4′-Acylated thymidine 5′-triphosphates: a tool to increase selectivity towards HIV-1 reverse transcriptase

Andreas Marx, Mario Amacker[1], Manuel Stucki[1], Ulrich Hübscher[1], Thomas A. Bickle[2] and Bernd Giese*

Department of Chemistry, University of Basel, St Johanns-Ring 19, CH-4056 Basel, Switzerland, [1]Department of Veterinary Biochemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland and [2]Division of Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

ABSTRACT

4′-Acylated thymidines represent a new class of DNA chain terminators, since they have been shown to act as post-incorporation chain-terminating nucleotides despite the presence of a free 3′-hydroxyl group. Here, we describe the action of the 4′-acetyl- (MeTTP) and 4′-propanoylthymidine 5′-triphosphate (EtTTP) on HIV-1 reverse transcriptase in RNA- and DNA-dependent DNA synthesis and on DNA synthesis catalyzed by the cellular DNA polymerases α, β, δ and ϵ. MeTTP exhibits a high selectivity towards HIV-1 reverse transcriptase. By the use of the bulkier propanoyl group as the 4′-substituent of the nucleoside 5′-triphosphate, selectivity towards HIV-1 reverse transcriptase could be increased without affecting substrate efficiency. Thus, 4′-modifications may serve as a tool to increase selectivity towards HIV-1 reverse transcriptase.

INTRODUCTION

DNA chain-terminating nucleoside analogs have found wide application in DNA sequencing techniques and antiviral chemotherapy. A common feature of chain-terminating nucleotides used in DNA sequencing procedures is lack of a 3′-hydroxyl functionality on the 2′-deoxyribose, such as in 2′,3′-dideoxyribonucleotides or in 7-deaza-2′,3′-deoxyguanosine (1,2). In antiviral chemotherapy to combat human immunodeficiency virus (HIV) infection, inhibition of reverse transcription catalyzed by HIV-1 reverse transcriptase (RT) is the mode of action of clinically approved nucleoside drugs. Chain termination is believed to be the primary mechanism by which the corresponding antiviral nucleoside analog 5′-triphosphates exert their anti-HIV activities. A common structural feature of these nucleoside analogs is either lack of a 3′-hydroxyl functionality as found in the 2′,3′-dideoxyribonucleosides [like ddI, ddC or 2′,3′-didehydro-2′,3′-dideoxyribonucleosides (e.g. D4T)] or its substitution by another function-

ality [like 3′-azidothymidine (AZT)] (3–10). Nucleoside 5′-triphosphates with other modifications at the 2′-deoxyribose moiety as well as a free 3′-hydroxyl group are known to be substrates for DNA polymerases (DNA pols) and also RTs. Incorporation of these nucleotide analogs, which include 4′-azidothymidine 5′-triphosphate (11), arabinofuranosyl nucleotides [like araC (cytarabine) and F-ara-A (fludarabine)] (12,13) and 2′,2′-difluorodeoxycytidine (gemcitabine) (14), do not lead to DNA chain termination, whereas inhibitors lacking the 3′-hydroxyl functionality do. Inhibition of reverse transcription is believed to proceed via a pseudo-chain-termination mechanism (11–14). The aim in the design of new antiviral nucleosides is to develop tools which lead to high selectivity towards the targeted enzyme.

Recently, we have shown that the 4′-acylated thymidines 4′-acetylthymidine 5′-triphosphate (MeTTP) and 4′-propanoylthymidine 5′-triphosphate (EtTTP) (for structures see Fig. 1) act as post-incorporation chain-terminating nucleotides on several DNA pols of the DNA pol I family and RTs despite the presence of a free 3′-hydroxyl group in the deoxyribose (15,16).

They represent a new class of DNA chain-terminating nucleotides even though they have a free 3′-hydroxyl group. We have found that the 4′-modification is an excellent tool to increase selectivity towards HIV-1 RT. In the present study, we describe the action of the 4′-acylated thymidines MeTTP and EtTTP on HIV-1 RT in RNA- and DNA-dependent DNA synthesis and on DNA synthesis catalyzed by the cellular DNA pols α, β, δ and ϵ. Both thymidine analogs were used as substrates by HIV-1 RT with similar efficiency. DNA pol α was able to use the methylketone MeTTP as a substrate, whereas the ethylketone EtTTP was not a substrate for DNA pol α. None of the other cellular DNA pols present in the nucleus (DNA pol β, δ and ϵ) were able to use the 4′-modified thymidine analogs. Thus, selectivity towards HIV-1 RT could be enlarged by changing the size of the 4′-substituent without affecting the substrate efficiency of the nucleotide for the lentiviral enzyme. These observations may be exploited to develop strategies for the design of new nucleosidic antiviral chemotherapeutics with higher selectivity.

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*To whom correspondence should be addressed. Tel: +41 61 267 1106; Fax: +41 61 267 1105; Email: giese@ubaclu.unibas.ch
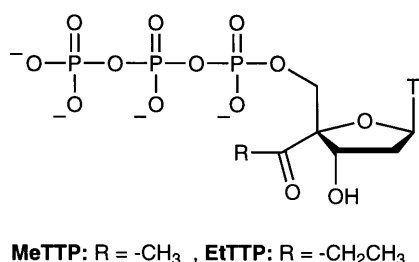
MeTTP: R = -CH₃ , EtTTP: R = -CH₂CH₃

Figure 1. Structures of the 4′-acylated thymidine 5′-triphosphates MeTTP and EtTTP.
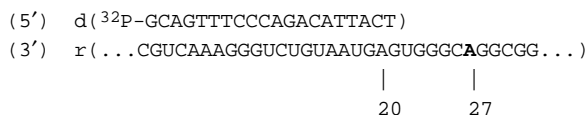
MATERIALS AND METHODS

Materials

MeTTP and EtTTP were synthesized according to the procedure described previously (15,17). Standard nucleotides and *Escherichia coli* 16S and 23S rRNA were purchased from Boehringer Mannheim. DNA oligonucleotides were prepared with a solid phase DNA synthesizer (Applied Biosystems) from β-cyano-ethyl-protected phosphoramidites, purified by the 'trityl-on' procedure, deprotected and purified by HPLC and PAGE. DNA primers were labeled at the 5′-end with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) and T4 polynucleotide kinase (New England Biolabs) (2). Human DNA pol β was purchased from Chimerx. HIV-1 RT was purified as described (18). Calf thymus DNA pols α, δ and ε were purified according to published procedures (19–21). Proliferating cell nuclear antigen (PCNA) was overexpressed and purified as described (22). One unit of DNA pol activity corresponds to incorporation of 1 nmol 2′-deoxyribonucleoside monophosphate into acid-precipitable material in 60 min at 37°C.

Enzyme reaction buffers

Buffer A: 20 mM Tris–HCl, pH 7.5, 6 mM MgCl₂, 40 mM KCl, 0.5 mM DTT. Buffer B: 20 mM K₂HPO₄/KH₂PO₄, pH 7.2, 10 mM MgCl₂, 0.5 mM DTT, 1.25 mg/ml BSA. Buffer C: 50 mM Tris–HCl, pH 8.7, 10 mM MgCl₂, 10 mM KCl, 1 mM DTT. Buffer D: 50 mM Tris–HCl, pH 6.5, 5 mM MgCl₂, 0.5 mM DTT, 1.25 mg/ml BSA.

DNA primer extension on 16S rRNA template

The 5′-end-labeled 20 base oligonucleotide primer was annealed to its complementary site on *E.coli* 16S rRNA (positions 116–135 using the deoxyribonucleotide numbering system of 23) by heating the DNA primer and a mixture of 16S and 23S rRNA to 80°C for 5 min and subsequent cooling to 25°C within 1 h.
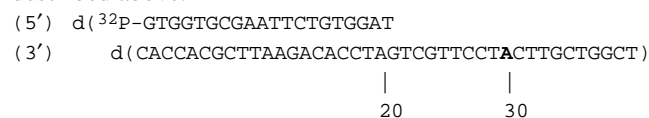
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(5′)  d(³²P-GCAGTTTCCCAGACATTACT)
(3′)  r(...CGUCAAAGGGUCUGUAAUGAGUGGGCAGGCGG...)
                          |        |
                          20       27
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Each DNA primer extension reaction mixture (10 μl) contained, dissolved in buffer A, 73 fmol labeled 20 base DNA primer, 0.5 μg *E.coli* 16S/23S rRNA mixture and 10 μM each dATP, dGTP and dCTP and various concentrations of TTP, MeTTP or EtTTP as indicated in the figure legends. The reactions

were initiated by addition of 0.3 U HIV-1 RT and incubated for 15 min at 37°C and analyzed in an 11% polyacrylamide denaturing sequencing gel (National Diagnostics). After electrophoresis the gel was transferred to filter paper (Whatmann 3MM) and dried at 80°C. The radioactivity in the DNA bands was quantified using a PhosphorImager (Molecular Dynamics). The relative velocities of incorporation of MeTTP and EtTTP were determined by dividing the radioactivity in the first T site (position 27) by radioactivity of the band 1 nt shorter (position 26), as previously described (8,9,24). The K_m and V_{max} values were then calculated from the Michaelis–Menten equation.

DNA primer extension on a DNA template

The 5′-end-labeled 20 base oligonucleotide primer was annealed to its complementary site on a 40 base oligonucleotide as described above.

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(5′)  d(³²P-GTGGTGCGAATTCTGTGGAT
(3′)       d(CACCACGCTTAAGACACCTAGTCGTTCCTACTTGCTGGCT)
                                 |        |
                                 20       30
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DNA primer extension reactions with HIV-1 RT contained, in a final volume of 10 μl in buffer A, 73 fmol labeled 20 base DNA primer, 230 fmol 40 base DNA template and 10 μM each dATP, dGTP and dCTP, various concentrations of TTP, MeTTP or EtTTP as indicated in the figure legends and 0.05 U HIV-1 RT. Incubation was performed for 15 min at 37°C and analyzed and quantified as described above. DNA primer extension reactions with DNA pols α and β were performed in buffers B and C respectively and 0.1 U enzyme was used. When DNA pol δ was used to perform DNA primer extension each reaction mixture (20 μl) contained, in buffer D, 36 fmol labeled 20 base DNA primer, 54 fmol 40 base DNA template, 90 ng PCNA and 20 μM each dATP, dGTP and dCTP, various concentrations of TTP, MeTTP or EtTTP as indicated in the figure legends and 0.01 U DNA pol δ. Incubation was performed for 15 min at 37°C and the products were analyzed and quantified as described above. When DNA pol ε was used to catalyze DNA synthesis the reaction mixtures (20 μl) contained, in buffer D, 36 fmol labeled 20 base DNA primer, 54 fmol 40 base DNA template and 30 μM each dATP, dGTP and dCTP and various concentrations of TTP, MeTTP or EtTTP as indicated in the figure legends. The reactions were initiated by addition of 0.4 U DNA pol ε, incubated for 15 min at 37°C and the products analyzed and quantified as described above.

RESULTS

DNA primer extension on RNA template catalyzed by HIV-1 RT

The action of the 4′-acylated thymidine 5′-triphosphates MeTTP and EtTTP (for structures see Fig. 1) on reverse transcription was studied using *E.coli* 16S rRNA (9) as template to which a 5′-^{32}P-labeled 20 base DNA primer was annealed. As shown in Figure 2, in the presence of dATP, dGTP and dCTP and in the absence of TTP, HIV-1 RT extended the primer six deoxyribonucleotides to form a main reaction product with a length of 26 nt (Fig. 2, lane 1).

When increasing concentrations of either MeTTP or EtTTP (1–100 μM) were added to the reaction mixture a decrease in the amount of the 26 base product and an increase in the 27 base product was observed (Fig. 2, lanes 2–15). This indicated that
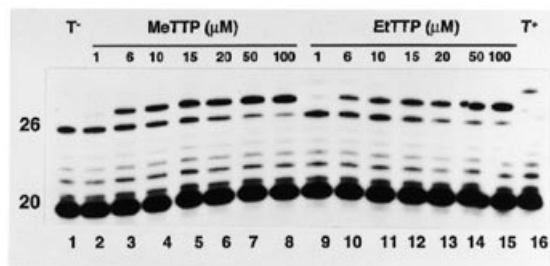
Figure 2. Incorporation of MeTTP and EtTTP into DNA by HIV-1 RT using rRNA template. The numbers on the left side of the figure indicate the length of oligonucleotide synthesized in primer extension. Lane 1, HIV-1 RT incubated with 10 μM each dATP, dCTP and dGTP and ^{32}P-labeled 20 base DNA primer annealed to 16S rRNA; lanes 2–8, as lane 1 but with 1, 6, 10, 15, 20, 50 and 100 μM MeTTP respectively; lanes 9–15, as lane 1 but with 1, 6, 10, 15, 20, 50 and 100 μM EtTTP respectively; lane 16, control reaction, as lane 1 but with 10 μM TTP.

Figure 3. Incorporation of MeTTP and EtTTP into DNA by HIV-1 RT using DNA template. The numbers on the left side of the figure indicate the length of oligonucleotide synthesized in primer extension. LM, line marker. Lane 1, HIV-1 RT incubated with 10 μM each dATP, dCTP and dGTP and ^{32}P-labeled 20 base DNA primer annealed to 40 base DNA template; lanes 2–10, as lane 1 but with 1, 6, 10, 15, 20, 50, 100, 150 and 200 μM MeTTP respectively; lanes 11–19, as lane 1 but with 1, 6, 10, 15, 20, 50, 100, 150 and 200 μM EtTTP respectively; lane 20, control reaction, as lane 1 but with 10 μM TTP.

HIV-1 RT was able to use both MeTTP and EtTTP as substrates for incorporation into the first T site in RNA-dependent DNA synthesis. Formation of the 27 base product was clearly due to incorporation of the thymidine analogs MeTTP and EtTTP into the nascent DNA strand, since no pausing at position 27 was observed when the reaction was performed in the presence of all four natural dNTPs (Fig 2, lane 16). Most of the cDNA products in the reaction shown in Figure 2, lane 16 ran higher on the gel and are therefore not visible. There were no indications that further DNA strand elongation occurs after incorporation of both 4′-modified thymidine analogs, despite the presence of a free 3′-hydroxyl group at the primer terminus. Both analogs acted as post-incorporation DNA chain terminators. The radioactivity in sites 26 and 27 was quantitated and the incorporation kinetics (K_m and V_{max}) were determined as described in Materials and Methods. As shown in Table 1, the K_m value for incorporation of MeTTP into the first T site (position 27) by HIV-1 RT was 2- to 3-fold less than that for incorporation of EtTTP, indicating that HIV-1 RT has a higher affinity for MeTTP. Interestingly, the substrate efficiency (V_{max}/K_m) (8,9,24) for MeTTP was the same as for the bulkier EtTTP.

DNA primer extension on DNA template catalyzed by HIV-1 RT

Recently we showed that DNA-directed incorporation of MeTTP by HIV-1 RT can result in a slower further elongation at the primer 3′-terminus when a high enzyme:DNA substrate ratio was employed (15,16). However, to quantitate nucleotide insertion kinetics the enzyme has to be saturated with its DNA substrate and therefore l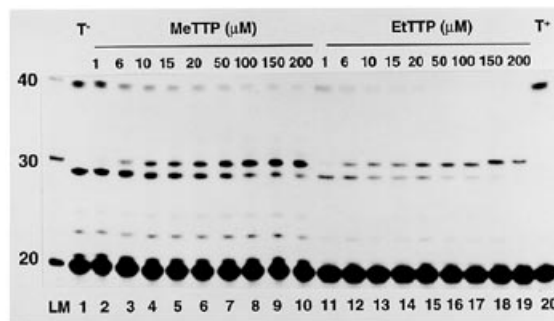ow enzyme:DNA substrate ratios have to be employed (8–10,24). Under these conditions, MeTTP acted as a post-incorporation DNA chain terminator in DNA-dependent DNA synthesis catalyzed by HIV-1 RT (Fig. 3). When the DNA 20 base primer/40 base template (for sequences see Materials and Methods) were incubated with HIV-1 RT in the presence of dATP, dGTP and dCTP without TTP, the 20 base primer was extended by 9 nt to form a major stop site (position 29), one position before the first T site (Fig. 3, lane 1).

The formation of longer reaction products indicated that some non-complementary dNTP molecules were incorporated into the first T site and subsequently the DNA primer strand was further elongated. This misincorporation tendency of HIV-1 RT confirms its known error-prone properties (25–28). When HIV-1 RT catalyzed primer extension in the presence of increasing concentrations of the 4′-acylated thymidine analog MeTTP or EtTTP (1–200 μM), increasing incorporation was observed by accumulation of 30 base reaction products (Fig. 3, lanes 2–19). Again, with the use of TTP instead of MeTTP or EtTTP (Fig. 3, lane 20) it could be confirmed that formation of the 30 base product can be ascribed to incorporation of the thymidine analogs. The kinetic parameters were determined as described above and are listed in Table 1. The K_m value for MeTTP incorporation with the DNA template is similar to the K_m value with the RNA template. For EtTTP, we found the K_m value and substrate efficiency in this reaction to be of the same order of magnitude as for MeTTP. The K_m value for incorporation of EtTTP in the DNA-dependent reaction turned out to be 3-fold less than found in the reverse transcription reaction.

Table 1. Kinetic parameters of MeTTP and EtTTP incorporation into DNA

Enzyme	Analog	16S rRNA template			40 base DNA template		
		K_m (μM)	V_{max}	V_{max}/K_m (per μM)	K_m (μM)	V_{max}	V_{max}/K_m (per μM)
HIV-1 RT	MeTTP	15	2.9	0.19	10	1.6	0.16
	EtTTP	42	5.6	0.13	12	2.4	0.20
DNA pol α	MeTTP	n.a.	n.a.	n.a.	338	0.72	0.002

n.a., not applicable, since DNA pol α does not copy RNA templates.
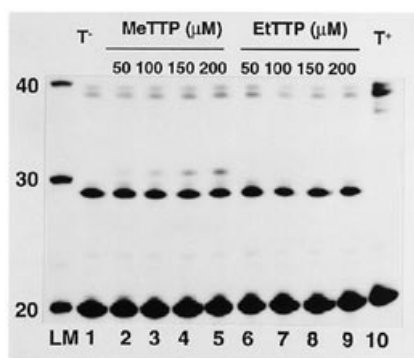
Figure 4. Action of MeTTP and EtTTP on DNA pol α. The numbers on the left side of the figure indicate the length of oligonucleotide synthesized in primer extension. LM, line marker. Lane 1, DNA pol α incubated with 10 µM each dATP, dCTP and dGTP and ^{32}P-labeled 20 base DNA primer annealed to 40 base DNA template; lanes 2–5, as lane 1 but with 50, 100, 150 and 200 µM MeTTP respectively; lanes 6–9, as lane 1 but with 50, 100, 150 and 200 µM EtTTP respectively; lane 10, control reaction, as lane 1 but with 10 µM TTP.

DNA primer extension catalyzed by cellular DNA pols

To evaluate the action of the 4′-acylated thymidines MeTTP and EtTTP on cellular DNA pols we used the DNA primer/template complex described above. When DNA pol α was used to perform primer elongation in the presence of dATP, dGTP and dCTP, formation of a 29 base DNA product as major reaction product was observed (Fig. 4, lane 1).

Only trace amounts of misincorporation were observed in this case. In the presence of increasing concentrations of MeTTP (50–200 µM) DNA pol α incorporated this analog into the nascent DNA strand (Fig. 4, lanes 2–5). The kinetic parameters were obtained as described above and are listed in Table 1. The K_m value for incorporation of MeTTP by DNA pol α is ~30-fold greater than that obtained with HIV-1 RT. The substrate efficiency of DNA pol α for MeTTP is ~100-fold lower than that for HIV-1 RT. Interestingly, no indication of post-incorporation chain

elongation could be obtained. Therefore, MeTTP acts as a chain terminator for DNA pol α as well, in spite of the free 3′-hydroxyl group. If primer elongation by DNA pol α was performed in the presence of the moderately bulkier EtTTP, no incorporation of the thymidine analog could be detected (Fig. 4, lanes 6–9). Thus, an increase in the bulk of the 4′-acyl modification of the analog by only one CH2 moiety ensures that this analog is no longer a substrate for DNA pol α. If, however, the three DNA pols β, δ and ε present in the nucleus were tested with the same concentrations of MeTTP and EtTTP and under optimal conditions for each enzyme, neither of the thymidine analogs was incorporated into the growing DNA strand by any of these other three cellular DNA pols (Fig. 5).

DISCUSSION

In spite of the fact that the 4′-acylated thymidines MeTTP and EtTTP bear a free 3′-hydroxyl group, this study indicates that their incorporation into DNA blocks further DNA chain elongation. DNA pols are thought to catalyze DNA polymerization by sequential conformational changes in the enzyme structure (29–31). HIV-1 RT is thought to undergo a conformational change after binding the nucleoside 5′-triphosphate, which positions the nucleotide for phosphodiester bond formation (31). Therefore, the binding affinity of the nucleotide substrate at the active site in the RT is crucial for incorporation. The K_m value represents a parameter for the binding affinity of the nucleotide at the active site (24). From the K_m values the differences in the free binding energies of MeTTP and EtTTP can be calculated (24). It turned out that the free binding energy of MeTTP for HIV-1 RT in reverse transcription is ~0.6 kcal/mol higher than for EtTTP. This may be due to the bulkier 4′-substituent, which may interfere with the enzyme or fix the sugar pucker conformation unfavorably for binding. However, the K_m values for DNA-directed DNA synthesis with MeTTP and EtTTP are about the same. The substrate efficiency of both substrates was found to be about the same for both reactions. These studies revealed that an increase in the size of the 4′-substituent by one methylene moiety did not have a significant effect on nucleotide binding and incorporation. In contrast, as shown recently (15,16), HIV-1 RT
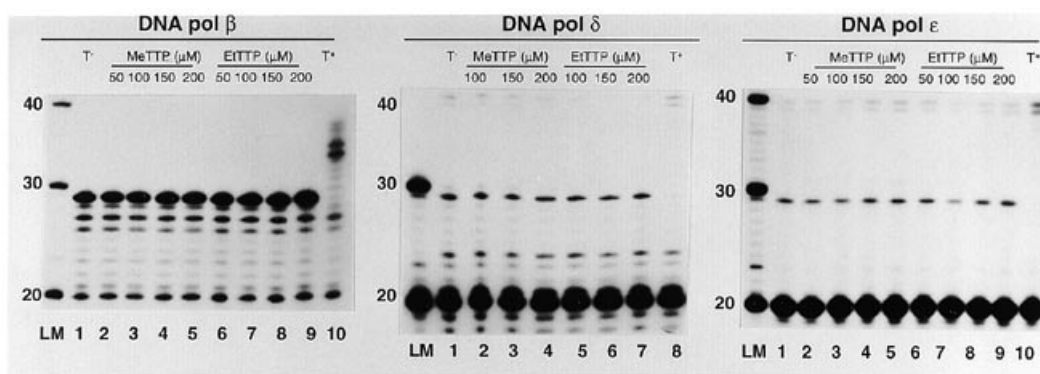

Figure 5. Action of MeTTP and EtTTP on DNA pols β, δ and ε. (**Left**) LM, line marker. Lane 1, DNA pol β incubated with 10 µM each dATP, dCTP and dGTP and ^{32}P-labeled 20 base DNA primer annealed to 40 base DNA template; lanes 2–5, as lane 1 but with 50, 100, 150 and 200 µM MeTTP respectively; lanes 6–9, as lane 1 but with 50, 100, 150 and 200 µM EtTTP respectively; lane 10, control reaction, as lane 1 but with 10 µM TTP. (**Center**) LM, line marker. Lane 1, DNA pol δ incubated with 20 µM each dATP, dCTP and dGTP, PCNA and ^{32}P-labeled 20 base DNA primer annealed to 40 base DNA template; lanes 2–4, as lane 1 but with 100, 150 and 200 µM MeTTP respectively; lanes 5–7, as lane 1 but with 100, 150 and 200 µM EtTTP respectively; lane 8, control reaction, as lane 1 but with 10 µM TTP. (**Right**) As in the left panel but with DNA pol ε.

is able to promote chain elongation after incorporation of the methylketone MeTTP when high enzyme quantities are used. The ethylketone EtTTP acts as a chain-terminating nucleotide upon incorporation. Thus, the size of the 4′-modification is less crucial for incorporation of the nucleotide by HIV-1 RT than for post-incorporation DNA elongation.

The K_m values obtained for incorporation of MeTTP and EtTTP are about the same and up to 50-fold greater than reported for TTP or antiviral nucleoside 5′-triphosphates such as ddTTP, AZTTP and D4TTP elucidated in similar assays (8,9). It should be emphasized that the kinetic values for incorporation of normal nucleotides is dependent on the sequence under evaluation (31). Therefore, a direct comparison of the kinetic values should be done with caution. In treatment of antiviral diseases, the nucleosidic drugs are applied as nucleosides and have to be transformed into the corresponding 5′-triphosphates by cellular kinases to be effective drugs. For further evaluation, the action of 4′-acylated thymidines on cellular kinases has to be studied.

As mentioned, selectivity towards a targeted enzyme is desirable in the design of new therapeutic agents. Therefore, we evaluated the action of MeTTP and EtTTP on four DNA pols (for reviews see 32–34) which are essential in cellular DNA replication (DNA pols α, δ and ε), in DNA repair processes (DNA pols β, δ and ε), in repair recombination (DNA pol ε; 35) and in V(D)J recombination (DNA pol δ; 36). It turned out that the 4′-acylated thymidine analogs were very poor substrates for all cellular DNA pols tested. Only DNA pol α was able to incorporate MeTTP, at ~100-fold less substrate efficiency than that obtained for HIV-1 RT. The bulkier EtTTP was not incorporated by DNA pol α. DNA pols β, δ and ε incorporated neither MeTTP nor EtTTP. Thus, an increase in size of the 4′-modification ensures that the thymidine analog EtTTP is not a substrate for incorporation by any cellular nuclear DNA pol. Since this structural modification does not change the sugar pucker conformation (17), the different reactivity of EtTTP to MeTTP can be ascribed to the increased bulk of the 4′-substituent.

Our studies demonstrate that the 4′-acylated thymidine analogs MeTTP and EtTTP are preferentially incorporated into DNA by HIV-1 RT. To assess the influence of the 4′-acyl modification on DNA synthesis catalyzed by HIV-1 RT and cellular DNA pols the size of the sugar pucker substituent was increased. Enlargement of the 4′-methylketone MeTTP by one methylene moiety to the 4′-ethylketone EtTTP resulted in rejection of the thymidine analog as a substrate for cellular nuclear DNA pols, without reducing its substrate efficiency for HIV-1 RT in comparison with MeTTP. Thus, the 4′-modification may serve as a tool to increase selectivity towards the lentiviral enzyme. These observations make 4′-modified nucleotides very interesting in the search for new nucleosidic drugs. Additionally, it would be quite significant if this strategy could also be applied to other positions of the sugar residue of nucleotides. Investigations along these lines are in progress.

ACKNOWLEDGEMENTS

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