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Highly selective action of triphosphate metabolite of 4′-ethynyl D4T: A novel anti-HIV compound against HIV-1 RT

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Abstract

2′,3′-Didehydro-3′-deoxy-4′-ethynylthymidine (4′-Ed4T), is a recently discovered nucleoside reverse transcriptase inhibitor (NRTI) showing a 5- to 10-fold greater anti-human immunodeficiency virus type 1 (HIV-1) activity and less cellular and mitochondrial toxicity than its parental compound, stavudine (D4T). It is also active against a variety of NRTI-resistant HIV-1 mutants under non-cytotoxic concentrations. In this study, the effects of 4′-Ed4TTP, which is the triphosphate metabolite of 4′-Ed4T, on HIV-1 reverse transcriptase (RT) activity were investigated. We found that 4′-Ed4TTP was a substrate of HIV-1 RT serving as a DNA chain terminator, and it inhibited the DNA polymerase activity of RT more efficiently than D4TTP. The value of $K_{i(4'\text{-Ed4TTP})}/K_{m(dTTP)}$ is 0.15 for DNA/RNA primer/template duplex (P/T), but 0.7 for DNA/DNA P/T, suggesting 4′-Ed4TTP inhibits RT more efficiently during RNA-dependent DNA synthesis than DNA-dependent DNA synthesis. 4′-Ed4TTP was also found to inhibit the 3TC (Lamivudine)-resistant RT mutant, M184V, with 3-fold less efficiency than the wild type (wt) RT. 4′-Ed4TTP showed much less inhibitory effects toward major host DNA polymerases. Overall, our results suggest that 4′-Ed4TTP is the active form for anti-HIV-1 activity via its inhibitory effect against RT.

Keywords: 2′,3′-Didehydro-3′-deoxy-4′-ethynylthymidine (4′-Ed4T); HIV-1; RT; Inhibition; NRTI

1. Introduction

HIV-1 RT has two distinct enzymatic activities, namely the RNA- or DNA-dependent DNA polymerase activity and the ribonuclease (RNase) H activity, both are essential for the replication of HIV-1 RNA genome (Goff, 1990). Shortly after HIV-1 infection into a permissive host cell, RT uses these two activities to convert the single-stranded RNA genome into a double-stranded DNA copy. The critical roles of RT in the HIV-1 replication cycle have made it a primary target for the development of many antiviral therapeutic agents including NRTIs and non-nucleoside RT inhibitors (NNRTIs) in the treatment of acquired immunodeficiency syndrome (AIDS) (Mitsuya et al., 1990). Among NRTIs, the dideoxynucleosides, such as AZT, D4T, ddC, ddI and 3TC, are a very important group of antiviral compounds (Hamamoto et al., 1987; Lin et al., 1987a,b). This group of compounds serves as DNA chain terminator after they are phosphorylated to their corresponding triphosphate forms by host cellular kinases and consequently incorporated into viral DNA by HIV-1 RT.

However, the application of this group of compounds is clinically limited due to their cytotoxicity since NRTIs are usually inhibitors and substrates of host DNA polymerases as well. For example, D4T, ddC and ddI can cause mitochondrial DNA damage through their incorporation into mitochondrial DNA by DNA polymerase γ (Chen and Cheng, 1989; Chen et al., 1991; Medina et al., 1994). Moreover, the rapid emergence of drug-resistant mutants is another major limitation for the use

Abbreviations: NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; D4T, 2′,3′-didehydro-3′-deoxythymidine (also known as stavudine); 4′-EdT, 4′-ethynyl D4T; AZT, 3′-azido-3′-deoxythymidine (also known as zidovudine); 3TC, β-L-2′,3′-dideoxy-3′-thiacytidine (also known as lamivudine); P/T, primer/template duplex

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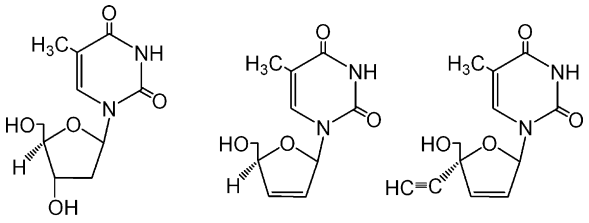

Fig. 1. Chemical structures of dT, D4T and 4′-Ed4T.

of this group of compounds. For example, a single amino acid substitution from Methionine-184 to Valine (M184V) of HIV-1 RT evokes resistance to 3TC up to 1000-fold (Schinazi et al., 1993); and the Q151M mutation in HIV-1 RT is associated with multidrug resistance towarding AZT, ddC and ddI (Ueno et al., 1995). These examples illustrate the urgent need in developing new compounds with less cytotoxicity and more potent antiviral effects, especially against the drug-resistant viral strains.

In the search for new antiviral compounds, 4′-Ed4T (Fig. 1), a novel D4T analog, was found to have 5-fold more anti-HIV-1 effect than its parent compound D4T. In addition 4′-Ed4T had a lower cellular and mitochondrial toxicity than D4T based on phenotypic drug susceptibility studies (Haraguchi et al., 2003; Dutschman et al., 2004; Nitanda et al., 2005) although the underlying mechanism was poorly understood. 4′-Ed4T was also found to be active against many drug-resistant HIV-1 strains, in which the RT bears amino acid substitutions such as M184V (3TC-resistant) and A62V/V75I/F77L/F116Y/Q151M (multidrug-resistant) (Nitanda et al., 2005). The potency of 4′-Ed4T against HIV-1 with M184V RT mutation is about 3–5 times less than that against wt HIV-1(Nitanda et al., 2005). Recent studies on the metabolism of 4′-Ed4T from our laboratory also showed that this compound could be phosphorylated stepwise to its mono-, di- and triphosphate forms by host kinases (Chih-Hung Hsu et al., unpublished results). The formation of 4′-Ed4TTP implies that 4′-Ed4T could be incorporated into viral DNA by HIV-1 RT and serve as a DNA chain terminator, in the same way as other dideoxynucleoside analogues.

The steady-state inhibition profile of D4TTP has been determined using the single-nucleotide incorporation assay by others (Ueno and Mitsuya, 1997). Here we performed the same assay to study the effects of 4′-Ed4T on RNA-dependent and DNA-dependent DNA syntheses by HIV-1 RT and compared with that of D4TTP. We also examined the effect of 4′-Ed4T on the RNase H activity of RT as well as the inhibitory effects of 4′-Ed4TTP on five major human DNA polymerases (pol α, β, γ, δ, ε).

2. Experimental procedures

2.1. Materials

4′-Ed4T was synthesized in the laboratory of Dr. Hiromichi Tanaka, School of Pharmaceutical Sciences, Showa University, Tokyo, Japan (Haraguchi et al., 2003); D4T was purchased from Sigma–Aldrich (St. Louis, MO). The mono-, di- and triphosphate forms of 4′-Ed4T and D4T were synthesized and purified following the protocol of Krishnan et al. (2002). The purity of these compounds was verified by HPLC analysis. [γ-^{32}P]ATP and [α-^{32}P]dTTP were purchased from NEN Life Sciences Company (Boston, MA). dNTPs were purchased from Amersham/Pharmacia (Piscataway, NJ). All other chemicals used were of analytical grade.

The DNA oligonucleotides (23, 36 and 60 mer) shown in Table 1 were synthesized and gel-purified by the Keck Facility at Yale University. The 36-mer RNA oligonucleotide (Table 1) was synthesized and gel-purified by New England Biolabs (Ipswich, MA). Activated calf thymus DNA was purchased from Sigma–Aldrich. T4 polynucleotide kinase was purchased from New England Biolabs. HIV-1 wt RT and M184V proteins as well as recombinant human DNA polymerase γ were purified according to previously published method (Murakami et al., 2004). DNA polymerase α in a 4-subunit protein complex including p180 (pol α), p68, p58 and p48 subunits purified from HeLa cells was a gift from Dr. Teresa Wang at Stanford University; recombinant human DNA polymerase β was kindly provided by Dr. Joann Sweasy at Yale University; recombinant human DNA polymerase δ (a 4-subunit complex), proliferating cell nuclear antigen (PCNA) as well as poly dA/Oligo dT were kindly provided by Dr. Bin Xie and Dr. Marietta Lee at New York Medical College. Human DNA polymerase ε was purified from H9 cells using a protocol described previously (Huang et al., 1990).

2.2. Preparation of DNA/RNA and DNA/DNA duplexes

For cold DNA/RNA and DNA/DNA P/T, the purified primer was annealed with the template in a molar ratio of 1:1.3 at 80 °C for 4 min and then 50 °C for 30 min. For radio-labeled DNA/RNA or DNA/DNA P/T, the primer was first labeled at 5′-end with γ-^{32}P-ATP by T4 polynucleotide kinase and then annealed to its complementary template.

2.3. Steady-state single nucleotide incorporation assays for K_m and k_{cat} determination by HIV-1 RT

All enzymatic reactions in this study were carried out in the standard reaction buffer containing 50 mM Tris–HCl, pH 8.0, 60 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA. Unless otherwise mentioned, all the concentrations reported in this study were final concentrations after mixing. In the steady-state single nucleoside incorporation assays for K_m and k_{cat} determination by HIV-1 RT, a mixture containing 250 nM 23/36 mer P/T (DNA/RNA or DNA/DNA duplex, with primer 5′-^{32}P labeled, see Table 1), and various concentrations of dTTP, D4TTP or 4′-Ed4TTP in the standard reaction buffer was pre-incubated at 37 °C, and the reaction was initiated by adding 2.5 nM wt or mutant RT. Aliquots (4 μl each) were removed at different times, and mixed with 2 μl gel loading buffer (98% formamide, 10 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue) to stop the reaction. The products were resolved by electrophoresis on a 20% polyacrylamide gel containing

Table 1
Sequences of oligonucleotide substrate used in this study

P/T	Sequence
DNA/RNA 23/36mer	5′-TCAGGTCCCTGTTCGGGCGCCAC-3′ 3′-CGAAAGUCCAGGGACAAGCCCGCGGUGACGAUCUCU-5′
DNA/DNA 23/36mer	5′-TCAGGTCCCTGTTCGGGCGCCAC-3′ 3′-CGAAAGTCCAGGGACAAGCCCGCGGTGACGATCTCT-5′
DNA/RNA 60/36mer	5′-TGGTTTCCCTTTCGCTTTCAGGTCCCTGTTCGGGCGCCACTGCTAGAGATTTTCCACACT-3′ 3′-CGAAAGUCCAGGGACAAGCCCGCGGUGACGAUCUCU-5′

50% urea, and then quantified by phosphorimaging (Molecular Dynamics or Bio-Rad).

2.4. Steady-state inhibition of the polymerase activities of RT and host DNA polymerases

For inhibition of polymerase activity of RT, the reaction mixture contained 250 nM 23/36 mer P/T (DNA/RNA or DNA/DNA duplex), various concentrations of [α-^{32}P]-dTTP and various concentrations of inhibitor. The reaction was initiated by adding 2.5 nM RT at 37 °C. Aliquots (4 μl each) were removed at different times, and mixed with 2 μl 0.5 M EDTA to stop the reaction. Then 3 μl of each sample was spotted onto DE81 filter paper. The filter paper was subsequently washed 3 times with 0.5 M Na_2HPO_4, pH 7.0, dried and exposed to a phosphor imager screen. The amount of incorporated dTTP was quantified by phosphorimaging.

Assays for inhibition of host DNA polymerases were performed similar to the RT inhibition assays described above, with 0.3 μM [α-^{32}P]-dTTP, various concentrations of inhibitor, a suitable amount of each kind of DNA polymerase and 0.1 mg/ml activated calf thymus DNA (except for assays to inhibit DNA polymerase δ, where 0.4 μM poly dA/oligo dT was used instead because of the low activity of pol δ with calf thymus DNA).

2.5. RNase H activity assay

RNA 36 mer (Table 1) was 5′-^{32}P labeled, annealed with either DNA 23 mer or DNA 60 mer (Table 1) to generate a DNA/RNA 23/36 mer with DNA recessed at the 3′-end or a DNA/RNA 60/36 mer with RNA recessed at the 5′-end. The reaction mixture contained 250 nM P/T, various concentrations of 4′-Ed4TMP, -DP, -TP or other nucleoside analogues (i.e. D4T). The reaction was initiated by adding 2.5 nM RT at 37 °C. Aliquots (4 μl each) were removed at different times, stopped and then gel-analyzed.

2.6. Data analysis

The steady-state kinetic parameters were determined from linear steady-state velocities using GraphPad Prism program (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Comparison of the inhibitory effects of 4′-Ed4TTP on RT activity during RNA-dependent and DNA-dependent DNA synthesis

Based on the structural similarity between 4′-Ed4T and D4T (Fig. 1), we anticipated that the anti-HIV activity of 4′-Ed4T would come from the inhibitory effect of its triphosphate form against HIV-1 RT, in the similar way as D4T. To prove this hypothesis, the effects of 4′-Ed4TTP on RNA-dependent DNA synthesis catalyzed by RT were investigated under steady-state condition. When 1 μM dTTP was used for DNA synthesis by RT, 4′-Ed4TTP inhibited RT activity with an IC_{50} value of 0.2 μM (Fig. 2A, upper panel), 50-fold less than D4TTP, which had an IC_{50} value of 10 μM (Fig. 2A, lower panel). While 4′-Ed4TMP and 4′-Ed4TDP showed no inhibition to RT activity with concentration up to 100 μM (data not shown). Then the type of inhibition was characterized by measuring the apparent K_m and k_{cat} values of dTTP at various concentrations of 4′-Ed4TTP. With DNA/RNA P/T, 4′-Ed4TTP competitively inhibited dTTP incorporation with a K_i of 0.026 μM, 7-fold lower than the K_m value of dTTP (Fig. 2B and Table 2).

Since HIV-1 RT catalyzes both RNA-dependent and DNA-dependent DNA syntheses, the K_i values of 4′-Ed4TTP for RT in both reactions were determined and compared. In addition, the K_i values of D4TTP were also determined under the same conditions for comparison. The results are summarized in Table 2. The value of $K_{i(compound)}/K_{m(dTTP)}$ was used as an index to evaluate the inhibition efficiency. A lower K_i/K_m value indicates more efficient inhibition. When a DNA/RNA P/T was used, the K_i/K_m value of 4′-Ed4TTP was 0.15, 5-fold lower than the K_i/K_m value when a DNA/DNA P/T was used, suggesting that 4′-Ed4TTP inhibits RNA-dependent DNA synthesis 5-fold more efficiently than it inhibits DNA-dependent DNA synthesis. Similarly D4TTP had a K_i/K_m value of 1.1 for DNA/RNA P/T, and 3.1 for DNA/DNA P/T. The D4TTP inhibition results were in agreement with those reported previously (Ueno and Mitsuya, 1997). Overall, the inhibitory efficiency of 4′-Ed4TTP was 7-fold higher than that of D4TTP with DNA/RNA P/T, and 4-fold higher with DNA/DNA P/T as judged by their K_i/K_m values.

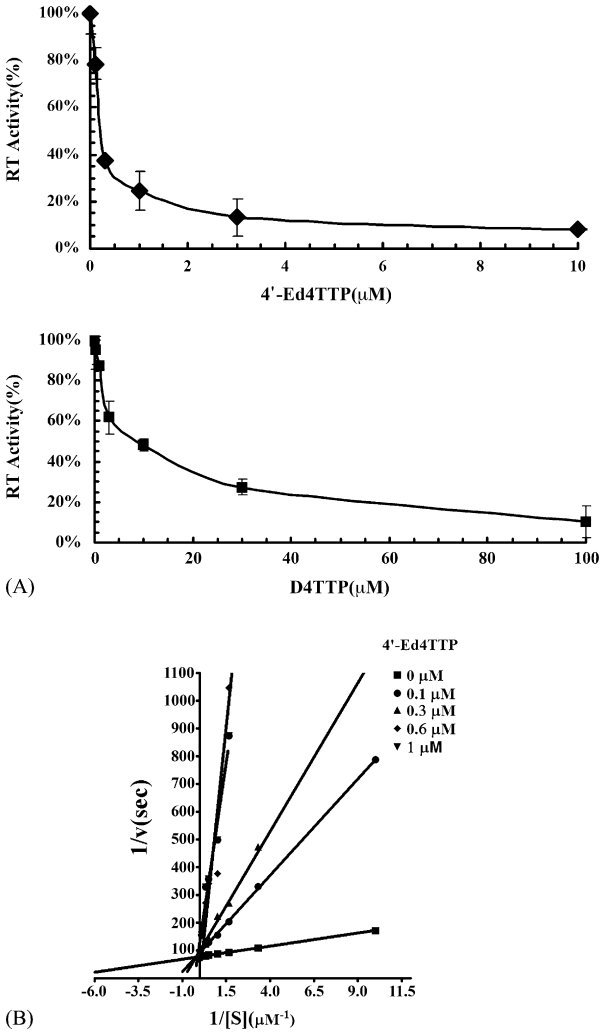

Fig. 2. Inhibition of RT by 4′-Ed4TTP and D4TTP. The steady-state inhibition of RT activity experiments were performed as described in Section 2. (A) Comparison of the inhibitory effect of 4′-Ed4TTP and D4TTP when 1 μM dTTP, 250 nM DNA/RNA 23/36 mer (Table 1) and 2.5 nM wt RT were used. The IC_{50} for 4′-Ed4TTP and D4TTP were 0.2 and 10 μM, respectively. (B) Lineweaver–Burk plots showed that 4′-Ed4TTP competitively inhibited dTMP incorporation by wt RT with a K_i value of 0.026 μM, 7-fold lower than the K_m value of dTTP. The experiments were performed as described in Section 2 with 250 nM DNA/RNA 23/36 mer as P/T.

3.2. Comparison of the inhibitory effects of 4′-Ed4TTP on wt and M184V mutant RT

Since our phenotypic drug susceptibility assay demonstrated that 4′-Ed4TTP was 3- to 11-fold less efficient against the 3TC-resistant HIV-1 mutant strain M184V than the wt HIV-1 strain ((Nitanda et al., 2005; Dutschman et al., unpublished results), it was of interest to examine the inhibitory effects of 4′-Ed4TTP on M184V mutant RT. Therefore, we compared the kinetic parameters of the wt RT and M184V mutant to better understand the correlation between drug susceptibility assays and kinetic studies. The results are summarized in Table 2. With a DNA/RNA P/T, the K_i/K_m value of 4′-Ed4TTP for M184V RT mutant was 0.42, showing a 3-fold increase compared to wt RT. With a DNA/DNA P/T, the K_i/K_m value of 4′-Ed4TTP for M184V mutant was 1.4, showing a 2-fold increase compared to wt RT. In contrast, D4TTP had decreased K_i/K_m values for M184V mutant compared to wt RT with both DNA/RNA and DNA/DNA P/T. These results were in good agreement with the results from drug susceptibility assays (Nitanda et al., 2005; Dutschman et al., unpublished results).

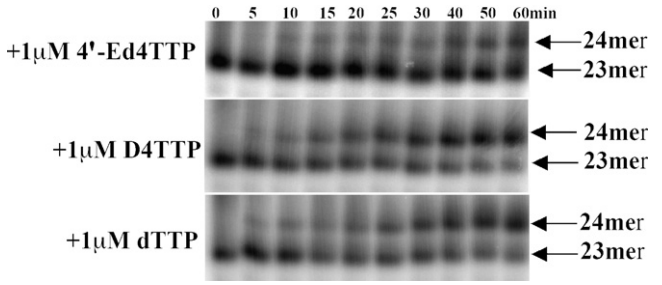

Fig. 3. Utilization of 4′-Ed4TTP, D4TTP and dTTP for primer extension by RT. The formation of 24 mer in the presence of 4′-Ed4TTP indicated that 4′-Ed4TTP was used by RT for DNA synthesis. The incorporation efficiency of 4′-Ed4TMP was lower compared with that of D4TMP and dTMP. The experiments were performed under single nucleotide incorporation assay condition with 250 nM DNA/RNA 23/36 mer, 5 nM RT and 1 μM 4′-Ed4TTP, D4TTP or dTTP, respectively, as described in Section 2.

3.3. Incorporation of 4′-Ed4TMP into the nascent DNA strand by HIV-1 RT

Since 4′-Ed4TTP competitively inhibited dTTP incorporation, it was necessary to know if 4′-Ed4TTP was a substrate of RT for DNA synthesis as well. We found that the DNA primer (23 mer) in the DNA/RNA P/T was elongated to a 24 mer by RT in the presence of 1 μM 4′-Ed4TTP (Fig. 3, upper panel) indicating that 4′-Ed4TMP was incorporated into DNA by RT, base-pairing with AMP from the template strand. However, the incorporation efficiency of 4′-Ed4TMP by RT was lower than that of D4TMP and dTMP (Fig. 3, middle and lower panels). A DNA primer with 4′-Ed4TMP at the 3′-end in a DNA/RNA

Table 2
Kinetic parameters for inhibition of wild type RT and M184V mutant by 4′-Ed4TTP and D4TTP

Enzyme, P/T	Wild type				M184V			
	DNA/RNA		DNA/DNA		DNA/RNA		DNA/DNA	
	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
D4TTP	0.19	1.1	3.7	3.1	0.070	0.44	1.2	2.4
4′-Ed4TTP	0.026	0.15	0.9	0.7	0.067	0.42	0.7	1.4
$K_{m(dTTP)}$ (μM)	0.17		1.2		0.16		0.52	

The K_i and K_m values represent means from at least three independent experiments with standard deviation less than 20%.

Table 3
Steady-state kinetic parameters for 4′-Ed4TMP, D4TMP and dTMP incorporation by wt RT and M184V mutant

Compound	Wild type RT			M184V		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
4′-Ed4TTP	0.16	0.008	0.05	0.28	0.006	0.02
D4TTP	0.12	0.021	0.18	ND[a]	ND	ND
dTTP	0.17	0.021	0.12	0.16	0.005	0.03

The K_m and k_{cat} values represent means from at least three independent experiments with standard deviation less than 20%.
[a] ND, not determined.

P/T could not be elongated in the presence of all four kinds of dNTPs indicating that 4′-Ed4TMP became a DNA chain terminator once it was incorporated into the DNA strand (data not shown).

The steady-state kinetic parameters for 4′-Ed4TMP incorporation by wt RT as well as by M184V mutant were determined with DNA/RNA P/T. The results were compared with the kinetic parameters obtained from D4TMP and dTMP incorporation by RT. As shown in Table 3, with wt RT, the K_m of 4′-Ed4TMP incorporation (0.16 μM) was identical to the K_m of dTMP incorporation, but slightly higher than the K_m of D4TMP incorporation. The k_{cat} of 4′-Ed4TMP incorporation was 0.008 s^{-1}, 2.5-fold less than the k_{cat} of D4TMP and dTMP incorporation (both were 0.021 s^{-1}). With M184V mutant, the K_m of 4′-Ed4TMP incorporation (0.28 μM) was 1.7-fold higher than the K_m of dTMP incorporation, while its k_{cat} was 0.006 s^{-1}, close to the k_{cat} of dTMP incorporation.

3.4. Inhibition of host DNA polymerases by 4′-Ed4TTP

In order to understand the much lower cellular toxicity of 4′-Ed4T compared with D4T, the effects of 4′-Ed4TTP on five major human DNA polymerases were also examined, including the recombinant DNA polymerases β, γ and δ as well as the endogenous DNA polymerase α in a 4-subunit complex purified from HeLa cells, and the DNA polymerase ε partially purified from H9 cells. As shown in Table 4, 4′-Ed4TTP exhibited no inhibition on DNA polymerase α, β and ε with the concentration of 4′-Ed4TTP up to 100 μM, and the IC_{50} value for pol δ was about 60 μM. It is worth noting that 4′-Ed4TTP could inhibit pol γ with an IC_{50} at about 100 μM, 100-fold greater than that of either D4TTP or ddTTP. These results indicate that 4′-Ed4TTP in general is a much weaker inhibitor against major host DNA polymerases compared with D4TTP and ddTTP.

Table 4
Action of 4′-Ed4TTP on major human DNA polymerases

DNA polymerase	IC_{50}(μM)[a,b]			
	4′-Ed4TTP	D4TTP	ddTTP	Aphidicolin
α	>100	>100	ND[c]	5
β	>100	1	0.3	ND
γ	~100	1	1	ND
δ	60	40	>100	ND
ε	>100	>100	ND	5

[a] The IC_{50} values represent means from at least three independent experiments with standard deviation less than 20%.
[b] When 0.3 μM dTTP was used in the assays.
[c] ND, not determined.

3.5. Effect of 4′-Ed4T on RNase H cleavage

Since the RNase H activity of HIV-1 RT removes the RNA template once the cDNA is synthesized, it was of interest to examine if 4′-Ed4T had any impact on the RNase H activity of RT. Two modes of RNase H cleavage have been described (Archer et al., 2001): (i) DNA 3′-end directed RNase H cleavage when DNA 3′-end is recessed; (ii) RNA 5′-end directed RNase H cleavage when RNA 5′-end is recessed. By annealing the 5′-[^{32}P] labeled 36 mer RNA with either a 23 mer DNA (to produce DNA 3′-end recessed P/T) or a 60 mer DNA (to produce RNA 5′-end recessed P/T), we were able to examine the effect of 4′-Ed4T on these two modes of RNase H cleavage. We compared the patterns of RNA cleavage products as well as the relative rates at which these products were formed under different concentrations of 4′-Ed4TMP, -DP and -TP forms (concentration range from 0 to 100 μM). Under these conditions there was no significant change in the product pattern or in the rate of product formation (data not shown). These results indicated that 4′-Ed4T had no significant impact on the RNase H activity of HIV-1 RT. Furthermore, by ^{32}P-labeling the 5′-ends of both the RNA template and the 23 mer DNA primer, we were able to monitor the RNase H activity as well as the polymerase activity of RT simultaneously when nucleoside triphosphates were present in the reaction. Our results showed that the rate of RNA template degradation by RNase H activity was much slower than the rate of dTMP, D4TMP or 4′-Ed4TMP incorporation under our standard assay conditions (data not shown). This was consistent with previous observations (Kati et al., 1992; Kerr and Anderson, 1997; Vaccaro et al., 2000), and it also suggested that minor degradation of RNA template would not interfere with the results obtained from DNA polymerization assays.

4. Discussion

4′-Ed4T was previously shown to be more active against HIV-1 replication and much less toxic to host cells than D4T by phenotypic drug susceptibility studies (Dutschman et al., 2004; Nitanda et al., 2005) with the underlying mechanism unclear. Our study is the first to elucidate the exact mechanism of its antiviral activity through detailed steady-state enzymatic analy-

ses. It is not surprising that 4′-Ed4T had no impact on the RNase H activity of RT, while its triphosphate metabolite, 4′-Ed4TTP exerts its anti-HIV-1 activity via an inhibitory effect on RT like other NRTIs. However, it is noteworthy that 4′-Ed4TTP, with the addition of a single ethynyl group at the 4′ position of D4TTP, differs from D4TTP regarding its inhibitory effects on RT and host DNA polymerases in several ways.

First, 4′-Ed4TTP showed much stronger inhibition on RT than D4TTP, especially with DNA/RNA P/T (Table 2). The K_i values for 4′-Ed4TTP and D4TTP to inhibit RT with DNA/RNA P/T was 35-fold lower than the K_i values with DNA/DNA P/T (Table 2) indicating that 4′-Ed4TTP and D4TTP bind to RT-DNA/RNA complex much tighter than they bind to RT-DNA/DNA complex. This is probably due to the conformation of a DNA/RNA P/T complexed with HIV-1 RT differs from that of a DNA/DNA P/T complexed with HIV-1 RT. It also suggests that 4′-Ed4TTP exerts its antiviral activity mostly through the inhibition of the reverse transcription of HIV-1 RNA genome other than through the inhibition of the subsequent DNA replication step by RT.

Extensive studies with 4′-substituted 2′-deoxy nucleosides demonstrated the superior potency of the 4′-ethynyl substitution against HIV-1 (Ohrui et al., 2000; Kodama et al., 2001; Haraguchi et al., 2003), implying strong binding of these nucleoside analogues to RT. Similarly, 1-(3-C-ethynyl-β-D-ribopentofuranosyl) cytosine (Ecyd or TAS-106), an analogue of cytidine, exerts strong antitumor activity through its triphosphate form, ECTP, which competitively inhibited human RNA polymerases (Tabata et al., 1997; Matsuda et al., 1999; Takatori et al., 1999; Shimamoto et al., 2001). However, it is still unclear how the inhibition efficiency of NRTIs on RT could markedly increase with 4′-ethynyl substitution. The crystal structure of RT-P/T-4′-Ed4TTP ternary complex should assist in addressing this question.

Second, in spite of the much lower K_i value for 4′-Ed4TTP to inhibit RT compared with that for D4TTP, the efficiency of 4′-Ed4TMP incorporation by RT with DNA/RNA substrate was 3-fold lower than that of D4TMP incorporation as judged by their steady-state k_{cat}/K_m values (Table 3). The difference between the inhibition and incorporation efficiencies of 4′-Ed4TTP implies that the binding of 4′-Ed4TTP to RT-P/T complex is a more important factor than actual 4′-Ed4TTP incorporation. Similarly the steady-state K_m value for AZTMP incorporation by RT is 100-fold higher than the K_i value for AZTTP to inhibit dTMP incorporation by RT (Kedar et al., 1990).

Third, 4′-Ed4TTP inhibited the 3TC-resistant RT mutant, M184V 2- to 3-fold less efficiently than wt RT; on the other hand, D4TTP inhibited M184V mutant about 2-fold more efficiently than wt RT (Table 2). The results of these inhibition assays were consistent with phenotypic drug susceptibility studies (Dutschman et al., 2004; Nitanda et al., 2005), further supporting the notion that 4′-Ed4TTP is the active metabolite targeting on HIV-1 RT. Similar results were also obtained with 4′-C-ethynyl ddCTP, which inhibited RT M184V mutant with less efficiency than wt RT (Siddiqui et al., 2004). With the crystal structure of 4′-C-ethynyl ddCTP available, both the D- and L-enantiomers of this compound were docked into the active site of HIV-1 RT-DNA/DNA-nucleotide ternary complex (Siddiqui et al., 2004). According to the computer modeling study, the 4′-ethynyl group was close to Met184 in wt RT, but in the model of M184V mutant, the 4′-ethynyl group of the D-enantiomer showed some negative steric interaction with Val184. When the L-enantiomer was docked into the M184V mutant, there was a steric clash between the ethynyl group and Val184, which was used to explain the lack of inhibition on M184V by the L-enantiomer. However, our kinetic measurements showed that the K_i values for wt and M184V RT with DNA/DNA P/T were similar (Table 1), suggesting 4′-Ed4TTP binding to RT was not disturbed by substitution of Met184 with Valine. Furthermore, this model was unable to explain why nucleoside analogues with the addition of 4′-ethynyl group showed such greatly increased inhibitory effect on RT compared to their parental compounds. Again the crystal structure of RT-P/T-4′-Ed4TTP ternary complex should provide answer to this question.

Last, 4′-Ed4TTP showed no or weak inhibitory effect on major host DNA polymerases. The triphosphate derivatives of many NRTIs are also substrates of human DNA polymerases, resulting in inhibition of cellular DNA synthesis. Martin et al. (1994) compared the effects of 16 NRTIs including D4T on the activities of host DNA polymerases α, β, ε and γ. Their study showed that D4TTP inhibited pol γ much more efficiently than it inhibited pol α and ε. This data was consistent with the conclusion from cell culture studies, which demonstrated that the cellular toxicity of D4T was related to mitochondrial DNA damage (Chen and Cheng, 1989; Chen et al., 1991; Medina et al., 1994; Johnson et al., 2001). In our study, we found that the IC_{50} values for 4′-Ed4TTP to inhibit pol γ and pol β were both at least 100-fold greater than that for D4TTP (Table 4), consistent with the observation that 4′-Ed4T caused much less cellular toxicity and mitochondrial DNA loss than D4T in cell culture studies (Dutschman et al., 2004). It will be of interest as well to investigate the mechanism underlying pol β and pol γ inhibition by D4TTP, given the lack of inhibition by its derivative, 4′-Ed4TTP.

In summary, 4′-Ed4TTP is a substrate of RT serving as a DNA chain terminator. Compared with D4TTP, it is a much more potent competitive inhibitor against RT with little inhibitory effect on major host DNA polymerases. The inhibitory effects of 4′-Ed4TTP on RT, further quantified herein, argue strongly for its role as the active metabolite of 4′-Ed4T against HIV-1.

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References

Archer, R.H., Wisniewski, M., et al., 2001. The Y181C mutant of HIV-1 reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors alters the size distribution of RNase H cleavages. Biochemistry 40 (13), 4087–4095.

Chen, C.H., Cheng, Y.C., 1989. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′,3′-dideoxycytidine. J. Biol. Chem. 264 (20), 11934–11937.

Chen, C.H., Vazquez-Padua, M., et al., 1991. Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. Mol. Pharmacol. 39 (5), 625–628.

Dutschman, G.E., Grill, S.P., et al., 2004. Novel 4′-substituted stavudine analog with improved anti-human immunodeficiency virus activity and decreased cytotoxicity. Antimicrob. Agents Chemother. 48 (5), 1640–1646.

Goff, S.P., 1990. Retroviral reverse transcriptase: synthesis, structure, and function. J. Acquir. Immune Defic. Syndr. 3 (8), 817–831.

Hamamoto, Y., Nakashima, H., et al., 1987. Inhibitory effect of 2′,3′-didehydro-2′,3′-dideoxynucleosides on infectivity, cytopathic effects, and replication of human immunodeficiency virus. Antimicrob. Agents Chemother. 31 (6), 907–910.

Haraguchi, K., Takeda, S., et al., 2003. Synthesis of a highly active new anti-HIV agent 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine. Bioorg. Med. Chem. Lett. 13 (21), 3775–3777.

Huang, P., Farquhar, D., et al., 1990. Selective action of 3′-azido-3′-deoxythymidine 5′-triphosphate on viral reverse transcriptases and human DNA polymerases. J. Biol. Chem. 265 (20), 11914–11918.

Johnson, A.A., Ray, A.S., et al., 2001. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J. Biol. Chem. 276 (44), 40847–40857.

Kati, W.M., Johnson, K.A., et al., 1992. Mechanism and fidelity of HIV reverse transcriptase. J. Biol. Chem. 267 (36), 25988–25997.

Kedar, P.S., Abbotts, J., et al., 1990. Mechanism of HIV reverse transcriptase: enzyme-primer interaction as revealed through studies of a dNTP analogue, 3′-azido-Dttp. Biochemistry 29 (15), 3603–3611.

Kerr, S.G., Anderson, K.S., 1997. Pre-steady-state kinetic characterization of wild type and 3′-azido-3′-deoxythymidine (AZT) resistant human immunodeficiency virus type 1 reverse transcriptase: implication of RNA directed DNA polymerization in the mechanism of AZT resistance. Biochemistry 36 (46), 14064–14070.

Kodama, E.I., Kohgo, S., et al., 2001. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob. Agents Chemother. 45 (5), 1539–1546.

Krishnan, P., Liou, J.Y., et al., 2002. Phosphorylation of pyrimidine L-deoxynucleoside analog diphosphates. Kinetics of phosphorylation and dephosphorylation of nucleoside analog diphosphates and triphosphates by 3-phosphoglycerate kinase. J. Biol. Chem. 277 (35), 31593–31600.

Lin, T.S., Schinazi, R.F., et al., 1987a. Antiviral activity of 2′,3′-dideoxycytidin-2′-ene (2′,3′-dideoxy-2′,3′-didehydrocytidine) against human immunodeficiency virus in vitro. Biochem. Pharmacol. 36 (3), 311–316.

Lin, T.S., Schinazi, R.F., et al., 1987b. Potent and selective in vitro activity of 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-didehydrothymidine) against human immunodeficiency virus. Biochem. Pharmacol. 36 (17), 2713–2718.

Martin, J.L., Brown, C.E., et al., 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. 38 (12), 2743–2749.

Matsuda, A., Fukushima, M., et al., 1999. A new antitumor nucleoside, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine (ECyd), is a potent inhibitor of RNA synthesis. Nucleosides Nucleotides 18 (4–5), 811–814.

Medina, D.J., Tsai, C.H., et al., 1994. Comparison of mitochondrial morphology, mitochondrial DNA content, and cell viability in cultured cells treated with three anti-human immunodeficiency virus dideoxynucleosides. Antimicrob. Agents Chemother. 38 (8), 1824–1828.

Mitsuya, H., Yarchoan, R., et al., 1990. Molecular targets for AIDS therapy. Science 249 (4976), 1533–1544.

Murakami, E., Ray, A.S., et al., 2004. Investigating the effects of stereochemistry on incorporation and removal of 5-fluorocytidine analogs by mitochondrial DNA polymerase gamma: comparison of D- and L-D4FC-TP. Antiviral Res. 62 (1), 57–64.

Nitanda, T., Wang, X., et al., 2005. Anti-human immunodeficiency virus type 1 activity and resistance profile of 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine in vitro. Antimicrob. Agents Chemother. 49 (8), 3355–3360.

Ohrui, H., Kohgo, S., et al., 2000. Syntheses of 4′-C-ethynyl-beta-D-arabino- and 4′-C-ethynyl-2′-deoxy-beta-D-ribo-pentofuranosylpyrimidines and -purines and evaluation of their anti-HIV activity. J. Med. Chem. 43 (23), 4516–4525.

Schinazi, R.F., Lloyd Jr., R.M., et al., 1993. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob. Agents Chemother. 37 (4), 875–881.

Shimamoto, Y., Fujioka, A., et al., 2001. Antitumor activity and pharmacokinetics of TAS-106, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine. Jpn. J. Cancer Res. 92 (3), 343–351.

Siddiqui, M.A., Hughes, S.H., et al., 2004. A 4′-C-ethynyl-2′,3′-dideoxynucleoside analogue highlights the role of the 3′-OH in anti-HIV active 4′-C-ethynyl-2′-deoxy nucleosides. J. Med. Chem. 47 (21), 5041–5048.

Tabata, S., Tanaka, M., et al., 1997. Anti-tumor mechanisms of 3′-ethynyluridine and 3′-ethynylcytidine as RNA synthesis inhibitors: development and characterization of 3′-ethynyluridine-resistant cells. Cancer Lett. 116 (2), 225–231.

Takatori, S., Kanda, H., et al., 1999. Antitumor mechanisms and metabolism of the novel antitumor nucleoside analogues, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine and 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)uracil. Cancer Chemother. Pharmacol. 44 (2), 97–104.

Ueno, T., Mitsuya, H., 1997. Comparative enzymatic study of HIV-1 reverse transcriptase resistant to 2′,3′-dideoxynucleotide analogs using the single-nucleotide incorporation assay. Biochemistry 36 (5), 1092–1099.

Ueno, T., Shirasaka, T., et al., 1995. Enzymatic characterization of human immunodeficiency virus type 1 reverse transcriptase resistant to multiple 2′,3′-dideoxynucleoside 5′-triphosphates. J. Biol. Chem. 270 (40), 23605–23611.

Vaccaro, J.A., Parnell, K.M., et al., 2000. Mechanism of inhibition of the human immunodeficiency virus type 1 reverse transcriptase by d4TTP: an equivalent incorporation efficiency relative to the natural substrate dTTP. Antimicrob. Agents Chemother. 44 (1), 217–221.