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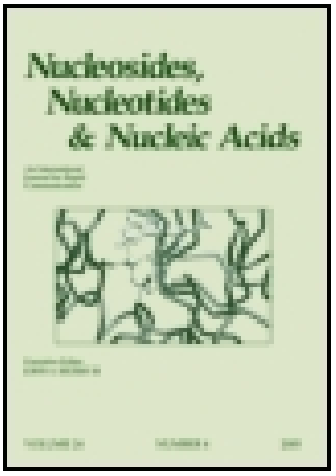

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Synthetic Nucleosides and Nucleotides. XXXI.[1] Inhibitory Effects of 2′-Deoxy-5-styryluridine 5′-Triphosphate Analogues on Retroviral Reverse Transcriptases and Higher Eukaryotic DNA Polymerases

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NUCLEOSIDES & NUCLEOTIDES, 13(6&7), 1247-1258 (1994)

SYNTHETIC NUCLEOSIDES AND NUCLEOTIDES. XXXI. [1] INHIBITORY EFFECTS OF 2'-DEOXY-5-STYRYLURIDINE 5'-TRIPHOSPHATE ANALOGUES ON RETROVIRAL REVERSE TRANSCRIPTASES AND HIGHER EUKARYOTIC DNA POLYMERASES[†]

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Abstract: Some new 5'-triphosphate derivatives of 2',3'-dideoxy-5-styryluridine analogues have been synthesized and examined for their inhibitory effects on retroviral reverse transcriptases and eukaryotic DNA polymerases. We describe here the mode of inhibition and the influence of the substituent at the 5-position of uracil ring of 2',3'-dideoxyUTP analogues.

Retroviral reverse transcriptase plays important roles in the replication of the retroviral genome. It is well known that 3'-azido-2',3'-dideoxythymidine (AZT) exerts its anti-human immunodeficiency virus (HIV) activity as a 5'-triphosphate by inhibiting the reverse transcriptase of HIV.[2,3] However, it has been shown that AZT may cause side effects such as bone marrow suppression[4,5] and mitochondrial myopathy[6] by long term treatment. These side effects are thought to be due to inhibition of host DNA polymerases, especially in γ-type.[2,7,8,9] Thus it is desired to develop new anti-HIV agents which inhibit HIV reverse transcriptase more efficiently and more selectively than host

[†] This paper is dedicated to Professor Morio Ikehara on the occasion of his 70th anniversary

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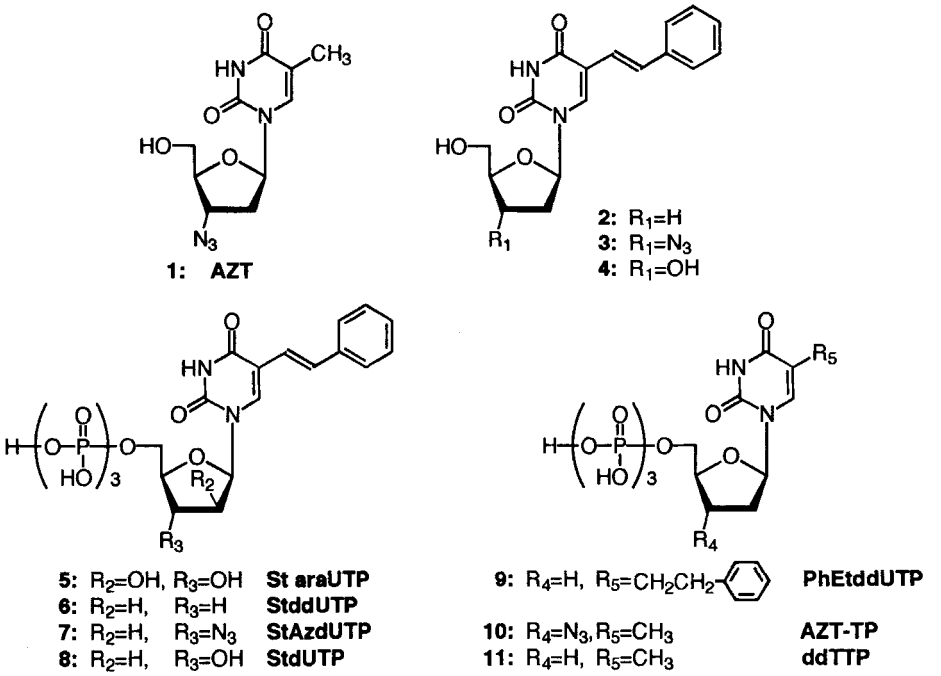
DNA polymerases. In a previous paper, we reported that *E*-5-styryl araUTP (**5**) and some *E*-5-(substituted)styryl araUTP's were potent inhibitor for DNA polymerase α and



herpesviral DNA polymerase.[10,11] These results suggest that steric and hydrophobic interaction of the substituent affects the affinity of the compound to various DNA polymerases. In order to obtain the information for the synthetic designing of selective inhibitor against HIV, we have synthesized some 2',3'-dideoxy-*E*-5-styryluridine analogues and reported that 2',3'-dideoxy-*E*-5-styryluridine (**2**) and 3'-azido-2',3'-dideoxy-*E*-5-styryluridine (**3**) were found to be active, with EC_{50} values of 10 and 5 μg/ml, respectively.[1] In the present paper, the inhibitory effects of their 5'-triphosphate derivatives on retroviral reverse transcriptases and eukaryotic DNA polymerases will be described.

MATERIALS AND METHODS

General procedure for phosphorylation[12,13] of 2'-deoxy-5-styryluridine analogues. A solution of 2'-deoxy-5-styryluridine analogue[1] (0.1 mmol) in triethyl phosphate (1 ml) was mixed with phosphorus oxychloride (0.05 ml) under cooling at −10°C. The mixture was stirred for 15 hr at 0–4 °C, and then mixed with 0.5 M sodium

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bicarbonate (10 ml) and chloroform (10 ml). The aqueous layer was diluted with water to a final volume of 50 ml. The solution was applied to a column of DEAE-cellulose (2.5 x 10 cm, bicarbonate form), which was eluted with a liner gradient (600 ml) of 0 to 0.3 M triethylammonium bicarbonate (pH 8.0). The main peak of UV absorption, eluted at 0.20 to 0.23 M (corresponding to the 5'-monophosphate) was collected and evaporated to dryness. The yields of 2',3'-dideoxy-*E*-5-styryluridine 5'-monophosphate (StddUMP) and 3'-azido-2',3'-dideoxy-*E*-5-styryluridine 5'-monophosphate (StAzdUMP) were 75 and 47%, respectively. A solution of 5'-monophosphate (0.03 mmol) (as the triethylammonium salt) in dimethylformamide (1 ml) was mixed with 1,1'-carbonyldiimidazole (24 mg, 0.15 mmol), and the mixture was stirred for 5 hr at room temperature. After treatment with 5 μl of methanol at room temperature for 30 min, it was mixed with tri-*n*-butylammonium pyrophosphate (0.6 mmol in 1 ml of dimethylformamide) under vigorous shaking. The mixture was stirred for 15 hr at room temperature, then the reaction mixture was evaporated to dryness. The residue was dissolved in 50 ml of water. Active carbon (Norit A) (0.4 g) was added to the solution and the mixture was stirred for 15 min. The mixture was filtered and the active carbon was washed well with water. The residue was eluted with 50% aqueous ethanol containing 3% ammonium hydroxide. The eluate was evaporated to dryness and the residue was dissolved in 20 ml of water. The solution was applied to a column of DEAE-cellulose (2.5 x 10 cm, bicarbonate form), which was eluted with a liner gradient (1,000 ml) of 0 to 0.6 M triethylammonium bicarbonate (pH 8.0). The fractions containing 5'-triphosphate were combined and evaporated to dryness. A portion of the residue was further purified by HPLC (*method A*). The yields of , 2',3'-dideoxy-*E*-5-styryluridine 5'-triphosphate (StddUTP, **6**) and 3'-azido-2',3'-dideoxy-*E*-5-styryluridine 5'-triphosphate (StAzdUTP, **7**) from corresponding 5'-monophosphate were 51 and 58%, respectively.

In a similar fashion, 2'-deoxy-*E*-5-styryluridine 5'-triphosphate (StdUTP, **8**) was synthesized from 2'-deoxy-*E*-5-styryluridine 5'-monophosphate[14] in 49% yield.

Preparation of 2',3'-dideoxy-5-(2-phenethyl)uridine 5'-triphosphate (PhEt-ddUTP, 9). A mixture of 500 OD_{308} units (0.029 mmol) of StddUTP (**6**) in 1 ml of water and 70 mg of 5% palladium on carbon was stirred under 1 atm of hydrogen gas at room temperature for 20 hr. The reaction mixture was filtered, and palladium-carbon was washed with 50 ml of 50% aqueous ethanol containing 3% ammonium hydroxide. The combined filtrates and washings were evaporated to dryness and the residue was dissolved in 20 ml of water. The solution was applied to a column of DEAE-cellulose (2.5 x 10 cm, bicarbonate form), which was eluted with a liner gradient (1,000 ml) of 0 to 0.5 M triethylammonium bicarbonate (pH 8.0). The fractions containing 5'-triphosphate were combined and evaporated to dryness. 144 OD_{267} units (0.016 mmol, 55%).

Chemical properties of 5-styryl dUTP's (6, 7 and 8) and PhEtddUTP (9). UV spectral and chemical properties of newly synthesized analogues including λmax, phosphate analyses and retention times on HPLC analyses are summarized in Table 1. The purities of compounds **6**, **7**, **8** and **9** were confirmed to be greater than 95% at 300 nm (**6**, **7** and **8**) or 270 nm (**9**).

Enzymes. DNA polymerase α was purified from calf thymus by immunoaffinity column chromatography,[15] and rat DNA polymerase β, expressed in *Escherichia coli* from recombinant plasmid, was purified as described previously[16]. Bovine DNA polymerase γ was purified from a crude mitochondrial fraction of bovine liver.[9] Bacterial recombinant HIV-1 reverse transcriptase, which showed two major polypeptides (51 and 66 kilodaltons) on SDS polyacrylamide gel electrophoresis, and recombinant Moloney murine leukemia virus (Moloney-MuLV) reverse transcriptase were purchased from Seikagaku Kogyo and Boehringer, respectively.

Chemicals. AZT-TP was synthesized as described,[17] and ddTTP was purchased from Wako Pure Chemical. dNTP's were purchased from Yamasa Shoyu.

High-performance liquid chromatography (HPLC).

Method A. The column employed was YMC Pack ODS A-302 (YMC Co., Ltd.) reversed phased column (4.5 mm I. D. x 15 cm L.). The solvent was a mixture of water, acetonitrile and 1 M triethylammonium acetate buffer (pH 7.0) (60:20:20, v/v/v). The flow rate was 1 ml/min. The analyses were performed at 45 °C.

Method B. The column employed was TSK-GEL DEAE-2SW (TOHSO Co., Ltd.) anion exchange column (4.5 mm I. D. x 25 cm L.). The solvent was 0.21 M potassium phosphate buffer (pH 6.95) containing 20 % acetonitrile. The flow rate was 1 ml/min. The analyses were performed at 45 °C.

Assays for DNA polymerase and reverse transcriptase activities. DNA polymerase activities were examined with poly(rA)-oligo(dT) ($(rA)_n$-$(dT)_{12-18}$) or activated calf thymus DNA as the template-primer under the optimum conditions for each DNA polymerase. These assay conditions were summarized in Table 2. After the addition of enzyme (0.01 to 0.05 units), incubation was carried out at 37 °C for 20 min. Then the reaction mixtures were chilled and transferred to DE81 ion-exchange papers (Whatman). The papers were washed with 5% Na_2HPO_4 (6 times). Radioactivity in the polynucleotides retained on the paper was measured. One unit of reverse transcriptase or DNA polymerase was defined as the amount of enzyme that incorporates 1 nmol of [^{3}H]dTMP into polymer per 60 min.

When kinetic analysis was performed, the concentrations of [^{3}H]dTTP and inhibitors were varied.

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TABLE 1. Analytical results and constants of synthetic componunds

Compound		UV, $\lambda_{max}^{H_2O}$ nm (ε)	(ε) P, Found	HPLC analyses (*Method B*) Retention times (min)
StddUTP	**6**	307 (17 400)	6 200	7.4
StAzdUTP	**7**	308 (18 000)	5 600	8.4
StdUTP	**8**	307 (17 600)	5 660	8.4
PhEtddUTP	**9**	267 (9 000)	3 600	5.4

TABLE 2. Assay conditions for reverse transcriptases and various DNA polymerases (Pol's)

DNA polymerase	template-primer and concn (μg/ml)	buffer	[^{3}H]dTTP [a] concn (μM)	unlabeled dNTP concn (μM)	divalent cation and concn (mM)	KCl concn (mM)
Reverse transcriptases	$(rA)_n$-$(dT)_{12-18}$ 20 : 10	50 mM Tris-HCl pH 8.3	50		Mn^{2+}, 0.5	50
	activated DNA [b] 100	50 mM Tris-HCl pH 8.3	50	other three dNTP's, 100 each	Mg^{2+}, 4	50
Pol α	activated DNA [b] 100	50 mM Tris-HCl pH 7.5	50	other three dNTP's, 100 each	Mg^{2+}, 4	
Pol β	$(rA)_n$-$(dT)_{12-18}$ 40 : 10	50 mM Tris-HCl pH 8.8	50		Mn^{2+}, 0.5	100
Pol γ	$(rA)_n$-$(dT)_{12-18}$ 40 : 10	40 mM KPi pH 7.5	50		Mn^{2+}, 0.5	50

All reaction mixtures (total volume 25 μl) contained 1 mM dithiothreitol and 100 μg/ml of bovine serum albumin.

a) The specific activities of [^{3}H]dTTP were 110 to 440 cpm/pmol.

b) Activated calf thymus DNA was used.

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RESULTS AND DISCUSSION

We measured the inhibitory effects of StddUTP (**6**), StAzdUTP (**7**), StdUTP (**8**) and PhEtddUTP (**9**) comparing with AZT-TP (**10**) and ddTTP (**11**) on HIV-1 and Moloney-MuLV reverse transcriptases and three eukaryotic DNA polymerases with poly(rA)-oligo(dT) or activated calf thymus DNA as the template-primer. As shown in Figure 1A and 1B, HIV-1 reverse transcriptase was inhibited strongly by dUTP analogues bearing styryl or 2-phenylethyl group at the 5-position (StddUTP (**6**), StAzdUTP (**7**), StdUTP (**8**) and PhEtddUTP (**9**)) when poly(rA)-oligo(dT) was used as the template-primer. Moloney-MuLV reverse transcriptase activity was also inhibited by these analogues(Figure 1C). However, the inhibitory effects of 2',3'-dideoxy analogues (ddTTP and StddUTP (**6**)) were weaker than those of 3'-azido analogues (AZT-TP and StAzdUTP(**7**)). In the both cases of HIV-1 reverse transcriptase and Moloney-MuLV reverse transcriptase, the hydrogenation of the styryl group of StddUTP (**6**) reduced the inhibitory effects. On the other hand, the inhibitory effects of StAzdUTP(**7**) on DNA polymerase β and γ were similar to that of AZT-TP (Figure 1D and 1E), whereas StdUTP (**8**) and StddUTP (**6**) were more potent inhibitory to both polymerases than AZT-TP. When activated DNA was used as the template-primer (FIG.2), HIV-1 reverse transcriptase was also inhibited by StddUTP (**6**) and StAzdUTP(**7**). However, these inhibitory effects were weaker than that of AZT-TP or ddTTP. In contrast, DNA polymerase α was inhibited only by StdUTP (**8**), and not or slightly inhibited by StddUTP (**6**) or StAzdUTP (**7**).

Next, Lineweaver-Burk analyses of the inhibitory effects of these analogues on reverse transcriptases and DNA polymerases were carried out. Typical results for HIV-1 reverse transcriptase is shown in Figure 3. All the dUTP analogues were essentially competitive with respect to the natural substrate dTTP for all the DNA polymerases and reverse transcriptases.

Based on these analyses, the inhibition constants (*Ki*'s) of each analogue for all the polymerases were determined and are summarized in Table 3. When poly(rA)-oligo(dT) was used as the template-primer, the *Ki*'s of StddUTP (**6**), StAzdUTP (**7**), ddTTP and AZT-TP for HIV-1 reverse transcriptase were found to be in the range of 0.03–0.05 μM (*Ki*/*Km*=0.002–0.004), no considerable difference is seen among these four analogues. Thus *Ki* values for these compounds were shown to be remarkably smaller than *Km* for dTTP (14 μM). Although the *Ki*'s of 3'-azido analogues (StAzdUTP (**7**) and AZT-TP) for Moloney-MuLV reverse transcriptase were also small (*Ki*/*Km*=0.002 and 0.004, respectively), *Ki*'s of 2',3'-dideoxy analogues (StddUTP (**6**) and ddTTP) were 10-times larger than those of the 3'-azido analogues. This means that Moloney-MuLV reverse transcriptase is about 10-times less sensitive to 2',3'-dideoxyUTP analogues than to 3'-

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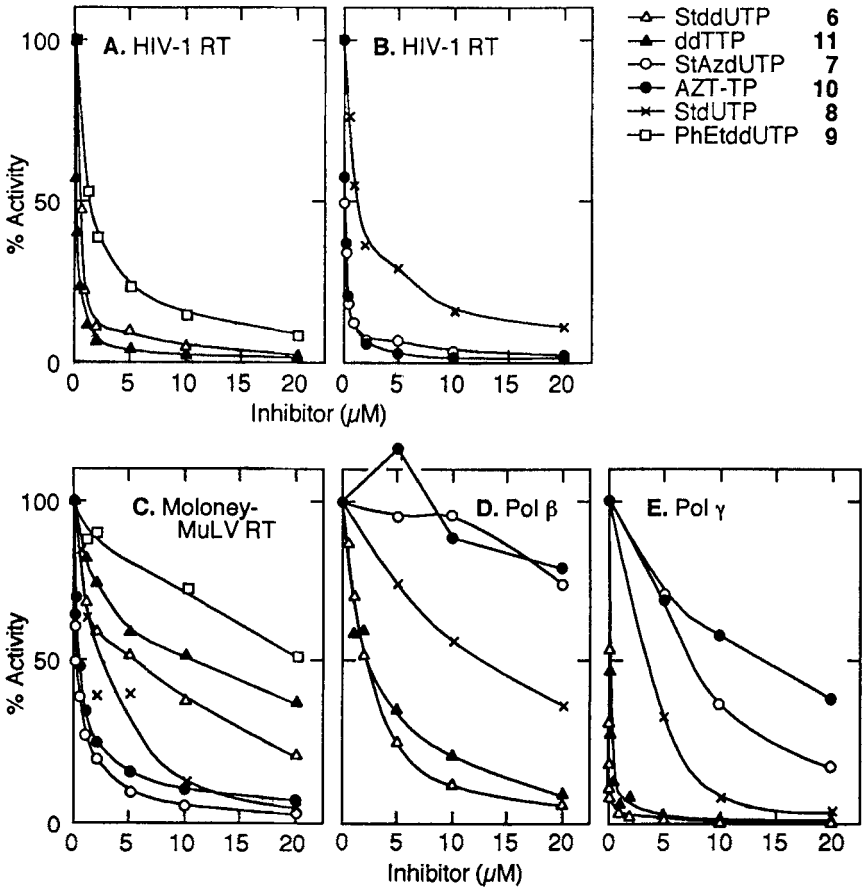

FIG. 1. Inhibitory effects of various 2',3'-dideoxy UTP analogues on retroviral reverse transcriptases (RTs) and eukaryotic DNA polymerases (Pol's).

The inhibitory effects of StddUTP (**6**), StAzdUTP (**7**), StdUTP (**8**), PhEtddUTP (**9**), AZT-TP and ddTTP on HIV-1 RT (**A** and **B**), Moloney-MuLV RT (**C**), DNA polymerases β (**D**) and γ (**E**) were measured with poly(rA)-oligo(dT) as the template-primer in the presence of 50 μM[^{3}H]dTTP.

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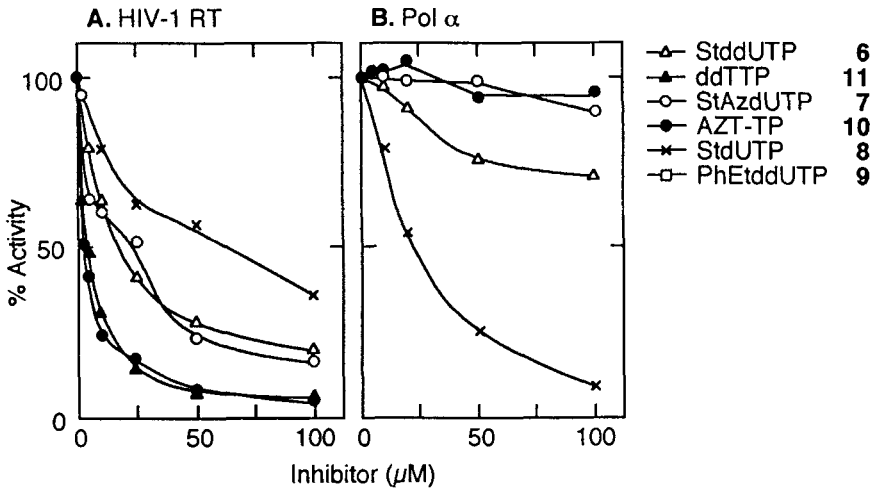

FIG. 2. Inhibitory effects of various 2',3'-dideoxy UTP analogues on HIV-1 reverse transcriptase (RT) and DNA polymerase α (Pol α).

The inhibitory effects of StddUTP (**6**), StAzdUTP (**7**), StdUTP (**8**), PhEtddUTP (**9**), AZT-TP and ddTTP on HIV-1 RT (**A**) and DNA polymerases α (**B**) were measured with activated DNA as the template-primer in the presence of 50 μM[^{3}H]dTTP.

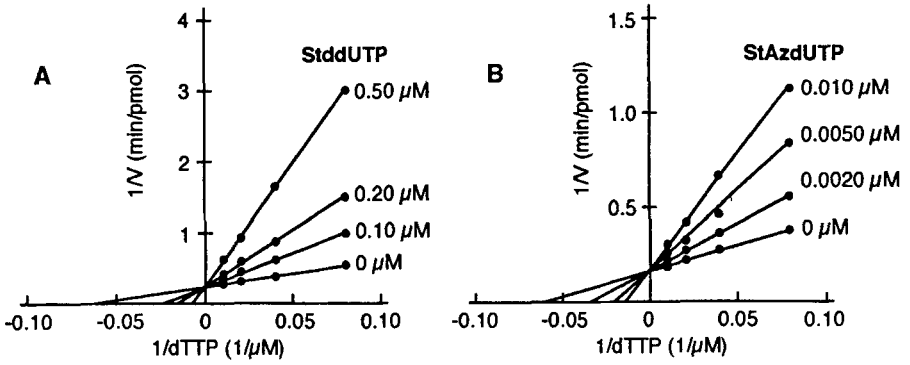

FIG. 3. Lineweaver-Burk plots for the inhibition of HIV-1 reverse transcriptase by StddUTP (**6**) (**A**) and StAzdUTP (**7**) (**B**). Poly(rA)-oligo(dT) was used as the template-primer.

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TABLE 3. Kinetic constants of various DNA polymerases (Pol's) and HIV-1 and Moloney-MuLV reverse transcriptases (RT's) for 2',3'-dideoxy-5-styrylUTP analogues, ddTTP and AZT-TP

Enzyme	dTTP	StddTTP (**6**)		ddTTP (**11**)		StAzdUTP (**7**)		AZT-TP (**10**)		StdUTP (**8**)		PhEtddUTP (**9**)	
Template-primer	*K*m (μM)	*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	*K*i (μM)	*K*i/*K*m	*K*i (μM)	*K*i/*K*m
HIV-1 RT													
$(rA)_n$-$(dT)_{12-18}$	**14**	**0.050**	*0.0036*	**0.040**	*0.0029*	**0.030**	*0.0021*	**0.040**	*0.0029*	**0.25**	*0.018*	**0.50**	*0.036*
Activated DNA	**1.1**	**0.12**	*0.11*	**0.037**	*0.034*	**0.12**	*0.11*	**0.040**	*0.036*	**0.40**	*0.36*	ND	
Moloney-MuLV RT													
$(rA)_n$-$(dT)_{12-18}$	**50**	**2.0**	*0.040*	**3.0**	*0.060*	**0.10**	*0.0020*	**0.20**	*0.0040*	**0.20**	*0.0040*	ND	
Pol α													
Activated DNA	**3.6**	ND		ND		ND		ND		**2.6**	*0.72*	ND	
Pol β													
$(rA)_n$-$(dT)_{12-18}$	**50**	**2.0**	*0.040*	**1.5**	*0.030*	ND		ND		**10**	*0.20*	ND	
Pol γ													
$(rA)_n$-$(dT)_{12-18}$	**4.0**	**0.0030**	*0.00075*	**0.020**	*0.0050*	**0.50**	*0.13*	**1.5**	*0.38*	**0.10**	*0.025*	**0.70**	*0.18*

ND: Not determined

azido-2',3'-dideoxyUTP analogues. Thus, a remarkable difference in the sensitivity to the analogues is seen between HIV-1 reverse transcriptase and Moloney-MuLV reverse transcriptase. This result suggests that other retrovirus except for HIV is not suitable for the screening of potential anti-HIV agent.

Interestingly, the substitution of methyl group of AZT-TP or ddTTP by styryl group at 5-position increased the inhibitory effect on DNA polymerase γ, in contrast, did not influence on their inhibitory effects for the reaction of HIV-1 reverse transcriptase when poly(rA)-oligo(dT) was used as the template-primer. However, this substitution slightly reduced the inhibitory effect on HIV-1 reverse transcriptase when activated DNA was used.

5-Phenethyl ddUTP (**9**) which is thought to have similar hydrophobicity at 5-position to that of StddUTP (**6**) was found to show approximately 10-times lower affinity for HIV-1 reverse transcriptase. This finding indicates that not only hydrophobicity but also steric hindrance of the substituent at the 5-position of dUTP analogues affects the affinity of the analogues for HIV-1 reverse transcriptase. In the case of DNA polymerase α, the similar result was obtained. Izuta and Saneyoshi reported that the inhibitory effects of 5-phenethyl araUTP was about 10-times less than that of 5-styryl araUTP (**5**).[10]

Since DNA polymerase γ is the most sensitive to the 2',3'-dideoxy-5-styryl UTP's among cellular DNA polymerases, we obtained and compared the ratios of (*Ki*/*Km* value for HIV-1 reverse transcriptase)/(*Ki*/*Km* value for DNA polymerase γ) as the selective index of the inhibitor for HIV-1 reverse transcriptase. The ratios of StddUTP (**6**), ddTTP, StAzdUTP(**7**) and AZT-TP were 4.8, 0.58, 0.016 and 0.0076, respectively. A smaller value of the ratio indicates selectivity for HIV-1 reverse transcriptase. Among these inhibitors, the value of the ratio of StAzdUTP (**7**) was very small. Therefore, StAzdUTP(**7**) seems to be a selective inhibitor for HIV-1 reverse transcriptase, although the value of StAzdUTP (**7**) was somewhat larger than that of AZT-TP.

Few studies have been reported on the inhibitory effects of 2',3'-dideoxyUTP analogues bearing bulky substituents at the C-5 position with respect to retroviral reverse transcriptase and eukaryotic DNA polymerases. StAzdUTP (**7**) is shown in the present study to be a selective and potent inhibitor for HIV-1 reverse transcriptase. However, anti-HIV activity of 3'-azido-2',3'-dideoxy-*E*-5-styryluridine (**3**) (EC_{50}=5 μg/ml) has been approximately 250-fold weaker than that of AZT.[1] Probably, 2',3'-dideoxyuridine analogues substituted with a styryl group at the C-5 position may be poorer substrates than AZT for cellular deoxynucleoside kinases. Recently, McGuigan et al reported that aryloxy phosphoramidite derivatives of AZT showed potent anti-HIV activity in the TK^- (thymidine kinase-deficient) mutant CEM cells in which AZT was virtually inactive, and these compounds were efficient prodrugs of AZT-MP, partially circumventing the first

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activation step (phosphorylation of AZT to its 5'-monophosphate) by cellular thymidine kinase.[18] We are also attempting to synthesize the novel 5'-monophosphate derivatives of 2',3'-dideoxy-5-styryluridines and evaluate their anti-HIV activity.

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