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Potent DNA Chain Termination Activity and Selective Inhibition of Human Immunodeficiency Virus Reverse Transcriptase by 2′,3′-Dideoxyuridine-5′-triphosphate

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SUMMARY

2′,3′-Dideoxyuridine (ddUrd) exhibits poor if any anti-human immunodeficiency virus (HIV) activity in ATH8 and MT-4 cells. This is in agreement with the failure of ddUrd to be efficiently anabolized intracellularly to its 5′-triphosphate metabolite. However, 2′,3′-dideoxyuridine-5′-triphosphate (ddUTP) proved to be a potent and selective inhibitor of the reverse transcriptase of HIV (K_i, 0.05 μM) and avian myeloblastosis virus (K_i, 1.0 μM). Bacterial DNA polymerase I, mammalian DNA polymerase α, terminal deoxyribonucleotidyl transferase, and Moloney murine leukemia virus reverse transcriptase were resistant to ddUTP. ddUTP is incorporated into the growing DNA chain principally at dTTP sites and inhibits further elongation. The potential of ddUTP as an anti-HIV therapeutic agent merits further investigation. However, to achieve this goal, it will be necessary to resort to techniques capable of delivering preformed phosphorylated ddUrd to the susceptible cells.

ddNs bearing the naturally occurring purine (adenine, hypoxanthine, guanine) or pyrimidine (thymine, cytosine, uracil) bases exhibit the property of blocking the replication of HIV *in vitro*, although the antiviral activities may vary widely between one ddN and another (1–3). The least effective ddN is ddUrd, which is virtually ineffective in its inhibitory effect against HIV replication (2, 3). Two obvious hypotheses can be proposed to explain the inactivity of ddUrd as an antiretrovirus agent: (i) ddUrd is not metabolized to its 5′-triphosphate derivative ddUTP or (ii) ddUTP is a poor inhibitor of HIV RT. In the present study we have attempted to distinguish between these possibilities. We found that ddUTP does not accumulate to any appreciable extent in human lymphocytes exposed to [^{3}H]ddUrd. However, the 5′-triphosphate of ddUrd proved to be, in fact, a preeminently potent inhibitor of HIV RT and acted as a powerful DNA chain terminator *in vitro*.

Materials and Methods

Chemicals. ddCyd was purchased from United States Biochemicals (Cleveland, OH); ddATP, ddCTP, ddGTP, and ddTTP were purchased from P-L Biochemicals. The corresponding 2′-deoxynucleotides dTTP, dATP, dCTP, and dGTP were obtained from Sigma Chemical Co. (St. Louis, MO).

Radiochemicals. [^{3}H]ddCyd (specific radioactivity, 8.3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). [^{3}H]dCTP (specific radioactivity, 24 Ci/mmol), [^{3}H]dTTP (specific radioactivity, 67 Ci/mmol), and [α-^{35}S]thio-dATP (specific radioactivity, 600 Ci/mmol) were from New England Nuclear (Boston, MA).

Cells. The human T lymphoblastoid cell line MOLT/4 and T lymphocyte cell line CEM were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56°, 30 min) fetal calf serum. The origin and cultivation of ATH8 and MT-4 cells have been described previously (4, 5).

Viruses. HIV (HTLV-III$_B$) was obtained from the culture supernatant of HTLV-III$_B$-producing H9 (H9/HTLV-III$_B$) cells, as previously described by Popovic *et al.* (6).

Enzymes. AMV RT, MMuLV RT, and calf thymus DNA polymerase α were obtained from Pharmacia (Piscataway, NJ). *Escherichia coli* DNA polymerase I and TdT were purchased from Sigma Chemical Co. DNA polymerase I (the Klenow fragment) was from Bethesda Research Laboratories (Gaithersburg, MD). HIV RT was purchased from Bionetics Research Inc.

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ABBREVIATIONS: ddN, 2′,3′-dideoxynucleosides; HIV, human immunodeficiency virus; ddUrd, 2′,3′-dideoxyuridine; ddCyd, 2′,3′-dideoxycytidine; RT, reverse transcriptase; AMV, avian myeloblastosis virus; MMuLV, Moloney murine leukemia virus; ddATP, 2′,3′-dideoxyadenosine-5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine-5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine-5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine-5′-triphosphate; TdT, terminal deoxyribonucleotidyl transferase; DTT, dithiothreitol; D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; AzddThd, 3′-azido-2′,3′-dideoxythymidine.

DNA and template primers. Poly(I)·oligo(dC)$_{12-18}$ was made by annealing the respective 12–18-base oligomer onto the poly(I) chain, as described previously (7). Poly(A)·oligo(dT)$_{15}$ was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Poly(dA-dT), poly(I), and oligo(dC)$_{12-18}$ were obtained from Pharmacia (Piscataway, NJ). M13 mp19 (+)-strand DNA and a 17-base synthetic M13 primer were from Bethesda Research Laboratories.

Synthesis of ddUrd. ddUrd was synthesized by the hydrolytic deamination of ddCyd in aqueous solution (8). Briefly, ddCyd was dissolved in a saturated solution of sodium bisulfite, pH 6.3, and incubated at 37° for 16 hr; the pH was adjusted to 8.9 with a saturated solution of barium hydroxide. After centrifugation at 12,500 rpm, the supernatant was applied to a C18 reverse phase column (ODS) (Beckman, Fullerton, CA) (5 μm, 4.6 mm × 25 cm). A gradient two-buffer system was used to separate ddUrd from ddCyd (buffer A, 0.5% acetic acid; buffer B, methanol; initial conditions, 100% A and 0% B; at 2 min, 90% A and 10% B; at 25 min, 85% A and 25% B). Fractions containing ddUrd were collected and freeze dried. The λ_{max} of ddUrd was 265.1 nm.

Synthesis of ddUTP. 2′,3′-Dideoxyuridine 5′-monophosphate triethylammonium salt (0.156 mmol) was dried by repeated evaporation from anhydrous pyridine. The residue was dissolved in dry dimethylformamide, whereafter 1,1′-carbonyldiimidazole (0.234 mmol) was added. The mixture was stirred for 24 hr at ambient temperature. A solution of tetrakis(tributylammonium)pyrophosphate (0.78 mmol) in dimethylsulfoxide was then added and the reaction mixture was stirred for 24 hr. Water was added and the solution was adjusted to pH 8 with triethylamine. After stirring for 1 hr at ambient temperature, the solution was extracted with ether and the aqueous layer was concentrated and applied to a column (75 × 2.5 cm) of DEAE-cellulose (DE 32, bicarbonate form; Bio-Rad, Richmond, CA). The products were eluted with a linear gradient of water (2.0 liter and 1.0 M triethylammonium bicarbonate (2.0 liter). Fractions containing the triphosphate, which eluted at 0.71–0.74 M, were pooled and evaporated. Residual triethylammonium bicarbonate was removed by successive addition and evaporation of ethanol and the remaining solid was dried over phosphorus pentoxide *in vacuo*. The product was dissolved in ethanol and a 1% solution of sodium iodide in acetone was added. The white precipitate that formed was collected by centrifugation. It was washed twice by suspension in acetone and dried over phosphorus pentoxide *in vacuo* [UV (ethanol): λ_{max} = 262 nm, ϵ = 10,200). High pressure liquid chromatography analysis of the product was performed on an anion-exchange column (Partisil SAX, 4.6 × 25 cm; Whatman Inc., Hillboro, OR), using 0.12 M phosphate buffer, pH 6.6, as eluent at a flow rate of 2.0 ml/min, and gave a single peak with a retention time of 13 min.

Inhibition of cell proliferation. Logarithmically growing MOLT/4 and CEM cells were suspended in RPMI 1640 medium, containing 10% (v/v) fetal calf serum and 2 mM L-glutamine, at a density of 2.5 × 10^5 cells/ml and were grown in 25-cm^2 Costar tissue culture flasks. After 48 hr at 37° in the presence of various concentrations of saline, ddUrd, or ddCyd, the cells were collected and counted in a Coulter counter (Coulter Electronics, Irvine, CA). The IC_{50} was defined as the concentration of drug that reduced cell growth by 50%.

Anti-HIV assay. Anti-HIV assays were performed as previously described (1–3, 9).

Intracellular metabolism of ddUrd and ddCyd. Logarithmically growing MOLT/4, CEM, and ATH8 cells were exposed to [^{3}H]ddUrd and [^{3}H]ddCyd, respectively at a concentration of 0.2 μM. After 24 hr at 37°, the cells were collected and washed twice with phosphate-buffered saline. The cell pellets were extracted with 10% trichloroacetic acid and centrifuged, and the supernatants were neutralized with tri-*n*-octylamine in Freon (1:4, v/v). The samples were applied to a radial compression column of Partisil-10 SAX for high pressure liquid chromatography analysis as previously described (10).

Enzymes assays. DNA polymerase reaction was assayed in a final reaction mixture of 25 μl, which contained 0.1 M Tris·HCl (pH 8.2), 2 mM DTT, 10 mM $MgCl_2$, 50 mM KCl, 1.25 μg of poly(dA)·oligo(dT), 4 μM [^{3}H]dTTP, and 2.5 units of DNA polymerase I or DNA polymerase α. RT assays were performed in two different template-primer systems: (i) poly(A)·(dT_{15}) (1.25 μg) was template-primer when [^{3}H]dTTP (0.7–2.5 μM) was the variable substrate and (ii) poly(I)·oligo (dC_{12-18}) (1.25 μg) served as the template-primer when [^{3}H]dCTP (0.2–4 μM) was the variable substrate; the reaction mixture (25 μl) contained 0.1 M Tris·HCl (pH 8.2), 2 mM DTT, 5 mM $MgCl_2$, 50 mM KCl, and 2.5 units of AMV RT or MMuLV RT or 1.0 unit of HIV RT. After a 60-min incubation at 37°, the reaction was terminated by heating at 95° for 2 min. Reaction products were separated from tritiated precursors by ascending 3 MM paper chromatography in a solvent system that contained ethanol/formic acid/H_2O (550:200:250, v/v), for 15 hr. The locations of polymerized products were determined by UV illumination; in all cases, they remained as sharp spots at the origin. The spots at the origins were excised, and radioactivity was measured as described previously (17).

TdT assay was performed in a 25-μl reaction mixture that contained 250 mM potassium cacodylate (pH 7.2), 8 mM $MgCl_2$, 0.3 mM $ZnSO_4$, 4 μM [^{3}H]dTTP, 1.0 μg of oligo(dT_{12}), and 5 units of TdT; the reactants were incubated at 37° for 60 min. Radioactive products were determined by the paper chromatographic system, as described above.

In the enzyme inhibition studies of DNA polymerase I, DNA polymerase α, RT, and TdT, the IC_{50} represents the inhibitor concentration that reduces enzyme activity by 50%.

The apparent K_m and K_i values were derived from Lineweaver-Burk plots by linear regression analysis.

M13/dideoxy sequence analysis. An M13 mp19 (+)*strand DNA, following annealing with a 17-base M13 universal primer, served as a template for nucleotide sequencing in a Sanger sequencing reaction, using the Klenow fragment of DNA polymerase I, and the RTs of AMV and HIV (11, 12). The reaction mixtures contained 60 mM Tris·HCl (pH 8.3), 75 mM NaCl, 7.5 mM $MgCl_2$, and 0.5 mM DTT.

Termination reaction with DNA polymerase I. A separate termination reaction mixture (6 μl) was prepared for each ddNTP. Each reaction mixture contained 2.5 μl of either termination mix A (200 μM ddATP, 25 μM dATP, and 250 μM dCTP, dGTP, dTTP), termination mix C (200 μM ddCTP, 25 μM dCTP, and 250 μM dGTP, dATP, dTTP), termination mix T (200 μM ddTTP, 25 μM dTTP, and 250 μM dATP, dCTP and dGTP), or termination mix U (250, 500, or 1000 μM ddUTP, 25 μM and dTTP, and 250 μM dATP, dCTP and dGTP) and 3.5 μl of the sequencing buffer containing 20 units of DNA polymerase I, 0.5 μM ^{35}S-labeled dATP, and 0.5 μg of M13 mp19(+) with 2 ng primer. After 5 min at 37°, the reaction was stopped by adding 4 μl of dideoxy stop solution [95% (v/v) deionized formamide, 10 mM Na_2EDTA, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol FF].

Termination reaction with AMV RT and HIV RT. A separate termination mixture (6 μl) was prepared for each ddNTP. Each reaction contained 2.5 μl of either termination mix A (1.26 μM ddATP, 12 μM dATP, and 50 μM dCTP, dGTP, and dTTP), termination mix C (1.26 μM ddCTP, 12 μM dCTP, 2 μM dATP and 50 μM dGTP and dTTP), termination mix G (1.26 μM ddGTP, 12 μM dGTP, 2 μM dATP, and 50 μM dCTP and dGTP), or termination mix U (0.55, 1.26, or 2.5 μM ddUTP, 12 μM dTTP, 2 μM dATP, and 50 μM dCTP and dGTP) and 3.5 μl of the sequencing buffer containing 0.5 μM ^{35}S-labeled dATP, 0.5 μg M13 mp19(+) with 2 ng primer, and 20 units of AMV RT or 1.0 unit of HIV RT. After 5 min at 37°, the reaction was stopped by adding 4 μl of dideoxy stop solution, as described above.

Gel electrophoresis. The termination reactions were heated to 80° for 5 min and subsequently quenched on ice immediately before loading. Samples (2 to 3 μl) of each termination reaction were applied to a 0.4-mm thick standard sequencing polyacrylamide gel [5.7% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 0.1 M Tris·HCl (pH 8.3), 0.09 M boric acid, 1 mM Na_2EDTA, and 7 M urea]. Electrophoresis proceeded for 1.5 hr at 1600 V. The gel was fixed, dried, and exposed to X-ray film at room temperature overnight.

Results

Examination of the antiviral activity of ddUrd. ddUrd was evaluated for its protective effect against HIV-induced cytopathogenicity in ATH8 and MT-4 cells. As shown in Fig. 1, ddUrd did not block the HIV replication at a concentration as high as 100 μM in ATH8 cells. Only at the highest concentration tested (625 μM in MT-4 or 1000 μM in ATH8 cells), ddUrd proved markedly inhibitory against HIV replication. However, at the latter concentrations, cell viability was markedly affected (Fig. 1). Chu and co-workers (13) and Baba *et al.* (14) found ddUrd effective at 96.8 and 48 μM in HIV-infected PBM and MT-4 cells, respectively. Thus, the anti-HIV activity of ddUrd depends, to some extent, on the type of cell line and/or the multiplicity of the viral infection. In contrast to ddUrd, ddCyd proved to be very effective in inhibiting HIV replication in ATH8 and MT-4 cells. Its ED_{50} (50% effective dose) was as low as 0.3–0.5 μM in ATH8 and MT-4 cells (1–3). Unlike ddUrd, whose IC_{50} values for MOLT/4, CEM, ATH8, and MT-4 cell proliferation were equal to or higher than 1000 μM, ddCyd reduced cell viability at markedly lower concentrations, depending on the cell line evaluated (IC_{50} for MOLT/4, CEM, ATH8, and MT-4 cells, 2.5, 5.0, 50, and 35 μM, respectively).

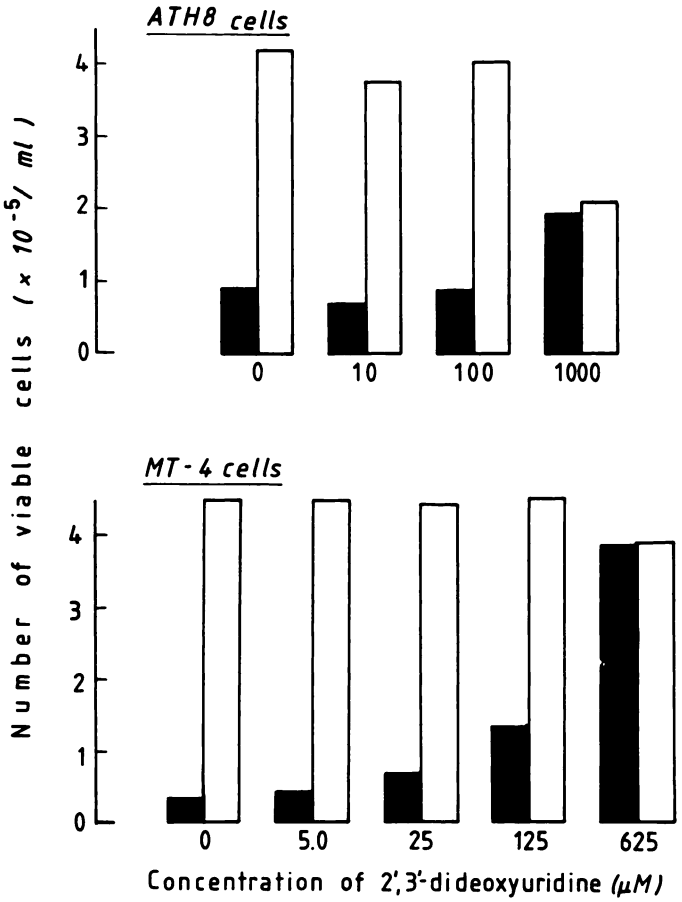

Fig. 1. Inhibition of the cytopathic effect of HIV by ddUrd in ATH8 and MT-4 cells. ATH8 cells (2×10^5) were preexposed to ddUrd (10, 100, or 1000 μM) for 24 hr, in RPMI 1640 medium containing 15% heat-inactivated fetal calf serum, 4 mM L-glutamine, 25% (v/v) lectin-depleted interleukin 2, 1% penicillin/streptomycin (v/v), and 20 units of human recombinant interleukin 2. Subsequently the cells were infected with HIV ($HTLV\text{-}III_B$) at 3000 viral particles/cell. Mock-infected controls were treated similarly. HIV-1- or mock-infected MT-4 cells were seeded in microtiter plates (200- μl wells) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 0.075% (w/v) sodium bicarbonate, and in the presence of the appropriate concentrations of test compound. On day 7 after infection for ATH8 cells or day 5 after infection for MT-4 cells, total viable cells were counted by trypan blue dye exclusion. □, Cell cultures treated with ddUrd only; ■, cell cultures treated with ddUrd 24 hr before infection with virus. The ddUrd source in the MT-4 cell experiment was derived through another procedure for chemical synthesis as published earlier (21). According to this procedure, 2'-deoxyuridine was used as the starting material. Therefore, this ddUrd preparation could not contain any ddCyd.

TABLE 1

Intracellular phosphorylation of 0.2 μM ddUrd and 0.2 μM ddCyd in human lymphocytes

Cell line	Phosphorylated metabolites					
	ddUMP	ddUDP	ddUTP	ddCMP	ddCDP	ddCTP
	nmol/10⁹ cells					
ATH8	0.002	0.004	0.002	2.10[a]	1.20[a]	0.50[a]
MOLT/4	0.005	0.005	0.002	0.082	0.082	0.25
CEM	0.004	0.004	0.004	0.73	0.75	0.18

[a] Data obtained in ATH8 cells at an initial concentration of 1 μM [^{3}H]ddCyd instead of 0.2 μM (see Ref. 15).

TABLE 2

Inhibitory effect of ddUTP against HIV, AMV, and MMuLV RTs

IC_{50} represents the ddUTP concentration at which RT activity was inhibited by 50% under the standard assay conditions. Values are mean ± standard error.

Template-primer	Substance	IC_{50}		
		HIV RT	AMV RT	MMuLV RT
		μM		
Poly(A)·oligo$(dT)_{15}$	dTTP	0.035 ± 0.002	0.85 ± 0.04	>20
Poly(I)·oligo$(dC)_{12-18}$	dCTP	1.0 ± 0.5	20 ± 1.10	>200

Intracellular metabolism of ddUrd. The intracellular phosphorylation of ddUrd was examined in MOLT/4, CEM, and ATH8 cells and compared with the metabolism of ddCyd (Table 1). ddCyd is well phosphorylated in ATH8, MOLT/4, and CEM cells. Levels of all three 5'-phosphorylated metabolites (i.e., ddCMP, ddCDP, and ddCTP) were easily detectable. In addition, the ddCDP-choline adduct, first reported in ATH8 cells by Cooney *et al.* (9), was present in the three cell lines studied. Intracellular levels of ddCTP in MOLT/4 and CEM cells amounted up to 0.18 to 0.25 pmol/10^9 cells (Table 1). The slightly higher ddCTP levels in ATH8 cells (0.50 nmol/10^9 cells) were overestimated, because the initial concentration of ddCyd in the ATH8 cell cultures was 5-fold higher (1 μM) than the initial ddCyd concentration in MOLT/4 and CEM cells. Moreover, Balzarini and co-workers (16) have shown that, for ddCyd doses between 0.2 and 10 μM, there is an almost linear relationship between the initial ddCyd dose and the eventual level of the corresponding 5'-triphosphate. Therefore, ddCTP levels in ATH8 cells should have been around 0.10 nmol/10^9 cells when an initial ddCyd dose of 0.2 μM was used. In contrast, poor if any metabolism (phosphorylation) of [^{3}H]ddUrd occurred. The intracellular levels were close to the detection limit. In fact, only the unphosphorylated nucleoside ddUrd was significantly present in the cell extracts, indicating that the nucleoside was taken up by the MOLT/4 cells.

Inhibition of RT by ddUTP. The inhibitory effects of ddUTP on the activities of AMV, MMuLV, and HIV RTs were compared in the two template-primer systems poly(A)·oligo$(dT)_{15}$ and poly(I)·oligo$(dC)_{12-18}$. Table 2 documents that the IC_{50} values of ddUTP in the template-primer poly(A)·oligo(dT_{15}) system varied markedly from one enzyme source to

TABLE 3

Inhibitory effect of ddUTP on DNA polymerase α, DNA polymerase I, and TdT

IC_{50} represents the inhibitory concentration at which enzyme activity was inhibited by 50% under our standard assay conditions. Values are mean ± standard error.

Enzyme	Template-primer	IC_{50}
		μM
DNA polymerase I	Poly(I)·oligo$(dT)_{12-18}$	>200
DNA polymerase α	Poly(I)·oligo$(dT)_{12-18}$	>200
TdT	Oligo(dT_{12})	27 ± 1.5

another. With an IC_{50} of 0.035 μM, ddUTP was an exceedingly potent and selective inhibitor of HIV. AMV RT was also markedly inhibited by ddUTP (IC_{50}, 0.85 μM) whereas MMuLV was virtually insensitive to the inhibitory effects of ddUTP (IC_{50}, >20 μM). In the template-primer poly(I)·oligo$(dC)_{12-18}$ system, the same activity spectrum among the different RTs was observed as in the poly(A)·oligo$(dT)_{15}$ system, but the IC_{50} values were markedly higher (Table 2).

Inhibition of DNA polymerase α, DNA polymerase I, and TdT by ddUTP. The effect of ddUTP on DNA polymerase α and DNA polymerase I was examined in the poly(A)·oligo$(dT)_{15}$ system and TdT was examined in the presence of oligo(dT_{12}). As evident from Table 3, both DNA polymerase I and DNA polymerase α were resistant to the inhibitory effect of ddUTP (IC_{50} values, >200 μM). TdT was modestly sensitive to inhibition by ddUTP (IC_{50}, 27 μM) (Table 3).

Kinetics of inhibition of RT by ddUTP. In order to clarify the nature of the inhibition of RTs by ddUTP, the HIV, AMV, and MMuLV RTs were subjected to a kinetic analysis in the poly(A)·oligo$(dT)_{15}$ system, at three different concentrations of ddUTP calculated to bracket the IC_{50} values of the compound. The experimental data were plotted by the method of Lineweaver and Burk and the K_i and K_m values are shown in Fig. 2 and Table 4. In all cases, the type of inhibition appeared to be competitive with respect to dTTP as the radiolabeled substrate. The K_m values of dTTP for the RTs of HIV, AMV, and MMuLV were within a narrow range (2.4, 4.6, and 5.4 μM, respectively). However, the inhibitory effect of ddUTP against HIV, AMV, and MMuLV RT (as reflected by their respective K_i values) differed markedly from one enzyme source to another. In fact, HIV RT was the most sensitive to the inhibitory effects of ddUTP (K_i, 0.05 μM). MMuLV RT was 400-fold less sensitive than HIV RT to ddUTP inhibition, whereas AMV RT affinity for ddUTP ranked in between. These data are in agreement with the IC_{50} values found for ddUTP against the different RTs (Table 1).

DNA chain termination by ddUTP. Inasmuch as ddUTP lacks a 3′-hydroxyl group, once it is incorporated into DNA it will prevent further chain elongation. Chain termination by ddUTP was tested using the sequencing procedure of Sanger *et al.* (17). DNA polymerase I (Klenow fragment), AMV RT, and HIV RT were used to extend a DNA primer that had been annealed to the M13 mp19 (+)-strand DNA. Synthesis was performed in the presence of ^{35}SdATP so that the newly formed DNA chain could be visualized by autoradiography after polyacrylamide gel electrophoresis. A comparison of the ddTTP and ddUTP lanes (Fig. 3) showed radiolabeled bands in identical positions, suggesting that incorporation of ddUTP into newly synthesized DNA strands resulted in chain termination at dTTP sites. The dose dependency of the chain termination (Fig. 3, *channels b* and *e*) suggested that a competitive mechanism (ddUTP versus dTTP) might be involved. When ddUTP and ddTTP were compared at an equimolar concentration (0.55 μM) for their chain-terminating effects against AMV RT and HIV RT, it was found that ddTTP reproducibly resulted in more intensively labeled bands than ddUTP in the AMV RT system. In contrast, ddUTP produced darker bands than ddTTP in the HIV RT system, suggesting a preferential chain-terminating effect of ddUTP against HIV RT. This result (Fig. 3, *channels c* and *f*) is in agreement with the kinetic constants of ddUTP versus HIV RT and AMV RT. The effect of ddUTP on chain termination using DNA polymerase I as the polymerizing enzyme was also evaluated (Fig. 4). Interestingly, ddUTP also induced mispairing at dG positions. Therefore, ddUTP seems to cause double chain-terminating effects, in that it is able to terminate the DNA chain both at a cytosine and a thymine residue (thus, at the site of a corresponding guanine and adenine base). These double chain-terminating effects were observed when not only ddUTP but also ddTTP was evaluated at high concentrations (Fig. 4). Thus, our data suggest that ddUTP under some experimental conditions might act as a dual-chain terminator.

Discussion

ddUrd is the only ddN containing a physiological base to exhibit poor if any inhibitory activity against HIV replication

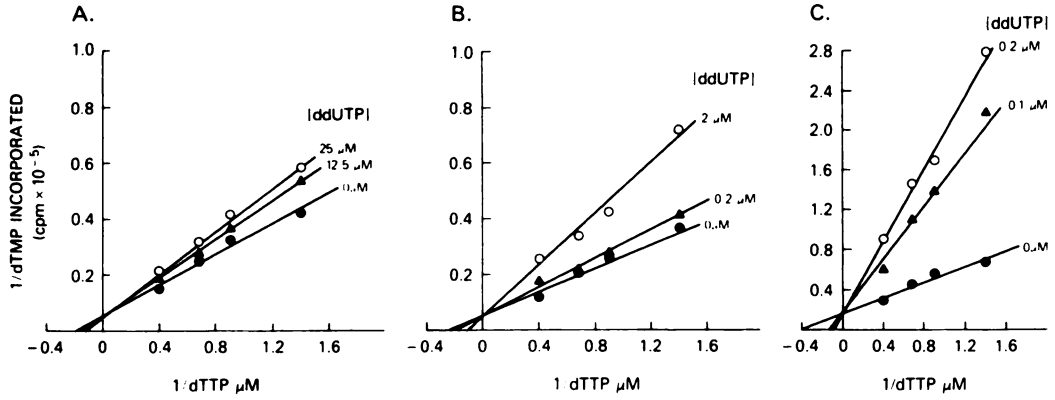

Fig. 2. Double-reciprocal plots of the inhibition by ddUTP of MMuLV RT (A), AMV RT (B), or HIV RT (C). ●, Control (without ddUTP); ○, in the presence of 25, 2, or 0.2 μM ddUTP for MMuLV, AMV, or HIV RT, respectively; ▲, in the presence of 12.5, 0.2, or 0.1 μM ddUTP for MMuLV, AMV, or HIV RT, respectively.

TABLE 4

Kinetic constants for AMV, MMuLV, and HIV RTs

Assays were performed as described in Materials and Methods. K_m and K_i values were determined from replots of the slopes of the Lineweaver-Burk regression lines. Values are the average of six independent determinations ± standard error.

Enzyme	Template-primer	Substrate	Inhibitor	Kinetic parameters		
				K_i	K_m	K_i/K_m
				μM	μM	
AMV RT	Poly(A)·oligo(dT)$_{15}$	dTTP	ddUTP	1.0 ± 0.05	4.6 ± 0.28	0.65
MMuLV RT	Poly(A)·oligo(dT)$_{15}$	dTTP	ddUTP	20.8 ± 0.46	5.4 ± 0.20	3.85
HIV RT	Poly(A)·oligo(dT)$_{15}$	dTTP	ddUTP	0.05 ± 0.02	2.4 ± 0.24	0.02

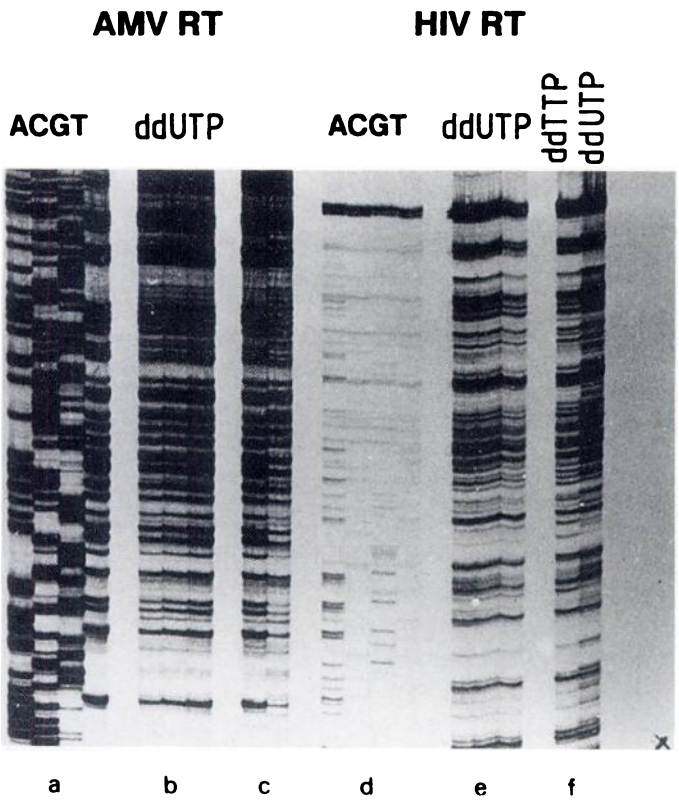

Fig. 3. Autoradiography of a 6% polyacrylamide/-7 M urea DNA sequencing gel. Chain termination assay reactions were performed with HIV RT or AMV RT as described in Materials and Methods. In *channel a* and *d*, *lanes A*, *C*, *G*, and *T* were performed with 1.26 μM ddATP, ddCTP, ddGTP, or ddTTP, respectively. In *channels b* and *e*, *lanes ddUTP* were performed with 0.55, 1.26, or 2.5 μM ddUTP, respectively (from *left* to *right*). In *channels c* and *f*, *lanes ddTTP* and *ddUTP* were performed with an equal concentration of ddTTP or ddUTP (0.55 μM).

in ATH8 and MT-4 cells (2, 3). Even when dialyzed serum has been used in the antiviral experiment (to avoid interference of thymidine that might be present in the serum), the anti-HIV activity of ddUrd was not significantly different (data not shown). In the present study, we have sought to investigate the molecular basis for this inactivity. Our results point conclusively to a failure to accumulate significant intracellular levels of ddUTP adequate to effectively inhibit the retroviral RT. This failure might conceivably stem from two causes, (i) a defect in anabolism of ddUrd to its corresponding 5′-triphosphate derivative or (ii) extensive enzymatic decomposition of any ddUTP formed to its corresponding unphosphorylated ddN. Some precedent for the latter possibility exists in the literature, because it is known that dUTP is subject to rapid and highly efficient intracellular hydrolysis. 2′-Deoxyuridine triphosphate hydrolase (dUTPase) purified from human lymphoid cells has been reported to act on ara-UTP, 6-aza-dUTP, 2′-fluoro-dUTP, and ddUTP (18). This catabolic step is most likely adopted by the cells to avoid extensive (mis)incorporation of this pyrimidine nucleotide into cell DNA (19). Although the potential action of UTPase on ddUTP levels in the cells has not been directly addressed in our study, it seems unlikely that this catabolic reaction is the main cause of the lack of any marked intracellular ddUTP pools. Indeed, if dUTPase should act on any hypothetic ddUTP formed in the lymphocyte cell lines studied, one might expect to find detectable intracellular levels of ddUMP. However, only the unphosphorylated ddN could be recovered from the cellular extracts to a marked extent. The presence of 5′-nucleotidase activity that should specifically act on ddUMP seems unlikely, because intracellular phosphorylation experiments on ddCyd, 3′-azido, 2′,3′-dideoxythymidine, and D4T do not suggest any unusual high or specific 5′-nucleotidase activity acting against the 5′-monophosphate derivatives of these ddN analogues. Thus, our findings argue against dUTPase as the cause of the lack of detectable ddUTP levels and also suggest that ddUrd is well taken up by the lymphocytes. Most likely, our findings on the lack of appreciable levels of 5′-mono-, 5′-di-, and 5′-triphosphate metabolites of ddUrd strongly suggest a highly efficient phosphorylation blockage of ddUrd at the nucleoside level (i.e., thymidine kinase). This hypothesis is in agreement with the recent findings of Balzarini *et al.* (3) that ddUrd lacks any appreciable substrate affinity for MOLT/4 and MT-4 thymidine kinase. Thus, in contrast to AzddThd, 2′,3′-dideoxythymidine, and ddCyd that were good to moderate substrates for the nucleoside kinases responsible for their initial activation (phosphorylation) (3, 16, 20), ddUrd seems not to be converted to its 5′-monophosphate derivative in the lymphocyte cells.

Although ddUrd is virtually inactive as an antiretroviral agent because of the lack of any marked anabolism of ddUrd, it is of remarkable interest that its 5′-triphosphate derivative proved to be an outstandingly potent inhibitor of HIV RT with poly(A)·oligo (dT)$_{15}$ as the template-primer and dTTP as the variable substrate. The avian and murine RTs were markedly less sensitive, which might reflect conformational differences in the substrate binding sites of these proteins. With poly(I)·oligo(dC)$_{12-18}$ as the template-primer and dCTP as the variable substrate, the IC_{50} of ddUTP was sharply (25 to 30-fold) increased (Table 2). This disparity makes it likely that ddUTP preferentially competes with dTTP, compared with dCTP. Based on these findings, we wondered whether ddUTP was also able to result in DNA chain termination with the RTs as the polymerizing enzymes. We found that ddUTP can indeed serve as a substrate for AMV RT, HIV RT, and DNA polymerase I (Klenow fragment) and that ddUTP should be considered as a specific and powerful DNA chain terminator. In this respect,

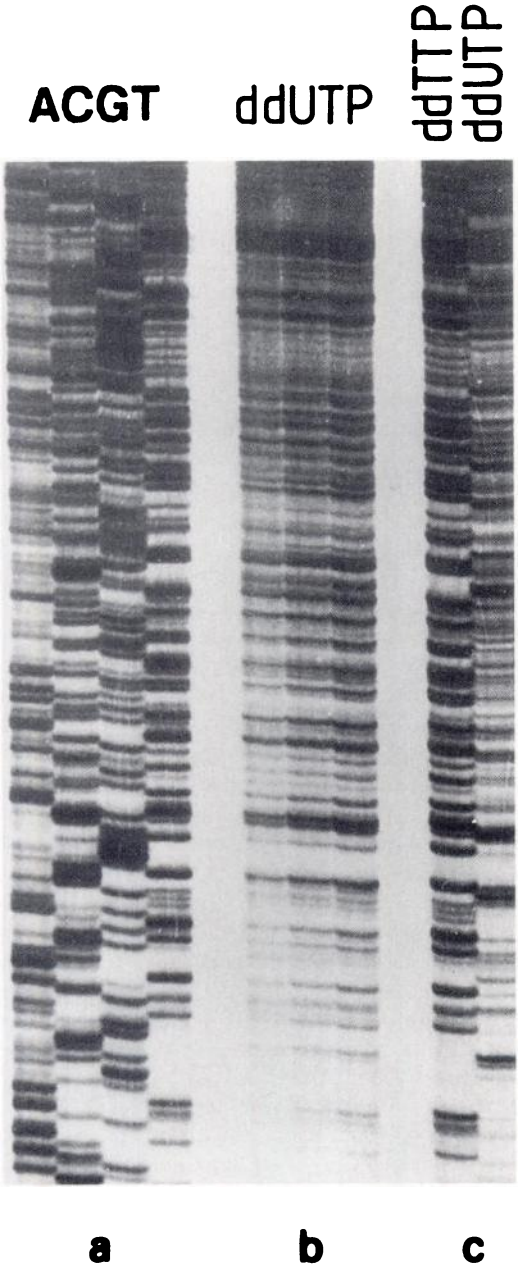

Fig. 4. Autoradiography of a 6% polyacrylamide/7 M urea DNA sequencing gel. Chain termination assay reactions were performed with DNA polymerase I (Klenow fragment) as described in Materials and Methods. In *channel a*, *lanes A*, *C*, *G*, and *T* were performed with 200 μM ddATP, ddCTP, ddGTP, or ddTTP, respectively. In *channel b*, the *ddUTP lanes* were performed with 100, 200, or 400 μM ddUTP (from *left* to *right*). *Channel c* was performed with equimolar concentrations of ddTTP or ddUTP (200 μM).

HIV RT was exceedingly sensitive to ddUTP. Inasmuch as the chain-terminating activity of a 2′,3′-dideoxynucleotide is a function of the affinity of a given DNA polymerase for this 2′,3′-dideoxynucleotide, the 3′-5′-exonucleotidase activity of the DNA polymerase (which can serve to excise the chain terminator), and the intrinsic processivity of the enzymes (which might determine the distribution of the length of the chain-terminating species), the differences in the chain-terminating potencies of ddTTP and ddUTP with HIV RT as the DNA-polymerizing enzyme might suggest therapeutic importance if ddUTP could be accumulated *in vivo*.

Because ddUrd is almost inert in its affinity for thymidine kinase and thus lacks any appreciable anti-HIV activity in cell culture, it would appear that the results recapitulated in the present paper would have only theoretical interest. However, if a technique could be devised to introduce preformed ddUTP into cells, as for example, through the aid of liposomes or prodrugs of ddUTP that facilitate penetration of ddUTP through the cellular membrane, it might be possible to capitalize in a pharmacologically meaningful way on the remarkably high potency of this nucleotide as a specific inhibitor of HIV RT and powerful DNA chain terminator. Therefore, attempts should be undertaken to synthesize novel alkyl or acetyl esters of ddUTP in order to decrease the polarity of the compound and facilitate its cellular uptake or to synthesize phosphonate derivatives of ddUTP in order to make the 5′-triphosphate of ddUrd resistant to enzymatic hydrolysis and to give it the opportunity to reach its target enzyme in the intact form. In this respect, it is relevant to reiterate that ddUTP was devoid of any inhibitory activity toward DNA polymerase α, the main replicative DNA polymerase of mammalian cells, which may stress the possible lack of cellular toxicity if ddUTP could be generated intracellularly at relatively high levels. It is unknown, however, whether ddUTP inhibits the DNA polymerases β or γ. If such inhibition occurs, it might result in an increased cell toxicity if ddUTP should be directly delivered into the virus-infected cells. However, higher intracellular ddUTP levels should not necessarily result in a higher cellular toxicity of the compound. Indeed, as has been shown by Balzarini *et al.* (15), much higher 3′-azido-2′,3′-dideoxythymidine-5′-triphosphate levels were present in murine L1210 cells than in human ATH8 cells, whereas the nucleoside analogue AzddThd was far less cytotoxic to L1210 than ATH8 cells. Moreover, although a rather close correlation was found between intracellular levels of the 5′-triphosphates of AzddThd and D4T and their antiretroviral effects, no such correlation was observed between the levels of the 5′-triphosphates of AzddThd and D4T and their cytotoxic effects (15, 22). Thus, it is worth considering the direct delivery of ddUTP into HIV-infected cells in order to increase the antiretroviral efficacy of ddUrd.

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