLE 1. Summary of chain termination effects of 3'-azido-3'-deoxythymidine 5'-triphosphate (1), 3'-deoxythymidine 5'-triphosphate (2), 3'-amino-3'-deoxythymidine 5'-triphosphate (3) and OXT-GTP (4) on several DNA polymerases.

Enzyme	Analogue (X= -P-O-P-O-P-O⁻)			
	 1	 2	 3	 4
DNA polymerase γ	−[a, c]	+[b, c]	−[a]	+[b]
DNA polymerase α	NI[d, e]	NI[d, e]	NI[d, e]	+[b, f]
DNA polymerase β	ND[g]	ND[g]	+[b, h]	NI[d, f]
HIV reverse transcriptase	+[b, c]	+[b, c]	+[b]	+[b, f]

a, not showing chain termination; b, showing chain termination; c, ref. (13); d, no inhibition; e, ref. (16); f, ref. (7); g, not determined; h, ref. (15)

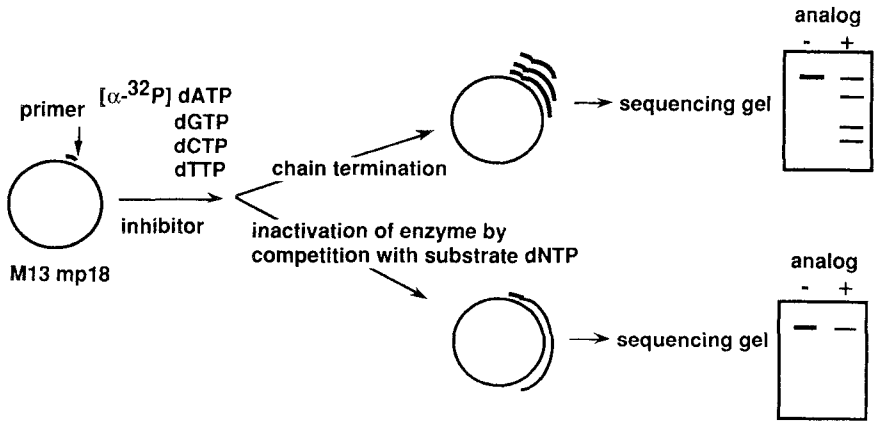


FIG. 1. System for the analysis of the products synthesized on singly primed M13mp18 single-stranded DNA. The method of DNA strand elongation assay is shown schematically. The singly-primed M13mp18 single-stranded DNA was used for a template primer, and the ^{32}P-labeled products were then subjected to 8% sequencing gel as described under "MATERIALS AND METHODS". If the analog causes the chain termination, short oligonucleotide bands are detected as described in the text.

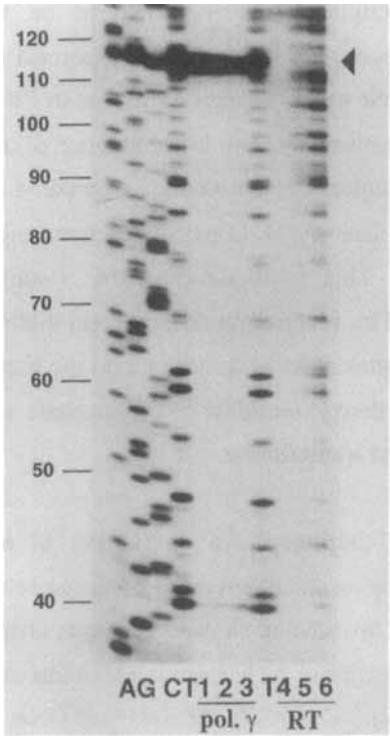


FIG. 2. Effects of 3'-amino-3'-deoxythymidine 5'-triphosphate on DNA strand elongation with DNA polymerase γ and HIV reverse transcriptase. The DNA strand elongation assay was performed with DNA polymerase γ (lanes 1-3) or HIV reverse transcriptase (lanes 4-6) in the absence (lanes 1 and 4) or presence of 3'-amino-3'-deoxythymidine 5'-triphosphate at the concentration of 50 μM (lane 2), 100 μM (lane 3), 5 μM (lane 5) or 10 μM (lane 6) as described in "MATERIALS AND METHODS". Lanes A, T, C and G are dideoxynucleotide sequencing of M13 single-stranded DNA with ddATP, ddTTP, ddCTP and ddGTP, respectively, the position of these nucleotide incorporation sites being indicated. The chain length of the products including primer (17mer) is shown at the left of the panel. The arrow head indicates the natural arrest site.

was performed with various concentrations of the inhibitor, no additional short fragment bands appeared, and the amount of natural arrest band decreased (FIG. 2, lanes 2 and 3). This suggests that the 3'-amino-3'-deoxythymidine 5'-triphosphate can not be incorporated into DNA by DNA polymerase γ. Thus, the main mechanism of inhibition of γ-polymerase by this analog may be the inactivation of enzyme by binding to the nucleotide substrate binding site of the enzyme.

HIV reverse transcriptase is also known to be inhibited by 3'-amino-3'-deoxythymidine 5'-triphosphate (17). We therefore examined the chain termination effect of this analog on HIV reverse transcriptase. As shown in FIG. 2 (lanes 5 and 6), short oligonucleotide bands which were not seen in the absence of inhibitor (lane 4), appeared according to the increasing amount of the analog. Compared with the dideoxynucleotide sequencing ladders (FIG. 2, lanes A, G, C and T), the termination sites were determined at adenine on the template. This result indicates that 3'-amino-3'-deoxythymidine 5'-triphosphate is incorporated by reverse transcriptase and inhibits further elongation from the 3'-end of the analog at sites opposite to adenine on the template. Thus, the inhibition mechanism of 3'-amino-3'-deoxythymidine 5'-triphosphate is different between DNA polymerase γ and HIV reverse transcriptase.

Chain termination by 5'-triphosphate derivative of oxetanocin G. We next examined the effect of 5'-triphosphate derivative of oxetanocin G (OXT-GTP) since we have previously found that this analog showed a unique chain termination mode in the DNA synthesis by DNA polymerase α, herpes simplex virus-coded DNA polymerase and HIV reverse transcriptase (7). The OXT-GTP inhibited DNA polymerase γ very weakly with a *K*i value of 32.5 μM and the inhibition mode was competitive with respect to the incorporation of dGTP (7). The inhibition mechanism of this compound was examined by the analysis of product with the singly primed M13mp18 single-stranded DNA as a template-primer. Results are shown in FIG. 3. Contrary to the case of 3'-substituted nucleotide analogs, the addition of OXT-GTP into the reaction gave the several short oligonucleotide bands in the DNA polymerase γ reaction (FIG. 3, lanes 1-3). The arrest sites seen in FIG. 3 are summarized schematically in FIG. 4. Interestingly, 5 of 7 major arrest bands appeared at the sites having the template nucleotide sequence of 3'-C<u>A</u>-5' (<u>A</u> is the arrest site) and the other two were the sites of 3'-CN<u>A</u>-5' (<u>A</u> is also the arrest site).

DISCUSSION

In the present study, we report the chain termination effects of two sugar-modified nucleotide analogs on DNA polymerase γ. All results, combined with our previous results (7, 13, 14), are summarized in TABLE 1. The *K*i value of 3'-amino-3'-deoxythymidine 5'-triphosphate (10 μM) was smaller than that of 3'-azido derivative (*K*i, 26 μM) (13), indicating that the amino group as 3'-substituent of dTTP has a higher affinity to γ-polymerase than the azido group. However, the inhibitory effect of this compound was much weaker than 3'-deoxythymidine 5'-triphosphate (*K*i, 0.4 μM).

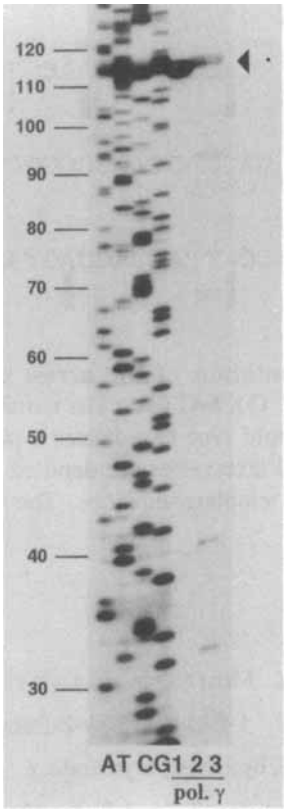


FIG. 3. Effects of OXT-GTP on DNA strand elongation with DNA polymerase γ. The DNA strand elongation assay was performed with DNA polymerase γ in the absence (lane 1) or presence of OXT-GTP at the concentration of 100 μM (lane 2) or 200 μM (lane 3) as described in "MATERIALS AND METHODS". Lanes A, T, C and G are dideoxynucleotide sequencing of M13 single-stranded DNA with ddATP, ddTTP, ddCTP and ddGTP, respectively, the position of these nucleotide incorporation sites being indicated. The chain length of the products including primer (17mer) is shown at the left of the panel. The arrow head indicates the natural arrest site.

The 3'-amino-3'-deoxythymidine 5'-triphosphate could not cause chain termination during DNA synthesis by DNA polymerase γ (FIG. 2). Similarly, another 3'-substituted nucleotide analog, 3'-azido-3'-deoxythymidine 5'-triphosphate, did not cause chain termination in the DNA polymerase γ reaction, while 3'-deoxythymidine 5'-triphosphate caused the strong chain termination (13, 14). This suggests that DNA polymerase γ can not incorporate a dTTP analog which has amino or azido group at the 3'-position instead of hydroxy group. Therefore, DNA polymerase γ can strictly distinguish each 3'-group of

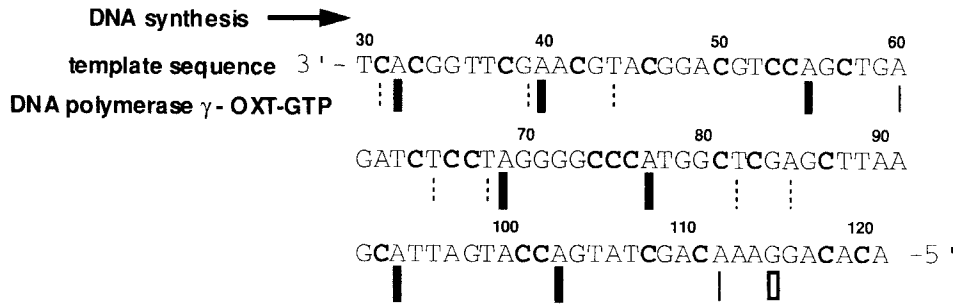


FIG. 4. Schematic representation of the arrest sites for DNA polymerase γ reaction in the presence of OXT-GTP. The termination bands seen in FIG. 3 are summarized schematically. A bold type C indicates a possible site at which oxetanocin analog is incorporated. Natural arrest sites are denoted by □. The chain length of the products are shown above the template sequence. The widths of the bar represent the intensities of the bands.

nucleotide during DNA synthesis. Most recently, Lewis *et al.* (18) have reported that the 5'-triphosphate derivative of 1-β-D-(2-deoxy-2-fluoroarabinofuranosyl)-5-iodouracil (FIAU) is incorporated into DNA by DNA polymerase γ. It may be interesting to compare the stringency of recognition between 2'- and 3'-groups of nucleotide analogs by DNA polymerase γ.

In the case of HIV reverse transcriptase, both 3'-amino- and 3'-azido-3'-deoxythymidine 5'-triphosphates were incorporated into DNA and caused the chain termination (FIG. 2). It is also reported that DNA polymerase β can incorporate 3'-amino-3'-deoxythymidine 5'-triphosphate into DNA (15). Thus, the action of 3'-amino- or azido-substituted dTTP analogs against DNA polymerase γ differ from those against HIV reverse transcriptase or DNA polymerase β.

We also examined the effect of OXT-GTP on DNA polymerase γ (FIG. 3). This analog showed the chain termination as well as 3'-deoxythymidine 5'-triphosphate, and the termination mostly occurred at the site having the template nucleotide sequence of 3'-C<u>A</u>-5' or 3'-CN<u>A</u>-5' (underlined A is the arrest site) (FIG. 4). This indicates that DNA polymerase γ could incorporate OXT-GTP into DNA opposite cytosine on the template. However, the elongation was blocked when thymidine was incorporated at one or two nucleotide downstream of oxetanocin. This result indicates that DNA polymerase can not utilize the 3'-hydroxy group of thymidine residue as a primer end, possibly due to the

conformational distortion of sugar backbone. However, at other sites at which OXT-GTP could be incorporated, such as 3'-CT-5' or 3'-CG-5' of the template sequence, the chain termination occurred very weakly. This suggests that γ-polymerase could further elongate the 3'-hydroxy group of the nucleotide next to oxetanocin. At present, it is unknown why DNA polymerase γ can hardly elongate from only thymidine next to oxetanocin as a primer end. The local conformation of DNA containing oxetanocin at the 5'-position of thymidine could be slightly different among those of deoxyadenosine, deoxyguanosine and deoxycytidine. On the other hand, in the case of other DNA polymerases including reverse transcriptase (7), the major arrest site on the template DNA is 3'-CN_1N_2-5' (N_1 or N_2 is arrest site) similar to the case of DNA polymerase γ, but no common base are found in N_1 or N_2. This indicates that these enzymes can not elongate the 3'-end of any nucleotide beyond one or two bases next to oxetanocin. Therefore, the action of OXT-GTP against DNA polymerase γ is different from other polymerases as well as 3'-amino-3'-deoxythymidine 5'-triphosphates.

In conclusion, we demonstrated that the action of some sugar-modified nucleotide analogs on DNA polymerase γ could be different from those on several DNA synthesizing enzymes, especially HIV reverse transcriptase. These results might provide useful information for developing a new type of nucleotide analog which specifically inhibits HIV reverse transcriptase, DNA polymerase γ, or other cellular polymerases.

ACKNOWLEDGMENT

We thank Dr. M. Saneyoshi of The Nishi-Tokyo University for providing 3'-amino-3'-deoxythymidine 5'-triphosphate. We also thank Drs. N. Shimada and M. Kitagawa of Nippon Kayaku Co., Ltd. for providing the triphosphate derivative of oxetanocin G.

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