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Inhibition of the reverse transcriptase from HIV by 3′-azido-3′-deoxythymidine triphosphate and its *threo* analogue

Lotta Vrang[1], Hervé Bazin[2], Gerald Remaud[2], Jyoti Chattopadhyaya[2] and Bo Öberg[3,*]

[1]*Department of Virology, Karolinska Institute, S-105 21 Stockholm,* [2]*Department of Bioorganic Chemistry, Biomedical Center, Box 581, Uppsala University, S-751 23 Uppsala, and* [3]*Department of Antiviral Chemotherapy, Research and Development Laboratories, Astra Alab AB, S-151 85 Södertälje, Sweden*

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Summary

3′-Azido-3′-deoxythymidine triphosphate (*erythro*) and its *threo* isomer were synthesized and investigated for their inhibition of HIV reverse transcriptase from virus isolate U 937/HTLV-III. The *erythro* isomer was a competitive inhibitor of the reaction directed by $(rA)_n(dT)_{12-18}$ and the K_i value was 0.0022 μM. The *threo* isomer was at least 100-fold less active. The inhibition was specific for dTMP incorporation, and dGMP incorporation using $(rC)_n(dG)_{12-18}$ as template/primer was not affected. The K_m value for dTTP varied between 0.7 μM and 1.7 μM. Reverse transcriptase from nine HIV isolates were tested for inhibition by the *erythro* isomer and only slight differences in sensitivity were observed.

HIV reverse transcriptase; 3′-Azido-3′-deoxythymidine triphosphate; Enzyme kinetics; Synthesis

Introduction

Antiviral drugs have mainly been directed against viral nucleic acid polymerases and have either been nucleoside analogues or pyrophosphate analogues (Öberg,

* *To whom correspondence should be sent.*
Present address: Department of Antiviral Chemotherapy, Research & Development Laboratories, Astra Alab AB, S-151 85 Södertälje, Sweden. Telephone: 755 329 80.

1983). The possibility of using the reverse transcriptase of the human immunodeficiency virus (HIV, formerly HTLV-III/LAV), the primary cause of AIDS (Barré-Sinoussi et al., 1983; Gallo et al., 1984) as a target for antiviral chemotherapy has recently attracted considerable attention. Some compounds have been considered sufficiently active against HIV to be tried clinically but present in vitro studies indicate that only a few will have any potential to be useful (see reviews by De Clercq and Balzarini, 1985; Hirsch and Kaplan, 1985; Öberg, 1986). Among the most selective inhibitors of HIV reverse transcriptase are phosphonoformate (foscarnet) (Öberg, 1986; Sandström et al., 1985; Sarin et al., 1985; Vrang and Öberg, 1986) and 3′-azido-3′-deoxythymidine (3′AZT, active as a triphosphate) (Mitsuya et al., 1985; Öberg, 1986). The mechanism of action of foscarnet has been published (Vrang and Öberg, 1986) but so far little has been reported on the mechanism of action of 3′-azido-3′-deoxythymidine triphosphate (*8*) (Mitsuya et al., 1985; St. Clair et al., 1985; Furman et al., 1985). We here report studies on the mechanism of inhibition of reverse transcriptase from HIV using two structural isomers of 3′-azido-3′-deoxythymidine triphosphate *6* and *8*, respectively, and also compare the sensitivity of reverse transcriptase from different virus isolates.

Materials and Methods

Chemicals

Deoxynucleoside triphosphates were from Sigma Chemical Co., St. Louis, MO. [^{3}H]dTTP and [^{3}H]dGTP were purchased from New England Nuclear Corp., Boston, MA. (Riboadenylic acid)$_n$ (deoxythymidylic acid)$_{12-18}$, ((rA)$_n$(dT)$_{12-18}$) and (ribocytidylic acid)$_n$ (deoxyguanylic acid)$_{12-18}$, ((rC)$_n$(dG)$_{12-18}$) were from Pharmacia, Uppsala, Sweden. Activated calf thymus DNA was prepared as described by Schlabach et al. (1971).

Enzymes

Tissue culture medium from Molt3 cells infected with virus strain U 937/HTLV-III, first clarified in low-speed centrifugation, was centrifuged at 132 000 × *g* for 30 min. The sedimented virus was disrupted in 50 mM Tris-HCl (pH 7.5), 35 mM KCl, 4 mM dithiothreitol, 1 mM disodium EDTA and 1.3% Triton X-100, and stored in working samples at −70°C until used. These preparations were kindly made by Bertil Eriksson and Johanna Sköld, Karolinska Institute, Stockholm. Reverse transcriptases from 8 patient isolates (numbered 1–8) were kindly prepared by Drs. E.-M. Fenyö and B. Åsjö in the same way as the U 937/HTLV-III isolate. Calf thymus DNA polymerase α was from Worthington Biochemical Corporation, Freehold, NJ.

Reverse transcriptase assay

The 100-μl reaction mixture contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM dithiothreitol, 6 mM $MgCl_2$, 50 μg of bovine serum albumin Fraction V, 1 μg of (rA)$_n$(dT)$_{12-18}$, or (rC)$_n$(dG)$_{12-18}$ or 125 μg activated DNA, the indicated

concentration of [^{3}H]dTTP (0.5–10 μM: specific activity, 5500–1100 cpm/pmol or [^{3}H]dGTP (2 μM, specific activity, 5900 cpm/pmol) and 10 μl enzyme preparation.

After incubation at 37°C for 40 min, 40 μl of the reaction mixture (in duplicate) were spotted on paper disks (Munktell No. 5; 24 mm) and washed 4 times in ice-cold 5% trichloroacetic acid/0.02 M pyrophosphate and 3 times in ethanol. The dried paper disks were counted in 3 ml of Econofluor scintillation solution. At saturation with respect to $(rA)_n(dT)_{12-18}$ and dTTP, 10 μl of enzyme preparation incorporated 20 pmol of dTMP in 40 min at 37°C. The enzyme reactions were linear with time in the experiments described.

Calf thymus DNA polymerase α assay

The 100 μl reaction mixture contained 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1.5 mM dithiothreitol, 45 μg bovine serum albumin Fraction V, 12.5 μg of activated calf thymus DNA, 0.2 mM each of dATP, dGTP and dCTP, [^{3}H]dTTP (1 μM, specific activity, 17900 cpm/pmol) and 1 unit of calf thymus DNA polymerase α.

Synthesis

^{1}H-NMR spectra were measured at 90 MHz with a Jeol FX 90Q spectrometer and Jeol GX 270 spectrometer at 270 MHz using tetramethylsilane as an internal standard in δ scale. ^{13}C-NMR spectra were recorded at 23.5 MHz using acetone-d_6 = 29.2 ppm as internal standard. ^{31}P-NMR spectra were recorded at 36 MHz using 85% phosphoric acid as an external standard in δ scale. UV absorption spectra were recorded with a Varian-Cary 2200 spectrophotometer. ^{15}N-NMR spectra were measured at 27.4 MHz using $CH_3{}^{15}NO_2$ as external reference (δ scale) in an inverted gated proton decoupled mode. The assignment of ^{15}N chemical shifts are in agreement with the literature (Martin et al., 1981).

*1-(3'-Azido-2',3'-dideoxy-5'-0-trityl-β-*D-threo*-pentofuranosyl)thymine (2)*

Compound *1* (884 mg, 1.57 mmol) was dissolved in dry tetrahydrofuran (25 ml) and a dry crystalline complex of potassium azide and 18-crown-6 (750 mg) added and refluxed under nitrogen for 20 h. The reaction mixture was cooled in ice-water followed by the addition of diethyl ether (10 ml); the crystalline precipitate thus obtained was filtered off. The filtrate was evaporated to give a gum which was dissolved in dichloromethane (50 ml) and washed twice with 75% saturated KCl solution, then with water and evaporated to give a white foam. This was dissolved in a mixture of hexane/dichloromethane (6:4, v/v) and purified over a short column of silica gel, washing the column with the same solvent mixture and then gradually increasing stepwise to pure dichloromethane to elute the title compound which was subsequently crystallized from diethyl ether (30 ml) (613 mg, 77%). The mother liquor upon evaporation gave a residue containing 50 mg of the title compound together with 7 mg of *4* which represent 1% of the total amount. Further elution with 2% ethanol/dichloromethane mixture gave 80 mg (11%) of *3*.

^{1}H-NMR ($CDCl_3$): 9.1 (br.s, 1H) NH; 7.15 (m, 16H) arom., H-6; 6.15 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, $J_{1',2'}$ = 2.8 Hz) H-1'; 4.23 (m, 1H) H-3'; 4.12 (dt, 1H, $J_{3',4'}$ = 3.8

Hz, $J_{4',5'} = J_{4',5''} = 5.8$ Hz) H-4'; 3.62 (dd, 1H, $J_{5',5''} = 10$ Hz) H-5'; 3.35 (dd, 1H) H-5''; 2.7 (ddd, 1H, $J_{2',3'} = 6.3$ Hz, $J_{2',2''} = 14.6$ Hz) H-2'; 2.14 (m, 1H) H-2''; 1.83 (s, 3H) methyl. IR (KBr): 2100 cm^{-1} (azido); UV (95% ethanol): λ_{max} 262 nm (ϵ = 8600); mp (diethyl ether): 127–130°C.

*1-(3'-Azido-2',3'-dideoxy-β-*D-threo-*pentofuranosyl)thymine (5)*

Compound *2* (540 mg) was dissolved in 80% acetic acid and the solution was refluxed for 10 min. The reaction mixture was cooled and the volatile matters were evaporated in vacuo and subsequently coevaporated with toluene (3 × 50 ml). The residue was then partitioned between toluene (20 ml) and water (40 ml). The aqueous layer was washed twice with toluene and once with hexane. The aqueous phase was concentrated and coevaporated with water and freeze-dried to give *5* (242 mg, 90%). MS (FAB^+): *m/z* 268 (27%, MH^+); UV (water): λ_{max} 267 nm (ϵ = 9100); IR (KBr): 2100 cm^{-1} (azido).

^{1}H-NMR (DMSO-d_6): 7.50 (d, 1H, J = 1.1 Hz) H-6; 6.03 (dd, 1H, $J_{1',2''} = 7.8$ Hz, $J_{1',2'} = 3.6$ Hz) H-1'; 4.46 (ddd, 1H, $J_{2'',3'} = 6.8$ Hz, $J_{3',2'} = 2.1$ Hz) H-3'; 4.01 (dt, 1H, $J_{3',4'} = 4.2$ Hz, $J_{4',5'} = J_{4',5''} = 5.6$ Hz) H-4'; 3.70 (d, 2H) H-5', 5''; 2.72 (ddd, 1H, $J_{2',2''} = 14.8$ Hz) H-2''; 2.06 (ddd, 1H) H-2'; 1.83 (s, 3H) methyl.

^{13}C-NMR (acetone-d_6): 164.1 (C-2), 150.8 (C-4), 134.8 (d, J = 179.5 Hz) C-6, 109.8 (C-5), 83.8 (d, J = 173 Hz) C-1', 82.7 (d, J = 148.5 Hz) C-4', 61.2 (C-3'), 60.2 (C-5'), 37.9 (t, J = 136 Hz) C-2', 12.1 (methyl).

^{15}N-NMR (H_2O): −136.7 (β-N); −167.9 (γ-N); −225.3 (N^3); −233.3 (N^1); −306.2 (α-N).

*1-(3'-Azido-2',3'-dideoxy-β-*D-erythro-*pentofuranosyl)thymine (7)*

Compound *4* (394 mg, 0.73 mmol) was treated with 80% acetic acid, as described for *5*, to give *7* (191 mg, 97%). MS (FAB^+): m/z 268 (13%, MH^+); UV (water): λ_{max} 267 (ϵ = 9600); IR (KBr): 2100 cm^{-1} (azido).

^{1}H-NMR (DMSO-d_6): 7.68 (d, 1H, J = 1.3 Hz) H-6; 6.10 (dd, 1H, $J_{1',2'} = 6.3$ Hz, $J_{1',2''} = 6.4$ Hz) H-1'; 4.41 (ddd, 1H, $J_{2'',3'} = 7.1$ Hz, $J_{3',2''} = 5.3$ Hz) H-3'; 3.82

*1-(3'-Azido-2',3'-dideoxy-β-*D-threo-*pentofuranosyl)thymine (5)*

Hz) H-5'; 3.59 (dd, 1H) H-5''; 2.38 (ddd, 1H, $J_{2',2''} = 14.1$ Hz) H-2''; 2.27 (ddd, 1H) H-2'; 1.78 (s, 3H) methyl.

^{13}C-NMR (acetone-d_6): 163.9 (C-2), 150.7 (C-4), 136.4 (d, J = 180 Hz) C-6, 110.1 (C-5), 84.8 (d, J = 149 Hz) C-4', 84.6 (d, 168.5 Hz) C-1', 61.5 (t, J = 142 Hz) C-5', 60.7 (d, J = 142.5 Hz) C-3', 37.1 (t, J = 135 Hz) C-2', 11.9 (methyl).

^{15}N-NMR (H_2O): −136.4 (β-N); −164.9 (γ-N); −225.3 (N^3); −235.1 (N^1); −303.4 (α-N).

*1-(3'-Azido-2',3'-dideoxy-5'-trisodium triphospho-β-*D-erythro-*pentofuranosyl) thymine (8)*

Compound *7* (10 mg, 37.4 μmol) was dissolved in dry trimethylphosphate (0.25 ml) and freshly distilled phosphorous oxychloride (40 μL) was added at 0°C and stirred for 3 h. Then tributylamine (0.25 ml) and a dry dimethylformamide solution of bis(tributylammonium)pyrophosphate (0.5 M, 2.5 ml) was added. The re-

action was quenched after 5 min by addition of triethylammonium hydrogen carbonate (TEAB) buffer (1 M, pH 7.3), stirring for 15 min at 20°C and evaporation to dryness. The residue was triturated with diethyl ether (3 × 20 ml) and evaporated to dryness. The residue was then dissolved in water and loaded onto a DEAE-Sephadex A25 column which was equilibrated with 0.01 M TEAB buffer. The column was subsequently eluted with a linear gradient of TEAB buffer (0.01–0.1 M, 300 ml each, followed by 0.1–0.6 M, 300 ml each): appropriate fractions were collected and freeze-dried; the residue was dissolved in water and passed through a column of Dowex Na^+ (Bio-Rad, AG 50W-X2, 200–400 mesh) and freeze-dried again to give *8* (15 mg, 70%).

^{1}H-NMR (D_2O): 7.63 (s, 1H) H-6; 6.16 (t, 1H, $J_{1',2'}$ = 6.8 Hz) H-1′; 4.47 (m, 1H) H-3′; 4.09 (d, 3H) H-4′,-5′ and −5″; 2.35 (q, 2H) H-2′ and −2″; 1.79 (s, 3H) methyl.

^{31}P-NMR (D_2O): −9.0 (d, J = 20.4 Hz) γ-P; −11.4 (d, J = 19.5 Hz) α-P; −22.6 (t) β-P; IR (KBr): 2100 cm^{-1}; UV (water): λ_{max} 267 nm (ε = 8400).

*1-(3′-Azido-2′,3′-dideoxy-5′-trisodium triphospho-β-*D-threo-*pentofuranosyl)-thymine (6)*

Compound *5* (10 mg, 37.4 μmol) was prepared under a condition analogous to *8*, to give *6* (13.5 mg, 60%).

^{1}H-NMR (D_2O): 7.63 (s, 1H) H-6; 6.05 (dd, 1H, $J_{1',2'}$ = 7.8 Hz, $J_{1',2''}$ = 2.7 Hz) H-1′; 4.42 (m, 1H) H-3′; 4.23 (d, 3H) H-4′, −5′ and −5″; 2.74 (ddd, 1H, $J_{2',3'}$ = 6.3 Hz, $J_{2',2''}$ = 15.5 Hz) H-2′; 2.19 (dd, 1H) H-2″; 1.80 (s, 3H) methyl.

^{31}P-NMR (D_2O): −5.3 (d, J = 20.4 Hz) γ-P; −9.1 (d, J = 18.6 Hz) α-P; −20.1 (t) β-P; IR (KBr): 2100 cm^{-1}; UV (water): λ_{max} 267 nm (ε = 7500).

Results

Chemistry

3′-Azido-2′,3′-dideoxy-5′-*O*-trityl-thymidine (*4*) was prepared, through the key intermediate 5′-*O*-trityl-2,3′-anhydrothymidine (*3*), using a procedure described in the literature (Glinski et al. 1984) and outlined in Fig. 1.

The literature procedure (Matsuda et al., 1980) describing the preparation of the corresponding *threo* analogue *2* in 47% yield, by heating the 3′-mesylate *1* with sodium azide in dry dimethylformamide at reflux temperature, did not work quite satisfactorily in our hands. The reason is that a direct intermolecular nucleophilic displacement of the 3′-mesylate *1* in pyrimidine nucleosides competes with the intramolecular reaction due to neighbouring groups giving a poor yield of the target compound *2*. A new procedure for the preparation of the *threo* analogue *2* was developed. Preparation of 1-(5′-trityl-3′-azido-2′,3′-dideoxy-β-D-threo-pentofuranosyl)thymine (*2*) involved a treatment of *1* with 1:1 crystalline complex of potassium azide and 18-crown-6 in dry tetrahydrofuran under nitrogen at reflux temperature for 20 h. Such a reaction condition gave the target compound in 74% crystalline yield, 11% of 5′-trityl-2,3′-anhydrothymidine (*3*) and ca. 1% of 5′-trityl-3′-azidothymidine (*4*).

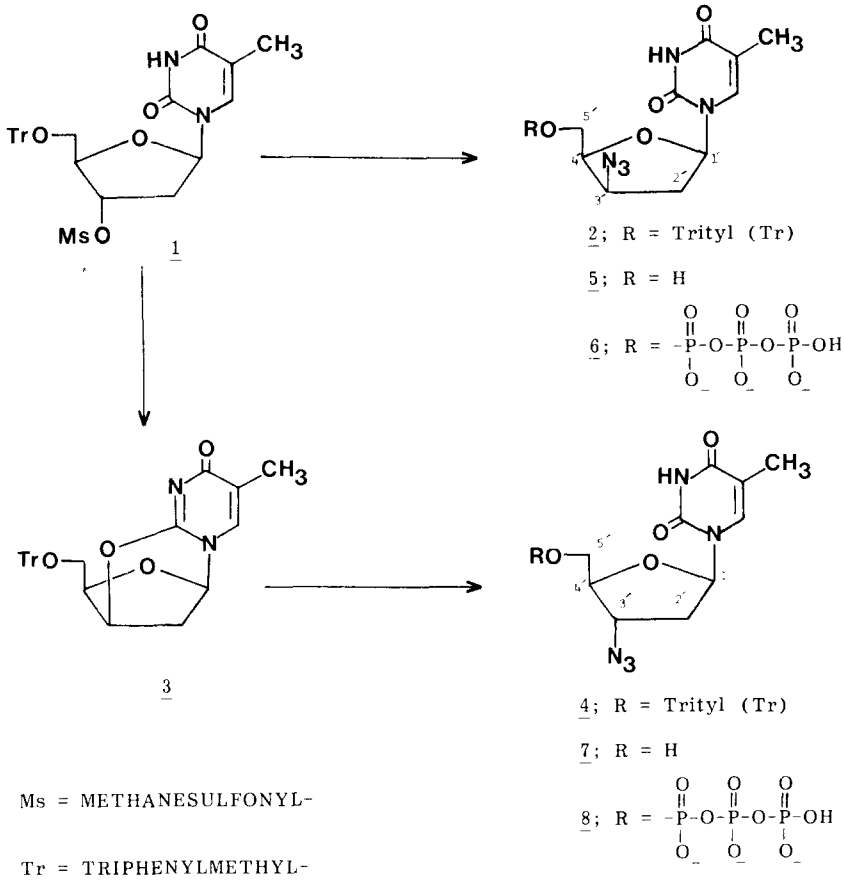

Fig. 1. Synthesis of 3′-azido-3′-deoxythymidine (*erythro*) (*8*) and its *threo* analogue (*6*).

The fully deprotected nucleosides *5* and *7* were obtained in 90 and 97% yields from the corresponding parent compounds by heating at reflux in 80% acetic acid for 10 min. Compounds *5* and *7* were subsequently converted to their triphosphates *6* and *8* in 70 and 60% yields, respectively, using phosphorous oxychloride for phosphorylation of the 5′-hydroxyl function followed by a condensation step with bis(tributylammonium)-pyrophosphate at 0°C as detailed in the experimental procedure (Yoshikawa et al., 1969). The *threo*isomer *6* was 99.5% pure and contained about 0.5% of the *erythro* isomer *8*.

Biochemistry

The effects of the two structural isomers of 3′-azido-3′-deoxythymidine triphosphate on HIV reverse transcriptase using different template/primers are presented in Table 1. Compound *8* was considerably more active than the isomer with the azido-group in "up" position as in compound *6*. Since *6* had a contamination of

TABLE 1

Inhibition of HIV reverse transcriptase by 3′-azido-3′-deoxythymidine 5′-triphosphate (*8*) and its *threo* isomer (*6*) using different template-primers

Template/primer	μM causing 50% inhibition[a]	
	8	*6*
$(rA)_n(dT)_{12-18}$	0.04	>1.7
$(dA)_n(dT)_{12-18}$	>20	>100
Activated DNA	25	>50
$(rC)_n(dG)_{12-18}$	>100	>100

[a] Inhibition assays were performed with 10 μM dTTP or dGTP as described in Methods.

about 0.5% *8* it seems likely that this accounts for the weak inhibition and *6* might totally lack effect on HIV reverse transcriptase. In the reverse transcriptase reactions only that using $(rA)_n(dT)_{12-18}$ as a template/primer was inhibited and no effect was observed when $(rC)_n(dG)_{12-18}$ was the template/primer. An inhibition of the reaction using activated DNA was seen but only with the "down" 3′-azido-3′-deoxythymidine triphosphate (*8*). The polymerase activity depending on $(dA)_n(dT)_{12-18}$ was not inhibited at the concentrations tested. Using DNA polymerase α and activated DNA a 50% inhibition was observed at 50 μM of *8* and >200 μM of *6* using the same conditions as for the HIV reverse transcriptase.

The sensitivity of reverse transcriptases from 8 patient isolates of HIV were compared to that of U 937/HTLV-III as shown in Table 2. It is evident that there is at least a twofold difference in sensitivity to inhibition by *8* between different reverse transcriptases. The inhibition was in all tested cases lower at a higher dTTP concentration during the assay.

Enzyme kinetic studies with HIV (U 937/HTLV-III) reverse transcriptase showed a competitive type of inhibition for 3′-azido-3′-deoxythymidine triphosphate (*8*) when the concentration of $(rA)_n(dT)_{12-18}$ was held constant and the dTTP con-

TABLE 2

Inhibition of HIV reverse transcriptase from different virus isolates by 3′-azido-3′-deoxtythymidine triphosphate (*8*)

Virus isolate	Inhibition (%)		
	At 0.6 μM dTTP		At 6 μM dTTP
	0.05 μM *8*	0.1 μM *8*	0.1 μM *8*
U 937/HTLV-III	73	81	69
1	45	65	–
2	38	51	39
3	75	–	–
4	67	–	–
5	66	81	67
6	77	88	75
7	56	70	–
8	52	67	51

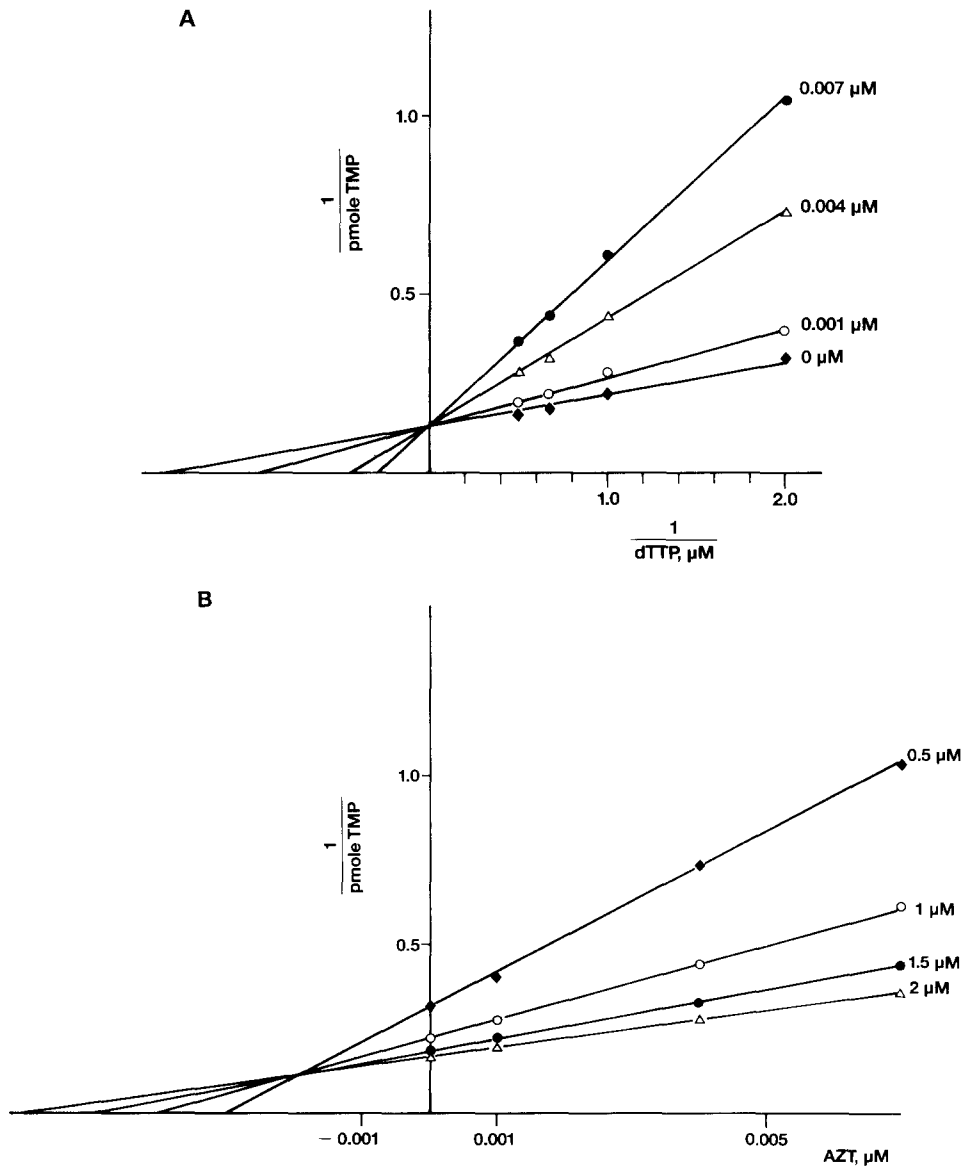

Fig. 2. Inhibition of HIV reverse transcriptase activity by 3′-azido-3′-deoxythymidine triphosphate (*erythro*) (*8*). $(rA)_n(dT)_{12-18}$ was used as template primer and dTTP as varied substrate. K_i value was 0.0019 μM and the K_m value 0.8 μM in this experiment. (A) Lineweaver-Burk plot: (◆) 0 μM, (○) 0.001 μM, (△) 0.004 μM and (●) 0.007 μM *8*. (B) Dixon plot: (◆) 0.5 μM, (○) 1 μM, (●) 1.5 μM and (△) 2 μM dTTP. Conditions and assay procedure were as described in Materials and Methods.

centration was varied (Fig. 2). The active form, with the 3′-azido group in "down" position, as in *8*, had a K_i value of 0.0022 μM (mean of five experiments) in the $(rA)_n(dT)_{12-18}$ directed reaction. The K_m for dTTP in the reverse transcriptase reaction directed by $(rA)_n(dT)_{12-18}$ varied between 0.7 and 1.7 μM in different experiments.

Discussion

It is not clear if an inhibitor of HIV reverse transcriptase will have any therapeutic effect on HIV infections but it seems likely that this type of inhibitors at least could prevent the infection of new cells (De Clercq and Balzarini, 1985; Hirsch and Kaplan, 1985; Öberg, 1986). While clinical evaluation of reverse transcriptase inhibitors is ongoing (see Yarchoan et al. (1986) for 3′-azido-3′-deoxythymidine) it is important to learn about their mechanism of action and to find new inhibitors.

3′-Azido-3′-deoxythymidine triphosphate (*8*) and its *threo* isomer (*6*) showed a considerable difference in activity against HIV reverse transcriptase (Table 1). Since dideoxythymidine triphosphate is an efficient inhibitor of reverse transcriptase (L. Vrang, unpublished observation) it seems likely that it is not the lack of a "down" 3′-azido group that makes compound *6* with an "up" 3′-azido group inactive. The "up" 3′-azido group could either directly cause steric hindrance for interaction with the enzyme or cause a conformational change of the whole molecule preventing the interaction. The results indicate that deoxythymidine derivatives with 3′-substituents in the "up" position may be less interesting than compounds with the 3′-group in the "down" position.

3′-Azido-3-deoxythymidine triphosphate (*8*) only blocked reverse transcriptase when dTTP was the substrate and did not inhibit the incorporation of dGMP. This result corresponds to the observation that 3′-azido-3′-deoxythymidine triphosphate (*8*) was a competitive inhibitor of a $(rA)_n(dT)_{12-18}$ primed reverse transcriptase activity (St. Clair et al., 1985). When a DNA template was used instead of an RNA template a less efficient inhibition of HIV reverse transcriptase was found (Table 1) and this has been reported also for phosphonoformate (Vrang and Öberg, 1986).

Inhibition of cellular DNA polymerase α required more than 1000-fold higher concentration than the inhibition of HIV reverse transcriptase when activated DNA and $(rA)_n(dT)_{12-18}$ respectively were used as templates. When activated DNA was used with both these enzymes a 2-fold difference was seen. It has been noted previously that *8* is a poor inhibitor of cellular DNA polymerase α but no data were given (Furman et al., 1985).

3′-Azido-3′-deoxythymidine triphosphate (*8*) caused a competitive inhibition of HIV reverse transcriptase (Fig. 2) when $(rA)_n(dT)_{12-18}$ was the template. The K_i value for the compound with the azido group in "down" position *8* was 0.0022 μM (mean of five experiments). The slight inhibition observed for *6* could be explained by a 0.5% contamination of *8*. The K_m value for thymidine triphosphate in this reaction varied between 0.7 and 1.7 μM showing that *8* had a better affinity

to the enzyme than dTTP while the affinity for the *threo* analogue of 3′-azido-3′-deoxythymidine triphosphate (*6*) was low, or absent. The results for *8* correspond well with the competitive type of inhibition mentioned by Furman et al. (1985) for HIV reverse transcriptase, but our K_i and K_m values were lower.

The reason for this difference can not be analyzed due to lack of information about experimental conditions. However, different HIV isolates show some difference in sensitivity of their reverse transcriptases, as indicated in Table 2. Even if the HIV genome has a conserved region coding for the reverse transcriptase some variation could be expected in analogy with the different sensitivities to polymerase inhibitors shown by different herpes simplex virus isolates (Field, 1983).

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