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Inhibition of HIV-1 reverse transcriptase by 5′-triphosphates of 5-substituted uridine analogs

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Summary

The 5′-triphosphates of some 5-substituted 2′-deoxyuridine analogs were investigated for their effects on purified recombinant reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) as well as cellular DNA polymerase α. The triphosphates were competitive inhibitors of the viral enzyme with dTTP as the variable substrate and poly(rA)oligo(dT) as template, and preferentially inhibited the viral polymerase. Ordering the compounds according to their decreasing binding affinities, as reflected by their increasing inhibition constants for the reverse transcriptase, gave nPrearaUTP > nPrdUTP > EtdUTP > nPredUTP > HMdUTP > CEdUTP.

Although nPredUTP was less inhibitory than nPrearaUTP under conditions of competitive inhibition, nPredUTP caused a time- and concentration-dependent inactivation of reverse transcriptase activity when preincubated with template. This inactivation was not reversed by excess dTTP. The decrease in template-primer activity did not occur with nPrearaUTP, but was shown with the chain-terminating 5′-triphosphates of 3′-fluoro- and 3′-azidothymidine. As nPredUTP, but not nPrearaUTP, was an alternative substrate, shown by the ability to support DNA synthesis in absence of competing substrate, the incorporation of nPredUTP into the primer-template apparently leads to increased inhibition of the enzyme.

Human immunodeficiency virus; Reverse transcriptase; Nucleoside analog; Chemotherapy

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Introduction

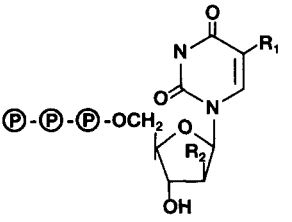
It is well appreciated that antiviral effects of nucleoside analogs, of which the 5′-triphosphates inhibit viral DNA polymerases, are cell-type dependent. This is due mostly to cell-type dependence of nucleoside phosphorylation and nucleotide dephosphorylation (Balzarini et al., 1988; De Clercq, 1982; Datema et al., 1987; Harmenberg et al., 1980). Indeed, a viral polymerase inhibitor not dependent on phosphorylation, phosphonoformate (foscarnet), shows less cell-type dependence than antiviral nucleoside analogs (Öberg, 1989).

The HIV-coded reverse transcriptase is a preferred target for antiviral agents, such as 5′-triphosphates of nucleoside analogs or pyrophosphates analogs (De Clercq, 1987; Mitsuya and Broder, 1987; Öberg, 1988). Also in the case of anti-HIV activities of nucleoside analogs the interplay of several enzymes in determining the pool size of the 5′-triphosphates is critical for antiviral activity. Therefore, analogs of nucleoside 5′-triphosphates able to penetrate cells, or inclusion of nucleoside analog 5′-triphosphates in synthetic lipid vesicles (drug-carrying liposomes) are worthy of investigation as alternatives to antiviral nucleoside analogs. The liposome-mediated drug delivery is of special interest, as in vivo these vesicles have a tendency to concentrate in cells of the mononuclear phagocyte system. To obtain information on the design of selective HIV-RT inhibitors we studied the interaction of 5′-triphosphates of some 5-substituted uridine analogs with this enzyme and with cellular DNA polymerase α. The reason for chosing this class of analogs is that several 5-substituted 2-deoxyuridine analog-5′-triphosphates can act as selective inhibitors of viral polymerases (De Clercq and Walker, 1984; De Clercq, 1984; Frank et al., 1986; Ruth and Cheng, 1981), and as shown here, this includes HIV-reverse transcriptase.

Materials and Methods

Chemicals

Unlabelled triphosphates (dATP, dCTP, dGTP, dTTP), DL-dithiothreitol (DTT), bovine serum albumin fraction V (BSA) and 5-hydroxymethyl-2′-deoxyuridine were obtained from Sigma. Poly(rA)·$(dT)_{12-18}$ and calf thymus DNA polymerase α (CTDNAPα) were purchased from Pharmacia. ^{3}H-dTTP (76.6 Ci/mmole) and ^{3}H-dATP (55.6 Ci/mmole) were from New England Nuclear Corp. 5-Ethyl-2′-deoxyuridine (EtdU), 5-(n-propyl)-2′-deoxyuridine (nPrdU), [E]-5-(1-propenyl)-2′-deoxyuridine (nPredU), 5-(1-propenyl)-1β-D-arabinofuranosyl uracil (nPrearaU) were synthesized as previously described (Stening et al., 1981). 5-(2-chloroethyl)-2′-deoxyuridine (CEdU) was obtained from Sandoz. 3′-Fluoro-3′-deoxythymidine triphosphate (FLTTP) and 3′-azido-3′-deoxythymidine triphosphate (AZTTP) were kind gifts from Drs L. Vrang and J. Chattopadhyaya. Activated DNA was prepared by treating calf thymus DNA (Sigma) with pancreatic DNase at room temperature for 15 min using the procedure of Baril et al. (1977).



R_1	R_2	Abbreviation
$-CH_2OH$	H	HMdUTP
$-CH_2CH_3$	H	EtdUTP
$-CH_2CH_2CH_3$	H	nPrdUTP
$-CH_2CH_2Cl$	H	CEdUTP
$-CH=CH-CH_3$	H	nPredUTP
$-CH=CH-CH_3$	OH	nPrearaUTP

Ⓟ = phosphate

Fig. 1. Structures of 5′-triphosphates of the 2′-deoxyuridine analogs investigated.

Purified human immunodeficiency virus (HTLV-IIIB) reverse transcriptase (HIV-RT) cloned in *E. coli* was obtained from Dr K. Moelling (Hansen et al., 1987).

For synthesis of triphosphates the reagents trimetylphosphate, phosphorous oxychloride, dimetylformamide (DMF) and tributylamine were obtained from Fluka Chemie AG. Bis(tributylammonium)pyrophosphate was prepared by mixing tributylamine (Bu_3N) and pyridinium pyrophosphate obtained by passing of sodium pyrophosphate through a column of a pyridinium cation exchanger (pyridinium Dowex 50 wx8) followed by extended lyophilization and evaporation several times from DMF. 5′-Triphosphates of 2′-deoxyuridine analogs were synthesized according to the method described by Yoshikawa et al. (1969) with some modifications (Tao et al., 1988). The compounds are shown in Fig. 1.

Enzyme assays

For HIV-RT assay the 50 µl reaction mixture contained 50 mM Tris-HCl buffer, pH 7.6, 100 mM KCl, 6 mM $MgCl_2$, 5 mM DTT, 275 µg/ml BSA, 2.5 µg/ml poly(rA)·$(dT)_{12-18}$, 0.26 µM [^{3}H]dTTP (or as indicated in the text) and HIV-RT. After 30 min incubation at 37°C 40 µl samples were spotted on paper discs (24 mm, Munktell, Sweden) and DNA was precipitated in cold 5% trichloroacetic acid with 0.1 M sodium pyrophosphate. The filters were washed three times in this solution and three times in 99.5% ethanol. After drying the radioactivity was measured in a liquid scintillation counter.

For the CTDNAPα assay, the 50 µl reaction mixture contained 100 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 5 mM DTT, 275 µ/ml BSA, 250 µg/ml activated CTDNA, 100 µM dATP, dGTP, dCTP, respectively, [^{3}H]dTTP (0.2–1.5 µM) and 0.0005 U of CTDNAPα. After 30 min of incubation at 37°C, 40 µl samples were taken and worked up as described above.

In the enzyme kinetic experiments a 20 min incubation time was used with saturated concentrations of poly(rA)·$(dT)_{12-18}$, the indicated concentrations of analogs and various concentrations of [^{3}H]dTTP. The kinetic constants, Km and Ki, were determined after linear regression analysis of the data in double reciprocal plots following the description of Lineweaver and Burk. All enzyme reaction rates were linear within experimental time.

Substrate substitution

The ability of analogs to support HIV DNA synthesis (directed by activated CT DNA) in the absence of each of four natural substrates respectively was estimated with 100 μM dNTP and analogs. The activity was compared with the activity obtained with 100 μM natural substrate added instead of the triphosphates of the thymidine analogs.

Effect on DNA primer efficacy

The analogs were preincubated with enzyme for different times (0–60 min) in a 100 μl reaction mixture containing rate limiting amount of template primer, in absence of dTTP. After the indicated times of incubation, the reaction mixtures were heated in a 56°C water bath for 30 min to inactivate the enzyme, and then slowly cooled down to room temperature for 30 min. After adding fresh enzyme and [^{3}H]dTTP, the incubation was continued for 30 min at 37°C. The activity was compared with a control in which water instead of the analog was preincubated with HIV-RT in the reaction mixture.

Results

Inhibition of HIV-RT by 5'-triphosphates of nucleoside analogs

The activity of HIV-RT, using poly(rA)·$(dT)_{12-18}$ as template and dTTP as substrate, was inhibited by the triphosphates of EtdU, nPrdU, nPredU and nPrearaU (Fig. 2). Kinetic experiments showed that the triphosphates were competitive inhibitors of the polymerisation reaction with respect to dTTP. This is shown for nPrearaUTP in Fig. 2. The inhibition constants (Table 1) show that nPrearaUTP was the most potent inhibitor, CEdUTP the least potent inhibitor, whereas the other triphosphates showed intermediary Ki values. Relative to inhibition of cellular polymerase α, nPrdUTP and nPrearaUTP were the most selective inhibitors.

Effect on primer template efficacy

For the most active inhibitor of HIV-RT, nPrearaUTP, and its close analog nPredUTP, we determined whether these nucleoside analog triphosphates could alter the template efficacy in the HIV-RT-catalyzed reaction. Their effects were compared with the effects of the triphosphates of 3'-fluoro-3'-deoxythymidine (FLT) and 3'-azido-3'-deoxythymidine (AZT), which are known chain-terminators in RT-catalyzed reactions (St. Clair et al., 1987; Matthes et al., 1985). Thus, the enzyme was preincubated for different times with template and various concentrations of nPredUTP, nPrearaUTP, FLTTP or AZTTP. The concentration of the template was rate-limiting. After different times of preincubation, the enzyme was heat-inactivated, and new enzyme plus substrate (dTTP) was added for a fur-

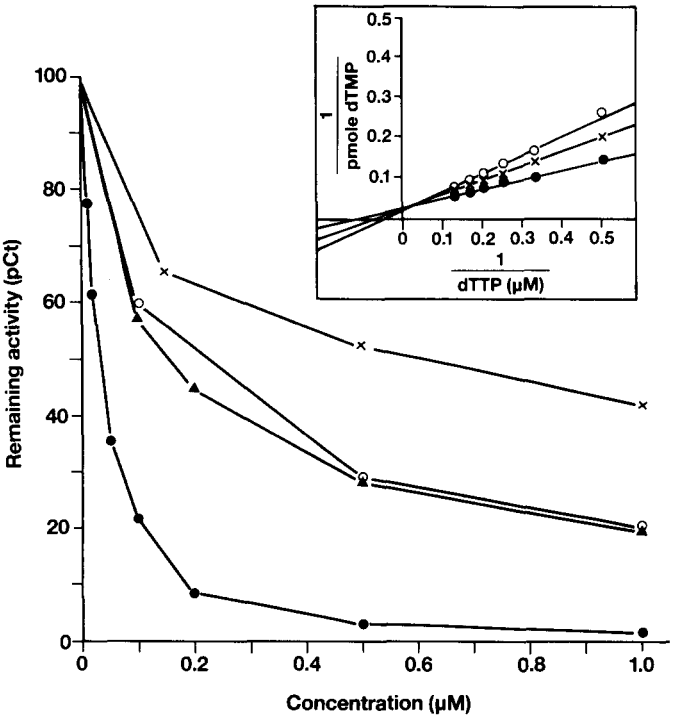


Fig. 2. Dose response curves for the inhibition of HIV-RT by 5′-triphosphates of 2′-deoxyuridine analogs. ×—×, nPredUTP, —○—, EtdUTP; △—△, nPrdUTP; •—•, nPrearaUTP. Insert: Lineweaver-Burk plot of a kinetic study with nPrearaUTP at 0 μM (•—•), 0.04 μM (×—×) and 0.08 μM (○—○).

ther 30 min incubation, and the remaining activity of the primer-template was determined. The amount of substrate added (216 μM, 28 × Km) after preincubation prevented the inhibitory effects of analogs on HIV-RT activity. The enzyme activity, expressed as percentage of the activity remaining when enzyme, template and substrate were incubated for the same period of time, was plotted as a func-

TABLE 1

Ki values and IC_{50}s of 5′-triphosphates of 2′-deoxyuridine analogs for HIV-RT and calf thymus DNAPα

Compound[a]	HIV-RT Ki (μM)	DNAPα IC_{50} (μM)
HMdUTP	0.80	1.2
EtdUTP	0.64	3.0
nPrdUTP	0.43	5.0
CEdUTP	1.2	1.8
nPredUTP	0.76	1.8
nPrearaUTP	0.08	0.7

[a]For abbreviations, see Fig. 1.
The Km value for dTTP was 8.3 μM for HIV-RT.

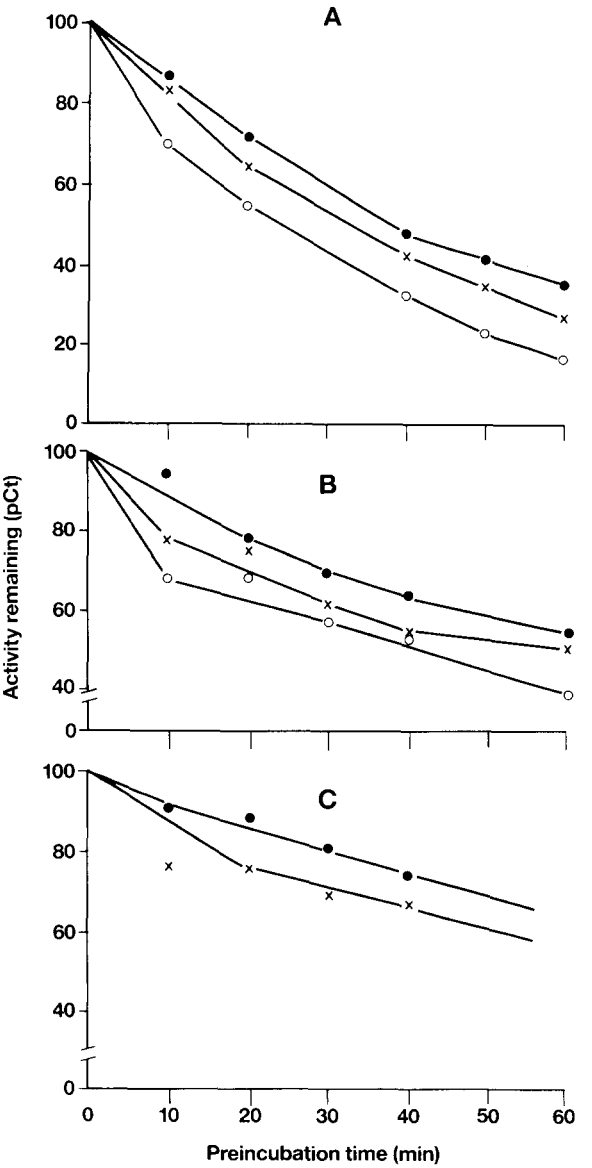


Fig. 3. Effects of nPredUTP (A), FLTTP (B) and AZTTP (C) on template efficiency in HIV-RT catalyzed reaction directed by poly(rA)·$(dT)_{12-18}$. HIV-RT was preincubated for different times with template and various concentrations of nPredUTP (A), FLTTP (B), AZTTP (C), respectively. After preincubation, the enzyme was heat-inactivated and new enzyme plus substrate (dTTP 216 μM) was added for a further 30 min incubation. The enzyme activities were determined and expressed as % of the control. In A: —●—, 4.0 μM (5 × Ki); —×—, 8.0 μM (10 × Ki); —○—, 16.0 μM (20 × Ki). In B: —●—, 0.035 μM (5 × Ki); —×—, 0.07 μM (10 × Ki); —○—, 0.14 μM (20 × Ki). In C: —●—, 0.03 μM (5 × Ki); —×—, 0.12 μM (20 × Ki).

TABLE 2

The effect of nPrearaUTP, nPredUTP, FLTTP and AZTTP on template efficiency in HIV-RT reactions directed by poly(rA)·$(dT)_{12-18}$

Inhibition* conditions	Activity remaining (%) after incubation at 10 × the Ki concentration of			
	nPrearaUTP	nPredUTP	FLTTP	AZTTP
E+I---T+S	100	89	96	76
E+I+T---S	100	52	33	58
E+T---I+S	100	93	85	85

*E, enzyme; I, inhibitor; T, template; S, substrate (dTTP).
HIV-RT was preincubated for 30 min as described for the RT assay in Methods but only with the components shown before the dashed line. After preincubation the reaction mixtures were complemented with the components shown after the dashed line and further incubated for 30 min. The enzyme activities are expressed as % of the activity without inhibitor.

tion of the preincubation time (Fig. 3A). This result shows that nPredUTP caused a time-dependent inactivation of priming activity, which – furthermore – increased with increasing concentration of the analog. The template efficacy was not altered when enzyme and template were preincubated with nPrearaUTP at concentrations up to 10 μM (125 × Ki), whereas with nPredUTP concentrations of 2 × Ki already caused a time-dependent inactivation of the primer (data not shown). As expected, preincubation of RT and template with the chain-terminators FLTTP and AZTTP also caused a time- and inhibitor-concentration dependent inactivation of the primer-template (Fig. 3B, C).

To determine whether the time-dependent inhibition of the polymerisation was solely dependent on altered template-efficacy, RT was preincubated with some ingredients of the reaction mixture and after a 30 min preincubation the reaction mixtures were complemented with the other components to obtain a complete reaction mixture incubated for 30 min. As shown in Table 2, strong inactivation by the triphosphates of nPredU, FLT and AZT occurred only in the combination where the template was preincubated with enzyme and inhibitor.

TABLE 3

Substrate substitution by nPredUTP and nPrearaUTP in HIV-RT reaction directed by activated DNA

Inhibitor	Activity (%), when omitting			
	dTTP	dCTP	dATP	dGTP
None	28	30	29	23
100 μM nPredUTP	69	29	0	0
100 μM nPrearaUTP	24	27	6	3

In incubations where dTTP or dCTP were omitted, [^{3}H]dATP was used as labelled substrate and in incubation where dATP or dGTP were omitted, [^{3}H]dTTP was used as labelled substrate.

nPredUTP is an alternative substrate

It is possible that the decreased template efficacy was caused by incorporation of nPredUMP into the template, whereas nPrearaUTP was not an alternative substrate. This question was addressed by using activated DNA as template, and studying DNA-dependent DNA synthesis by HIV-RT. nPredUTP, but not nPrearaUTP, under these conditions caused a time- and concentration-dependent inactivation of enzyme activity similar to that described for the reverse transcription (results not shown).

We determined whether nPredUTP and nPrearaUTP could function as alternative substrates, by studying their ability to support DNA synthesis when omitting either dTTP, dCTP, dATP or dGTP. When omitting dTTP a low DNA polymerase activity was observed with dATP as the labelled substrate. Addition of nPredUTP (100 μM) partly restored the enzyme activity, but addition of nPrearaUTP did not (Table 3). This indicates that nPredUTP but not nPrearaUTP can substitute for dTTP in the RT-catalysed reaction. When dATP or dGTP was omitted both analogs further decreased the residual incorporation of dTMP from dTTP into DNA.

Discussion

The 5′-triphosphates of several 5-substituted uridine analogs were investigated for their effects on purified recombinant HIV-1 RT and CTDNAPα activities. For HIV-1 RT all the triphosphates in Fig. 1 were competitive inhibitors with dTTP as variable substrate and poly(rA)·$(dT)_{12-18}$ as template, and the triphosphates preferentially inhibited the viral polymerase. Ordering the compounds according to their decreasing binding affinities, as reflected by their increasing inhibition constants (Ki) for HIV-1 RT gave nPrearaUTP > nPrdUTP > EtdUTP > nPredUTP > HMdUTP > CEdUTP. Thus changing the sugar moiety from 2′-deoxyribose to arabinose, as in nPredUTP and nPrearaUTP, resulted in a ten-fold increase in inhibitory activity for HIV-1 RT. The difference for the Ki values of the other 5′-triphosphate analogs are not so large and a distinction between those for HMdUTP, EtdUTP and nPredUTP would require further determinations. It should be noted that the triphosphates of AZT and FLT (St. Clair et al., 1987; Matthes et al., 1985) are considerably more potent than the compounds studied here.

Although nPredUTP was less inhibitory than nPrearaUTP under conditions of competitive inhibition, nPredUTP caused a time- and concentration-dependent decrease of DNA synthesis with both poly(rA)·$(dT)_{12-18}$ and activated CT DNA as templates. This decrease in template-primer activity did not occur with nPrearaUTP but was also shown with chain-terminators FLTTP and AZTTP (Chidgeavadze et al., 1986; St. Clair et al., 1987; Matthes et al., 1985). As nPredUTP, but not nPrearaUTP, is an alternative substrate, as shown by the ability to support DNA synthesis in absence of competing substrate, the incorporation

of nPredUTP into the template-primer apparently leads to increased inhibition of DNA synthesis. Thus, despite the presence of a 3′-OH group, nPredUTP when incorporated into DNA or into the synthetic template decreases the efficiency of the primer-templates. This is at variance with the results obtained by Ruth and Cheng (1981) with HSV-2 DNA polymerase. However, the structural requirements of the inhibitors for the HSV-2 and HIV-1 polymerase are different as well (Ruth and Cheng, 1981).

Taken together, our results indicate that 5′-triphosphates of 5-substituted 2′-deoxyuridine analogs are inhibitors of HIV-RT, and that further studies on this class of compounds to find even more potent inhibitors or enzyme-inactivators should be pursued.

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