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Effects of β-L-3′-azido-3′-deoxythymidine 5′-triphosphate on host and viral DNA polymerases

Abdesslem Faraj [a,*], Abdelaziz Moulay El Alaoui [a], Gilles Gosselin [b], Jean-Louis Imbach [b], Casey Morrow [c], Jean-Pierre Sommadossi [a]

[a] *Department of Pharmacology, Center for AIDS Research, The Comprehensive Cancer Center, and Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

[b] *Laboratoire de Chimie Organique Biomoleculaire de Synthèse, UMR 5625 CNRS, case courrier 008, Université Montpelier II, Place Eugène Bataillon, 34095 Montpelier, Cedex 5, France*

[c] *Department of Microbiology, Center for AIDS Research, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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Abstract

We have previously reported that several β-L-thymidine analogues including β-L-3′-azido-3′-deoxythymidine (β-L-AZT), β-L-3′-fluoro-2′,3′-dideoxythymidine (β-L-FLT) and β-L-2′,3′-didehydro-2′,3′-dideoxythymidine (β-L-D4T) did not inhibit HIV replication in human peripheral blood mononuclear (PBM) cells whereas their corresponding β-D-counterparts are known as potent and selective anti-HIV agents [Faraj et al., 1997. Nucleosides and Nucleotides 16, 1287–1290]. In order to gain insight on the lack of antiviral activities of these β-L-derivatives, in vitro enzymatic steady state studies were conducted in the present study with β-L-AZT. β-L-AZT 5′-triphosphate (L-AZTTP) was chemically synthesized and found to moderately inhibit wild-type HIV reverse transcriptase (HIV-1 RT) with a K_i value of 2 μM; while lacking any inhibitory effect towards human DNA polymerase α, β or γ. However, the inhibitory effect of L-AZTTP towards HIV-1 RT was very modest (266-fold less potent) when compared to its isomer β-D-AZT 5′-triphosphate (D-AZTTP) which exhibits a K_i value of 0.0075 μM and this finding was further confirmed by DNA chain termination assay. These data suggest that the absence of antiviral activity of the parent β-L-AZT may in part be explained by the poor inhibition of the targeted viral enzyme by L-AZTTP, the active metabolite. Finally, L-AZTTP was found to lack affinity for the mutant RT at position 184 (M184V) demonstrating that this mutation confers resistance not only to β-L-2′,3′-dideoxycytidine analogs as previously reported by our group [Faraj et al., 1994. Antimicrob. Agents Chemother. 38, 2300–2305] but as well as to β-L-2′,3′-dideoxythymidine analogs.

Keywords: β-L-AZTTP; WT HIV-1 RT; M184V HIV-1 RT, Chain termination

Recently, the importance of chirality and its influence on the selectivity of nucleoside analogs were recognized with the demonstration that some cytidine analogs with the unnatural β-L configura-

* Corresponding author. Tel.: +1-205-9345217; fax: +1-205-9754871.

E-mail address: abdesslem.faraj@ccc.uab.edu (A. Faraj).

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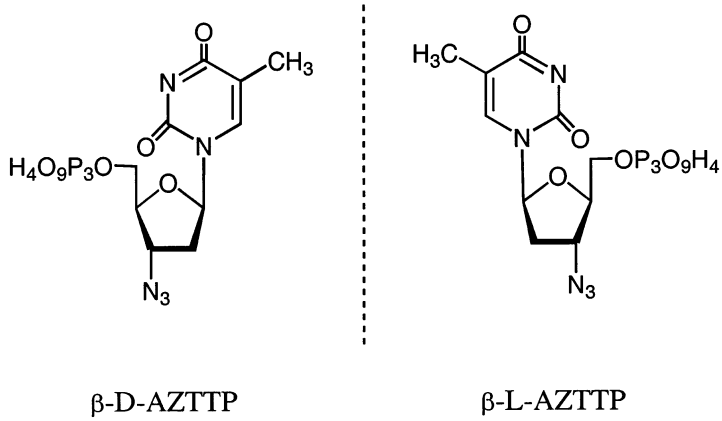
tion exhibited potent in vitro anti-HBV and/or anti-HBV activities (For review, Wang et al., 1998). This new generation of antiviral compounds has produced several potent and safer drugs with the leading compounds being β-L-2′,3′-dideoxy-3′-thiacytidine (3TC). 3TC was approved by the US Food and Drug Administration as lamuvidine for the first-line treatment of AIDS in combination with β-D-AZT and it is actually the world's first oral antiviral treatment of chronic HBV as it was approved recently as Epivir-HBV in the USA and other countries in the world.

In contrast to the extensive studies performed on β-L-cytidine analog derivatives, only limited interest has emerged from the other pyrimidine series, the β-L-thymidine analogs. This probably results, in part, from the reported absence of anti-HIV activity by β-L-2′,3′-dideoxythymidine (β-L-ddT) (Lin et al., 1994), β-L-2′,3′-didehydro-2′,3′-dideoxythymidine (β-L-D4T) (Mansuri et al., 1991) and β-L-3′-azido-3′-deoxythymidine (β-L-AZT) (Wengel et al., 1991) combined with a potential decreased inhibition of HIV-1 reverse transcriptase (RT) by the 5′-triphosphates of β-L-thymidine analogs as compared to their corresponding β-D enantiomers (Van Draanen et al., 1992; Yamaguchi et al., 1994). However, HIV RT inhibition with β-L-thymidine 5′-triphosphates analogs was highly variable and it was unclear whether observed differences resulted from the HIV RT strain used or assay conditions (Furman et al., 1995). Our group has recently reported that several β-L-3′-substituted-2′,3′-dideoxythymidine analogs including β-L-AZT, β-L-3′-amino-2′,3′-dideoxythymidine (β-L-AMT), β-L-3′-fluoro-2′,3′-dideoxythymidine (β-L-FLT) and β-L-D4T lacked both in vitro anti-HIV-1 and anti-HBV activities (Faraj et al., 1997). These data suggested that these β-L-thymidine analogs may not be efficiently intracellulary delivered inside the cells, phosphorylated to their 5′-triphosphate derivatives and/or the 5′-triphosphate derivatives may be poor inhibitors of the viral target protein. To investigate the later hypothesis, β-L-AZT 5′-triphosphate (L-AZTTP) was chemically synthesized (Fig. 1) and its effects on HIV-1 reverse transcriptase (RT) as well as human DNA polymerase α, β and γ were investigated. Moreover, studies using cloned and expressed mutant RT at position 184 (substitution of methionone to valine [M184V]) were performed under steady-state conditions and compared to the wild type (WT) parental HIV-1 RT to assess whether that mutation confers resistance to other pyrimidine analogs as reported with cytidine analogs (Faraj et al., 1994).

β-L-AZTTP was prepared by a standard phosphorylation method from unprotected β-L-AZT as previously described for others nucleoside analog (White et al., 1989). D-AZTTP was obtained from Moravek Biochemicals Co. (Brea, CA). Human DNA polymerases α and β were purchased from Molecular Biology Resources, Inc



β-D-AZTTP β-L-AZTTP

Fig. 1. Chemical structures of β-D- and β-L-enantiomers of 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate (AZTTP).

Table 1
Comparative inhibitory activities of β-L-AZTTP and β-D-AZTTP on wild type HIV-1 RT and mutant (M184V) HIV-1 RT[a]

Inhibitor	K_i[b] (μM)	
	WT HIV-1 RT	M184V HIV-1 RT
β-L-AZTTP	2 ± 0.5	>50
β-D-AZTTP	0.0075 ± 0.003	0.025 ± 0.005

[a] Assays were performed as described in the material and methods section. Each set of data represents the arithmetic mean value ± standard deviation of at least three independent experiments.

[b] K_i values were determined using poly(rA).oligo(dT)$_{15}$ as template-primer for recombinant (p66/p55)WT or M184V HIV-1 RT. Inhibitor parameters were analyzed by Dixon plot analysis. Under these conditions, the calculated mean K_m for dTTP was 0.9 μM for WT RT and 1.2 μM for M184V RT.

(Milwaukee, WI) and Human DNA polymerase γ was a gift from Dr William Parker (Southern Research Institute, Birmingham, AL). In vitro viral and cellular polymerases assays were conducted as previously described (Faraj et al., 1994). Briefly, assays for RT kinetics studies contained 60 mM Tris (pH 8.0), 10 mM $MgCl_2$, 40 mM KCl, 2.50 mM DTT, 0.10 mg/ml BSA, 20 μM [^{3}H]dTTP, 2 μM poly(rA).oligo(dT)$_{15}$ and various concentrations of inhibitor in a final volume of 40 μl. The solution was equilibrated at 37°C and the reaction was initiated with the addition of 0.2 μg of the wild type or mutant recombinant RT. All the assays were performed for 30 min at 37°C. Reaction mixtures for DNA polymerase a contained 50 mM Tris-HCl pH 8.0, 10 mM magnesium chloride, 1.0 mg/ml of BSA, 1.0 mM dithiothreitol, 10 μg activated calf thymus DNA, 50 μM of each dATP, dCTP, and dGTP, and various concentrations of [^{3}H]-dTTP. Human DNA polymerases β and γ assays were carried out in the same buffer as described above for DNA polymerase α except that 60 mM Tris–HCl at pH 8.7, 100 mM potassium chloride, and 15% (v/v) glycerol were used for DNA polymerase β and 100 mM potassium chloride for DNA polymerase γ. L-AZZTP and D-AZTTP were present at various concentrations. Reaction was initiated by the addition of 1.5 U of DNA polymerase α, β or γ. All of reaction mixtures were incubated for 60 min at 37°C and aliquots of 40 μl were spotted onto Whatman DE81 filters. The filters were washed three times with 125 mM Na_2HPO_4 buffer, rinsed once with water and once with ethanol. Filters were subsequently dried and counted in 4 ml of scintillation fluor liquid in a Beckman LS TA 5000. In vitro steady-state rates were determined by linear regression analysis. The experiments were performed under conditions leading to linear reaction rates. The amount of product formed was proportional to the incubation time of the substrate intervals studied. Dixon plot analysis was used to determine inhibition constants. Sequencing procedures and acrylamide gel electrophoresis were performed as recommended in the kit. Specifically, the nucleotide analogue reaction mixture contained 60 mM Tris (pH 8.0), 40 mM KCl, 1 mM DTT, 2 μM dTTP, 2 μM dCTP, 2 μM dGTP, 5 mCi/ml [^{32}P]dATP (3000 Ci/mmol), 0.15 pmole of single stranded M13mp18(+), 3.50 pmol of 17-base primer (universal primer-40), and various concentrations of inhibitor. The control did not contain any drug or dideoxynucleoside triphosphate. The reaction mixture was initiated by adding 0.5 μg of wild type or mutant HIV-1 RT, in a total volume of 5.5 μl. Samples were incubated for 15 min at 37°C and chased for an additional 15 min at 37°C with 0.5 μl of a solution containing the four natural dNTP to a final concentration of 10 μM. The reactions were terminated by the addition of 4 μl of formamide stop solution, and the DNA reaction products were analyzed by using autoradiographs of wedge polyacrylamide sequencing gels.

Although we have recently reported that β-L-AZT is indeed inactive against HIV replication in PBM cells as well as against HBV replication in 2.2.15 cells (Faraj et al., 1997), we currently find that the corresponding 5′-triphosphate derivative (L-AZTTP) inhibited moderately recombinant wild type (WT) HIV-1 RT by using the synthetic poly(rA).oligo(dT)$_{15}$ as template primer with K_i value of 2 μM, when compared to its natural isomer D-AZTTP which exhibits a K_i value of 0.0075 μM (Table 1). Interestingly, in vitro assays with HIV-1 RT in the presence of bovine serum albumin (BSA) showed that the inhibition by

β-L-AZTTP was 10-fold less potent than in the absence of BSA suggesting that L-AZTTP may interact directly with BSA (data not shown). Kinetic studies under Michaelis–Menten conditions showed that L-AZTTP inhibited the WT HIV-1 RT in a non competitive manner in respect to dTTP incorporation (Table 1), suggesting that L-AZZTP does not effect the combination of the substrate dTTP with HIV-RT but effects only the activity when all the enzyme is in the ES (enzyme-substrate) form. In addition, the inhibitory effect of L-AZTTP towards viral polymerases was specific since L-AZTTP did not inhibit human DNA polymerase α, β or up γ to 100 μM (Table 2). The moderate effect of L-AZTTP towards HIV-1 RT was subsequently visualized by using a modified Sanger sequencing procedure (Fig. 2). L-AZTTP acted as a DNA chain terminator toward HIV-1 WT RT at position corresponding to deoxythymidine residues, however, this effect was less potent than the one observed with D-AZTTP since chain terminations were present at greater extent with β-D-AZTTP at 1 μM indicating more premature DNA chain terminations as compared to L-AZTTP at 10 μM (Fig. 2). The cellular metabolism of radiolabeled L-AZT in PBMC would provide us with useful information regarding the intracellular pools of L-AZT metabolites;

Table 2
Comparative inhibitory activities of β-L-AZTTP and β-D-AZTTP on human DNA polymerases α, β and γ[a]

Inhibitor	K_i[b] (μM)		
	DNA polα	DNA polβ	DNA polγ
β-L-AZTTP	>100	>100	>100
β-D-AZTTP	>100	40 ± 5	ND[c]

[a] Assays were performed as described in the material and methods section. Each set of data represents the arithmetic mean value ± standard deviation of at least three independent experiments.

[b] K_i values were determined using calf thymus activated DNA as template-primer for human DNA polymerase α or β or γ. Inhibitor parameters were analyzed by Dixon plot analysis. Under these conditions, the calculated mean K_m of human DNA polymerase α, β and γ for dTTP was 1.5, 2 and 0.8 μM, respectively.

[c] ND, not determined.

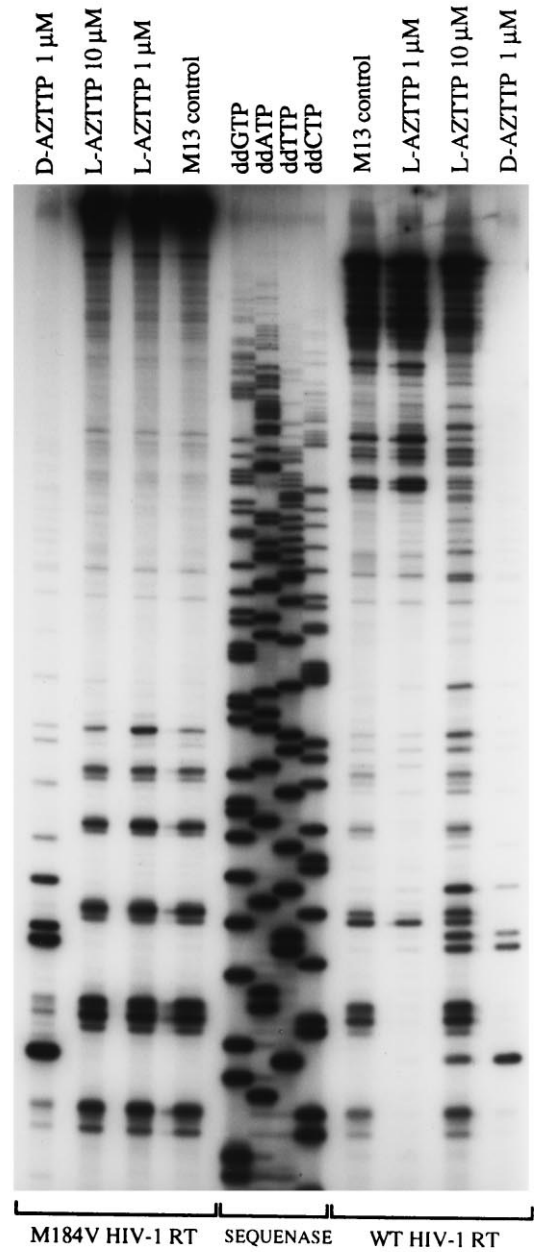


Fig. 2. Autoradiograph of chain-terminating sequencing reactions with β-D-AZTTP and β-L-AZTTP using wild type (WT) or mutant (M184V) HIV RT. Assays were performed as described in the material and method section.

however, even if the active metabolite L-AZTTP pools reached a significant levels inside the cells and based on our data, the poor inhibition of HIV RT by L-AZTTP may in part explain the lack of antiviral activity of β-L-AZT.

Mutation of the RT at codon 184 from methionine to valine (M184V) has been generated through in vitro selection with 3TC and other β-L-cytidine analogs and this mutation is involved in the emergence of in vitro drug-resistance seen with these β-L-nucleoside enantiomers (Schinazi et al., 1993; Van Draanen et al., 1994). We have previously reported that while the WT RT did not differentiate between β-D- and β-L-cytidine 5′-triphosphate analogs, the M184V RT specifically recognized the β-D-cytidine 5′-triphosphate analogs (Faraj et al., 1994). β-L-AZTTP was shown in the present study to have a good affinity for the WT RT ($K_i = 2$ μM), but was not an inhibitor for the mutant M184V RT up to a concentration of 50 μM, whereas, β-D-AZTTP was recognized by both the WT and mutant RT (Table 1). This effect was further confirmed by a DNA chain termination assay since L-AZTTP was not recognized as substrate by the mutant M184V HIV-1 RT up to 10 μM, whereas D-AZTTP acted as a potent DNA chain terminator towards both the wild type and the mutant HIV-1 RT (Fig. 2). Furman et al. (1995) have reported similar data with D-ddTTP and D-D4TTP which were recognized by M184V RT with an affinity of a 1100- and 8-fold, respectively, when compared to their β-L-enantiomers. The mutation at M184 of HIV RT probably induces a conformational change in the active site of the enzyme that results in an enantioselectivity with respect to β-L-2′,3′-dideoxycytidine and β-L-2′,3′-dideoxythymidine analogs.

In conclusion, these data demonstrate that the lack of anti-HIV activity of β-L-AZT is probably due in part by the poor inhibition of the viral HIV RT. Finally, the mutation M184V seems to affect the affinity of the RT for β-L-2′,3′-dideoxycytidine analogs as well as for β-L-2′,3′-dideoxythymidine analogs.

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