Differential Patterns of Intracellular Metabolism of 2′,3′-Didehydro-2′,3′-dideoxythymidine and 3′-Azido-2′,3′-dideoxythymidine, Two Potent Anti-human Immunodeficiency Virus Compounds*

Jan Balzarini, Piet Herdewijn, and Erik De Clercq

From the Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

3′-Azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) are potent and selective inhibitors of human immunodeficiency virus replication in MT-4 and ATH8 cells. They are also inhibitory to the replication of murine retroviruses, *i.e.* Moloney murine sarcoma virus-induced transformation of C3H cells. In MT-4 cells AZT is readily phosphorylated to its 5′-monophosphate, while the 5′-di- and 5′-triphosphates are generated to a 200–600-fold lower extent than the 5′-monophosphate. D4T is phosphorylated in MT-4 cells to its 5′-monophosphate at a 300–600-fold lower extent than AZT. The phosphorylation of AZT in the thymidine kinase-deficient cell line (Raji/TK⁻) is severely depressed, while D4T phosphorylation is only slightly diminished in Raji/TK⁻ as compared to Raji/0 cells. D4T has a 10-fold lower affinity for phosphorylation by crude MT-4 cell extracts than AZT (K_m, 142 and 14 μM, respectively), and the V_{max} for phosphorylation of D4T is only 5% that of AZT. D4T is phosphorylated by MT-4 cell extracts about 180-fold less efficiently than AZT (V_{max}/K_m, 0.06 for D4T, as compared to 11 for AZT), and this is consistent with the differences found in the amounts of phosphorylated products of D4T and AZT formed in intact MT-4 cells. The 5′-triphosphates of AZT and D4T are equipotent in their inhibitory effects on the reverse transcriptases from human immunodeficiency virus and Moloney murine leukemia virus.

Several pyrimidine 2′,3′-dideoxynucleoside analogues (ddNs)[1] (*i.e.* 2′,3′-dideoxycytidine (ddCyd, D2C) (1, 2), 2′,3′-didehydro-2′,3′-dideoxycytidine (ddeCyd, ddddCyd, D4C) (2–4), 3′-azido-2′,3′-dideoxythymidine (AzddThd, AZT) (5–8), 2′,3′-didehydro-2′,3′-dideoxythymidine (ddeThd, ddddThd, D4T) (3, 9, 10), 3′-azido-2′,3′-dideoxyuridine (AzddUrd) (11, 12), 3′-fluoro-2′,3′-dideoxythymidine (FddThd) (6, 12–14), and 3′-fluoro-2′,3′-dideoxyuridine (FddUrd) (12)) have been recognized as potent and selective inhibitors of the replication of human immunodeficiency virus (HIV), the etiologic agent of the acquired immunodeficiency syndrome (AIDS). The 2′,3′-dideoxyribonucleoside analogues are assumed to be targeted at the virus-encoded RNA-directed DNA polymerase (reverse transcriptase). To exert their antiretroviral activity, the ddNs should be phosphorylated intracellularly to their 5′-triphosphate (ddNTP) metabolites. The phosphorylation steps are catalyzed by cellular enzymes, as has been shown for AZT in H9, ATH8, and Molt/4F cells (7, 15, 16), for ddCyd in ATH8 and Molt/4F cells (16–19), and for FddThd in Ehrlich ascites carcinoma, MT-4, CEM, and H9 cells (13, 14, 20). The nucleoside kinases (*i.e.* dThd kinase and dCyd kinase) convert the ddNs (*i.e.* AZT and ddCyd, respectively) to their 5′-monophosphates. However, the affinity of the ddNs for their respective nucleoside kinases may differ considerably from one compound to another (*i.e.* K_m value of AZT for H9 cell dThd kinase, 3 μM (15); K_m value of FddThd for Ehrlich ascites carcinoma cell dThd kinase, 11.2 μM (20); K_m value of ddCyd for Molt/4F cell and KB cell dCyd kinase, 200 and 220 μM, respectively (7, 19)) and determines the eventual intracellular levels of the ddNTPs.

In an attempt to gain a better insight in the metabolism and mechanism of antiretrovirus action of the ddNs, we investigated the intracellular phosphorylation of two structurally related and potent anti-HIV compounds (AZT and D4T) in MT-4 cells. This cell line is exquisitely sensitive to the inhibitory effects of 2′,3′-dideoxythymidine analogues (*i.e.* AZT and D4T) on HIV replication and would therefore seem useful to investigate in parallel the intracellular metabolism of AZT and D4T. D4T has not previously been studied for its metabolic fate within the cell.

[1] The abbreviations used are: ddNs, pyrimidine 2′,3′-dideoxynucleoside analogues; ddCyd, D2C, 2′,3′-dideoxycytidine; ddeCyd, ddddCyd, D4C, 2′,3′-didehydro-2′,3′-dideoxycytidine; AzddThd, AZT, 3′-azido-2′,3′-dideoxythymidine; ddeThd, ddddThd, D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; AzddUrd, 3′-azido-2′,3′-dideoxyuridine; FddThd, 3′-fluoro-2′,3′-dideoxythymidine; FddUrd, 3′-fluoro-2′,3′-dideoxyuridine; HIV, human immunodeficiency virus; MSV, Moloney murine sarcoma virus; MLV, Moloney murine leukemia virus; HPLC, high performance liquid chromatography; AZT-TP, AZT 5′-triphosphate; AZT-TP, AZT 5′-triphosphate; ddThd, 2′,3′-dideoxythymidine; AZT-MP, AZT 5′-monophosphate; AZT-DP, AZT 5′-diphosphate; D4T-MP, D4T 5′-monophosphate; D4T-DP, D4T 5′-diphosphate.

MATERIALS AND METHODS

Cells—The origin and cultivation of MT-4, ATH8, and C3H cells have been described previously (21–23). MT-4 cells were a gift from Dr. N. Yamamoto (Yamaguchi University, Yamaguchi, Japan). Both MT-4 and ATH8 cells represent immortalized helper/inducer T cell clones, obtained by cloning a normal T4 cell line in the presence of tumor cells derived from a patient with adult T cell leukemia or lethally irradiated HTLV-I-producing MJ tumor cells, respectively. C3H cells represent a continuously growing murine embryo fibroblast cell line. Cultivation and characterization of the Raji/0 cell line and its dThd kinase-deficient derivative (Raji/TK⁻) has been described previously (24).

Viruses—Human immunodeficiency virus (HIV) was obtained from the culture supernatant of H9 cells persistently infected with

HTLV-III$_B$ (derived from a pool of American patients with AIDS) and kindly provided by Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD) (25). Moloney murine sarcoma virus (MSV) was prepared from tumors obtained in 3-day-old NMRI mice that were inoculated intramuscularly with the virus (26). Moloney murine leukemia virus (MLV) was from Electronucleonics (Bethesda, MD).

Compounds—3'-Azido-2',3'-dideoxythymidine (AZT) and 2',3'-didehydro-2',3'-dideoxythymidine (D4T) were synthesized according to previously published methods (6). Their formulae are shown in Fig. 1. The 5'-triphosphate derivative of AzddThd was synthesized as described earlier (27, 28). The 5'-triphosphate of D4T was prepared following essentially the same synthetic procedure. The other reagents used were of the highest quality available.

Radiochemicals—[methyl-^{3}H]AZT (specific radioactivity, 10 Ci/mmol) and [methyl-^{3}H]D4T (specific radioactivity, 20 Ci/mmol) were obtained from Moravek Biochemicals Inc., Brea, CA. [methyl-^{3}H]dThd (specific radioactivity, 40 Ci/mmol) was from the Radiochemical Centre Amersham (Amersham, United Kingdom).

Antiretroviral Assays—The procedures for measuring anti-HIV activity in ATH8 and MT-4 cells and anti-MSV activity in C3H cells have been described previously (2, 3, 22, 29, 30).

Cytostatic Assays—Cytostatic effects of the compounds were assessed by measuring inhibition of cell proliferation. The experimental procedures have been described previously (3, 12). Briefly, exponentially growing MT-4, CEM, and H9 cells were seeded in 200-μl microplate wells and incubated in the presence of varying concentrations of the test compound (50,000 cells/well). After an incubation period of 3, 4, and 5 days the cell number was determined in a Coulter counter (12). ATH8 cells were seeded at 10^5 cells/culture tube (2 ml/tube) in the presence of the test compound, and after 7 days of incubation the number of living cells was determined in a blood cell counting chamber using the trypan blue dye exclusion technique (3).

Metabolism of [methyl-^{3}H]AZT and [methyl-^{3}H]D4T in Human MT-4 and Raji Cells—The metabolism of the radiolabeled compounds was monitored according to previously established procedures (16, 17). Briefly, the MT-4 cells were seeded at 2-4 × 10^6 cells/ml and incubated with varying concentrations of the radiolabeled compounds (see footnote to Table III). At different time intervals (see footnote to Table IV), cells were centrifuged, washed, and precipitated with cold trichloroacetic acid (10%). After centrifugation, the supernatants were neutralized with tri-*n*-octylamine in Freon. HPLC analysis of the neutralized cell extracts was carried out using a Partisil-SAX radial compression column. A linear gradient of 0.007 M ammonium dihydrogen phosphate (pH 3.80) to 0.25 M ammonium dihydrogen phosphate + 0.50 M KCl (pH 4.5) was used. The different fractions of the eluate were assayed for radioactivity in a toluene-based scintillant.

Phosphorylation of [methyl-^{3}H]D4T and [methyl-^{3}H]AZT was also examined in Raji/0 cells and its dThd kinase-deficient Raji/TK$^-$ counterpart, and H9 cells. The procedure was essentially the same as that described for the MT-4 cells.

Effect of AZT and D4T on the Intracellular Radiolabeled [methyl-^{3}H]dTMP, [methyl-^{3}H]dTDP, and [methyl-^{3}H]dTTP Pools in MT-4 Cells—MT-4 cells were seeded at 400,000 cells/ml in 5-ml tissue culture bottles for 24 h in the presence of different concentrations of AZT or D4T (0, 50, 500 μM). Then, 0.04 μM [methyl-^{3}H]dThd (10 μCi/5-ml tissue culture bottle) was added to the cell cultures, and after 2 h, the trichloroacetic acid-soluble intracellular material was analyzed by thin layer chromatography in a mixture of isopropyl alcohol:NH$_3$:H$_2$O (6:3:1) and by HPLC using the same separation method as described above.

Enzyme Assays—Cell extracts containing dThd kinase were pre-pared from exponentially growing MT-4 cells, which were first washed twice with phosphate-buffered saline at 4 °C and then homogenized by sonication. The suspensions were clarified by centrifugation at 100,000 × g for 60 min. In the enzyme experiments, [methyl-^{3}H]dThd, [methyl-^{3}H]AZT, or [methyl-^{3}H]D4T served as the radiolabeled substrates. Substrate concentrations were as follows: for [methyl-^{3}H]dThd, 5, 10, 25, and 50 μM; for [methyl-^{3}H]AZT, 2, 5, 10, 20, and 40 μM; and for [methyl-^{3}H]D4T, 20, 40, 100, 200, and 250 μM. The K_m and V_{max} values for the radiolabeled substrates were derived from Lineweaver-Burk plots, using a linear regression analysis program. In the competition assays, [methyl-^{3}H]AZT and [methyl-^{3}H] D4T were incubated in the presence of 10 μM unlabeled dThd or dTTP. The other experimental conditions used for the dThd kinase assay were as previously described (31).

Inhibition of the HIV- or MLV-associated reverse transcriptase by the 5'-triphosphates of AZT (AZT-TP) and D4T (D4T-TP) was determined as previously described (27). Inhibition of reverse transcriptase by the test compounds was estimated during the period that the reverse transcriptase activity increased linearly. In the MLV reverse transcriptase experiments, the endogenous viral RNA served as the template. In the HIV reverse transcriptase assays, exogenous poly(A)·oligo(dT)$_{12-18}$ served as the template. DNA polymerase α was obtained from calf thymus (Pharmacia, Uppsala, Sweden). The reaction mixture (40 μl) contained 4 μl of activated DNA (0.5 mg/ml), 4 μl of bovine serum albumin (5 mg/ml), 2 μl of a mixture of dGTP, dCTP, and dATP, 2 mM), 4 μl of [methyl-^{3}H]dTTP (4 μCi; 30 μM), 6 μl of distilled water, 4 μl of a mixture containing 200 mM Tris-Cl (pH 7.5), 30 mM MgCl$_2$, and 2 mM dithiothreitol, 8 μl of the appropriate inhibitor concentrations (final concentrations: 500, 100, 20, 4 μM) and 12 μl of enzyme (0.024 unit).

Determination of the Lipophilicity of Test Compounds—To estimate the lipid solubility of 2'-deoxythymidine (dThd), 2',3'-dideoxythymidine (ddThd), D4T, and AZT, the partition of the compounds between 1-octanol and 10 mM potassium phosphate buffer, pH 7.4, was measured as previously described (30). Briefly, a 50 μM concentration of the test compound in 10 mM potassium phosphate buffer was thoroughly mixed with an equal volume of 1-octanol for 30 min. Then, the mixture was further equilibrated at room temperature for 60 min, UV absorption was measured for the aqueous and alcoholic liquid phases, and the percentage of the test compound present in each liquid phase was calculated based on their absorption maxima. Alternatively, the R_F values of dThd, ddThd, D4T, and AZT were determined by thin layer chromatography on Silica Gel M5735 with a mixture of chloroform:methanol (92.5:7.5).

Tumor Formation in NMRI Mice Inoculated Intramuscularly with Moloney Murine Sarcoma Virus (MSV)—NMRI mice were inoculated at 2 days after birth with MSV (day 0). AZT and D4T were administered daily by the intraperitoneal route at a dose of 125 or 25 mg/kg/day, starting 2 h before virus inoculation (day 0) and continued until day 5. Tumor appearance and mortality associated herewith were recorded daily. There were 10 mice per group.

RESULTS

Antiretroviral and Cytotoxic Effects of AZT and D4T—AZT and D4T were evaluated for their antiretroviral effects in human MT-4 and ATH8 cells infected with HIV and murine C3H cells infected with MSV (Table I). Both AZT and D4T proved to be potent and highly selective inhibitors of HIV replication in MT-4 cells. AZT showed an ED$_{50}$ (50% effective dose) of 0.005 μM, whereas D4T was 10-fold less active. Since the CD$_{50}$ (50% cytotoxic dose) of AZT and D4T was 8.1 and 19 μM, respectively, AZT could be considered as 3-fold more selective as an anti-HIV agent than D4T in MT-4 cells. In ATH8 cells, however, AZT and D4T were about 480- and 80-fold less effective against HIV than in MT-4 cells, while their cytotoxic effects were diminished only by a factor of 5. In murine C3H cells AZT was 100-fold more effective in inhibiting MSV cell transformation than was D4T (ED$_{50}$, 0.023 and 2.1 μM, respectively), while neither compound was toxic for C3H cells at 200 μM (Table I).

When the cytostatic effects of AZT and D4T were evaluated against a number of human T cell lines (*i.e.* MT-4, H9, CEM, ATH8), remarkable differences were noted (Table II). AZT was considerably more inhibitory to the growth of MT-4 and
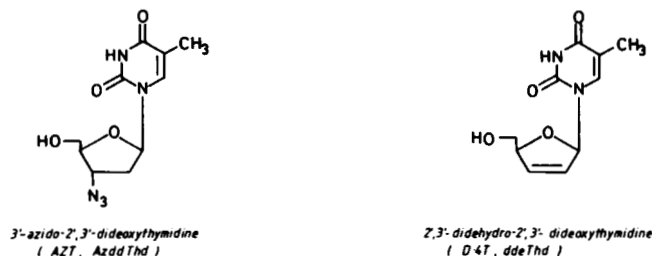

3'-azido-2',3'-dideoxythymidine
(AZT, AzddThd)

2',3'-didehydro-2',3'-dideoxythymidine
(D4T, ddeThd)

FIG. 1. **Structural formulae of 3'-azido-2',3'-dideoxythymidine (*AzddThd, AZT*) and 2',3'-didehydro-2',3'-dideoxythymidine (*ddeThd, D4T*).**

TABLE I

Inhibitory effects of AZT and D4T on HIV-induced cytopathogenicity in human MT-4 and ATH8 cells and MSV-induced transformation of murine C3H cells

Compound	MT-4 cells		ATH8 cells		C3H cells	
	$ED_{50}{}^a$	$CD_{50}{}^b$	$ED_{50}{}^a$	$CD_{50}{}^b$	$ED_{50}{}^c$	MTC^d
	μM		μM		μM	
AZT	0.005 ± 0.001	8.1 ± 1.8	2.4[e]	40[e]	0.023 ± 0.01	>200
D4T	0.05 ± 0.001	19 ± 3.6	4.1[e]	110[e]	2.1 ± 0.7	>200

[a] 50% effective dose, or dose required to inhibit HIV-induced destruction of MT-4 or ATH8 cells by 50%.

[b] 50% cytotoxic dose, or dose required to inhibit MT-4 or ATH8 cell proliferation by 50%.

[c] 50% effective dose, or dose required to inhibit MSV-induced transformation of C3H cells by 50%.

[d] Minimal toxic concentration, or lowest dose required to cause a microscopical alteration of normal C3H cell morphology.

[e] Data taken from Ref. 3.

TABLE II

Cytostatic effects of AZT and D4T on MT-4, ATH8, H9, and CEM cells

Compound	Cell line	50% cytostatic dose			
		Day 3	Day 4	Day 5	Day 7[a]
		μM		μM	
AZT	MT-4	53 ± 9	42 ± 7	36 ± 10	
	H9	>1000	>1000	>1000	
	CEM	582 ± 73	>1000	>1000	
	ATH8				40
D4T	MT-4	112 ± 13	84 ± 12	41 ± 18	
	H9	264 ± 70	243 ± 19	228 ± 13	
	CEM	138 ± 56	143 ± 15	158 ± 29	
	ATH8				110

[a] Data taken from Ref. 3. Due to the long time that the ATH8 cells required for proliferation, the inhibitory effects of the compounds on ATH8 cell growth were measured only after 7 days of incubation.

TABLE III

Phosphorylation of 1 μM [methyl-³H]AZT and 1 μM [methyl-³H] D4T in MT-4 cells as a function of incubation time

Time of incubation	Phosphorylated products[a]		
	5'-Monophosphate	5'-Diphosphate	5'-Triphosphate
h	nmol/10⁹ cells		
AZT			
3.5	155	1.30	0.68
7	164	1.53	0.71
24	100	1.96	0.80
48	147	1.34	0.72
72	95	0.43	0.15
D4T			
3.5	0.47	0.06	0.23
7	0.29	0.09	0.24
24	0.21	0.10	0.23
48	0.24	0.07	0.35
72	0.15	0.03	0.14

[a] Data represent average values for two separate experiments.

ATH8 cells than for CEM or H9 cells. The antiproliferative effect of AZT on MT-4 cells slightly increased upon longer incubation times (Table II). Less striking differences were noted in the cytostatic effects of D4T on the different T cell lines. The CD_{50} values of D4T for H9, CEM, and ATH8 cells ranged from 112 to 264 μM. The antiproliferative effect of D4T on MT-4 cells significantly increased upon longer incubation times (Table II).

Phosphorylation of [methyl-³H]AZT and [methyl-³H]D4T in MT-4 Cells following Different Incubation Times—The metabolism of radiolabeled AZT and D4T by MT-4 cells was followed upon incubation of the cells with 1 μM radiolabeled compounds for 3.5, 7, 24, 48, and 72 h (Table III). A typical HPLC chromatogram for the intracellular phosphorylation of [methyl-³H]AZT and [methyl-³H]D4T is depicted in Fig. 2. AZT was extensively metabolized to its 5'-monophosphate derivative (AZT-MP) in MT-4 cells. Upon incubation with 1 μM AZT, the intracellular AZT-MP levels measured after 3.5–48 h ranged from 100 to 164 nmol/10⁹ cells and slightly decreased to 95 nmol/10⁹ cells after 72 h. In contrast, relatively small levels of the 5'-diphosphate (AZT-DP) (1.3–1.9 nmol/10⁹ cells) and 5'-triphosphate (AZT-TP) (0.68–0.80 nmol/10⁹ cells) were detected within the first 48 h of incubation; and after 72 h, the AZT 5'-di- and 5'-triphosphate levels decreased to 0.43 and 0.15 nmol/10⁹ cells, respectively. The accumulation of AZT 5'-monophosphate in MT-4 cells contrasts sharply with the low levels found for the D4T 5'-monophosphate (D4T-MP) (Table III). The D4T 5'-triphosphate (D4T-TP) levels were similar to those detected for D4T-MP. The intracellular D4T-TP levels were only 2–3-fold lower than the AZT-TP levels after 24–48 h incubation, and the 72-h levels of D4T-TP were similar to the AZT-TP levels (Table III).
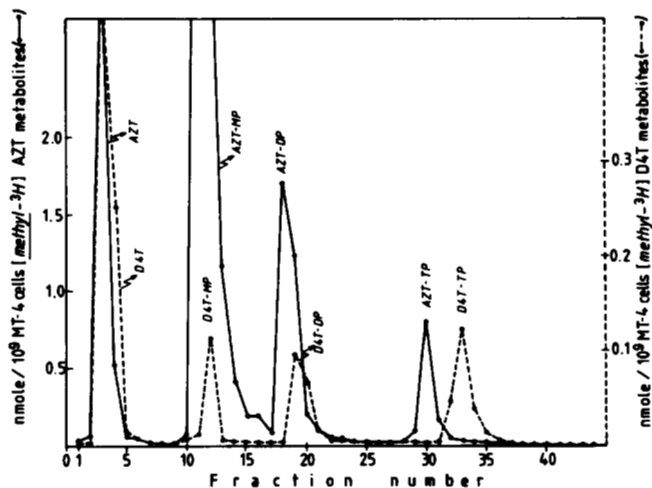
In Fig. 2, the phosphorylation pattern of AZT and D4T

FIG. 2. **Ion exchange (Partisil-SAX) HPLC elution profile of a 10% trichloroacetic acid extract of MT-4 cells incubated for 24 h with 1 μM [*methyl*-³H]AZT (●——●) or 1 μM [*methyl*-³H]D4T (●- - -●).** The column was equilibrated and developed with 0.007 M ammonium phosphate (pH 3.80) for 6 min, followed by a linear gradient to 0.25 M ammonium phosphate + 0.50 M KCl (pH 4.5) over the next 20 min, and finally by 20-min isocratic elution with the latter buffer.

following 24-h incubation with MT-4 cells is presented. No metabolites other than the 5'-mono-, 5'-di-, and 5'-triphosphates of AZT and D4T were detected. Identification of the 5'-mono-, 5'-di-, and 5'-triphosphate metabolites of AZT and D4T was ascertained by spiking the cell extracts with the chemically synthesized 5'-mono- and 5'-triphosphates of AZT and D4T. The radiolabeled peaks that were tentatively iden-

TABLE IV

Phosphorylation of [methyl-³H]AZT and [methyl-³H]D4T in MT-4 cells upon 24-h incubation as a function of the input concentrations of the nucleosides

Compound	Initial concentration	Nucleoside	5′-Monophosphate	5′-Diphosphate	5′-Triphosphate
	µM		*nmol/10⁹ cells[a]*		
AZT	50	219	1212	5.6	1.6
	10	35	377	4.0	1.1
	1.3	4.0	70	3.1	1.0
	0.53	1.7	26	1.6	0.54
	0.37	1.6	21	1.4	0.45
D4T	50	93	2.4	1.7	2.3
	10	19	0.77	1.0	1.0
	1.1	2.0	0.13	0.16	0.22
	0.25	0.44	0.03	0.06	0.06

[a] Data represent average values for two separate experiments.

TABLE V

Phosphorylation of 1 µM [methyl-³H]AZT and 1 µM [methyl-³H] D4T in human lymphoblast Raji/0 and Raji/TK⁻ cells upon 24-h incubation

Compound	Concentration of 5′-phosphorylated metabolites[a]	
	Raji/0	Raji/TK⁻
	nmol/10⁹ cells	
AZT	2.52	0.704
AZT-MP	31.7	0.053
AZT-DP	1.01	<0.005
AZT-TP	0.721	0.007
D4T	1.91	1.77
D4T-MP	0.038	0.025
D4T-DP	0.011	0.006
D4T-TP	0.017	0.015

[a] Data represent average values for two separate experiments.

TABLE VI

Effect of dThd and dCyd on the phosphorylation of 1 µM [methyl-³H] AZT and 1 µM [methyl-³H]D4T in MT-4 cells

Compound	5′-Phosphorylated metabolites[a]			
	As such	Upon addition of		
		25 µM dThd	1000 µM dCyd	250 µM dThd + 1000 µM dCyd
		nmol/10⁹ cells		
AZT	4.25	2.88	2.15	1.62
AZT-MP	100	42.0	18.6	1.19
AZT-DP	1.96	1.12	0.717	0.036
AZT-TP	0.795	0.427	0.313	0.022
D4T	1.35	0.621	0.762	0.658
D4T-MP	0.201	0.041	0.025	0.007
D4T-DP	0.098	<0.005	0.005	0.006
D4T-TP	0.231	0.019	0.014	<0.005

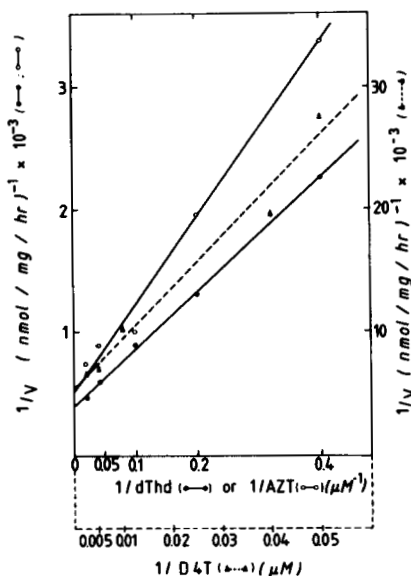[a] Values are the mean of two separate experiments.

FIG. 3. **Double-reciprocal (Lineweaver-Burk) plots for dThd (O——O), AZT (●——●), and D4T (▲－－▲) phosphorylation by MT-4 cell extracts.**

tified as the 5′-mono- and 5′-triphosphate of AZT and D4T coincided with the authentic 5′-mono- and 5′-triphosphates of AZT and D4T on the chromatogram. Upon treatment of the cell extracts with alkaline phosphatase, the radiolabeled peaks identified as the phosphorylated derivatives of AZT and D4T disappeared while the nucleoside forms accumulated (data not shown). Similarly, after phosphodiesterase had been added to the cell extracts, the D4T 5′-tri- and 5′-diphosphate peaks significantly diminished in size, whereas the D4T 5′-monophosphate peak increased (data not shown).

Phosphorylation of [methyl-³H]AZT and [methyl-³H]D4T in MT-4 Cells as a Function of Different Input Concentrations—MT-4 cells were incubated with [methyl-³H]AZT and [methyl-³H]D4T at concentrations ranging from 0.25 to 50 µM. Formation of the 5′-mono-, 5′-di-, and 5′-triphosphate metabolites of AZT and D4T increased with higher initial concentrations of the nucleosides.

While the intracellular levels of AZT-MP increased proportionally with increasing concentrations of the input AZT, or about 60-fold when the initial AZT concentration increased from 0.37 to 50 µM, AZT 5′-diphosphate and 5′-triphosphate levels increased only by a factor of 4–5-fold under the same experimental conditions. In contrast, when the initial concentration of D4T was raised from 0.25 to 50 µM, phosphorylated products (5′-mono-, 5′-di-, and 5′-triphosphate) were formed at a 30–80-fold greater extent (Table IV). The D4T-TP levels were inferior to the AZT-TP levels if the initial concentration of the nucleosides was lower than 1 µM, equal to the AZT-TP levels if the initial nucleoside concentrations were 1–10 µM and superior to the AZT-TP levels if the initial nucleoside concentrations were 50 µM. Essentially the same results were obtained in H9 cells (data not shown).

Metabolism of [methyl-³H]AZT and [methyl-³H]D4T in Raji/0 and dThd Kinase-deficient Raji/TK⁻ Cells—Phosphorylation of [methyl-³H]AZT and [methyl-³H]D4T was examined in the human B-lymphoblast Raji/0 cell line and its dThd kinase-deficient counterpart Raji/TK⁻ (Table V).

AZT was phosphorylated in Raji/0 cells about 2–3-fold less

TABLE VII

Intracellular [methyl-³H]dTTP levels and ratios of intracellular [methyl-³H]dTMP/([methyl-³H]dTDP + [methyl-
³H]dTTP) levels in MT-4 cells preincubated with AZT and D4T prior to pulse labeling with 0.04 µM [methyl-³H]
dThd for 2 h

Data represent average values for two to three separate experiments.

Compound concentration	Intracellular levels of [*methyl-*³H]dTTP		Ratio $\dfrac{[methyl\text{-}^3H]dTMP}{[methyl\text{-}^3H]dTDP + [methyl\text{-}^3H]dTTP}$	
	AZT	D4T	AZT	D4T
µM	*pmol/10⁹ cells*			
0	160	160	0.71	0.71
50	24.5	164	8.54	0.96
500	3.6	273	17.0	1.77

TABLE VIII

Inhibitory effects of AZT-TP and D4T-TP on the activity of MLV-
and HIV-associated reverse transcriptase and DNA polymerase α

Compound	50% inhibitory concentration			
	MLV		HIV 30-min assay	DNA polymerase α 30-min assay
	30-min assay	60-min assay		
	µM	*µM*	*µM*	*µM*
AZT-TP[a]	1.15 ± 0.4	0.51 ± 0.3	0.43	>500
D4T-TP	1.63 ± 0.4	1.18 ± 0.2	0.84	175 ± 117

[a] Data taken from Ref. 27.

TABLE IX

Partition coefficients (P) between 1-octanol and 10 mM potassium
phosphate buffer and R_f values in chloroform:methanol (92.5:7.5) on
silica gel TLC for dThd, ddThd, D4T, and AZT

Compound	R_F[a]	P[a]
dThd	0.124 ± 0.059	0.067 ± 0.005
ddThd	0.339 ± 0.088	0.233 ± 0.005
D4T	0.325 ± 0.091	0.154 ± 0.008
AZT	0.431 ± 0.082	0.964 ± 0.038

[a] Data represent average values (±S.D.) for at least two to three separate experiments.

efficiently than in MT-4 cells, and D4T was phosphorylated 10-fold less efficiently in Raji/0 than MT-4 cells (compare the data in Tables IV and V). In the dThd kinase-deficient cell line Raji/TK⁻, AZT was 500–1000-fold less extensively phosphorylated to its 5′-mono-, 5′-di-, and 5′-triphosphate than in Raji/0 cells. AZT-DP levels were even under the detection limit (0.005 nmol/10⁹ cells). In contrast, D4T phosphorylation proceeded at about the same rate in Raji/TK⁻ as in Raji/0 cells (Table V). These data suggest a crucial role for cellular dThd kinase in the phosphorylation of AZT but not D4T in Raji cells.

Phosphorylation of [methyl-³H]dThd, [methyl-³H]AZT, and [methyl-³H]D4T by MT-4 Cell Extracts—Different concentrations of [*methyl-*³H]dThd, [*methyl-*³H]AZT, and [*methyl-*³H]D4T were incubated with MT-4 cell extracts under assay conditions stable for optimal dThd kinase activity. Michaelis-Menten constants (K_m) and maximal velocity (V_{max}) values were calculated from Lineweaver-Burk diagrams (Fig. 3). dThd and AZT were phosphorylated to a comparable extent by the MT-4 cell extracts, while D4T was phosphorylated much less efficiently. The V_{max} values for phosphorylation of dThd, AZT, and D4T were 190, 153, and 8.2 nmol/mg of protein/h, respectively. The K_m values were 13.5, 13.8, and 142 µM, respectively. Addition of 10 µM dThd or 10 µM dTTP considerably decreased the phosphorylation of radiolabeled AZT or D4T by MT-4 cell extracts (data not shown). When the V_{max}/K_m ratio (14, 11, and 0.06, respectively) was taken as a parameter for the phosphorylation rate of dThd, AZT,

and D4T, it appeared that AZT was phosphorylated to a similar extent as dThd, while D4T was phosphorylated about 200-fold less efficiently.

Effect of dThd and dCyd on the Phosphorylation of [methyl-³H]AZT and [methyl-³H]D4T in MT-4 Cells—As shown in Table VI, addition of 250 µM dThd (in the presence of 1000 µM dCyd to avoid cytotoxicity of dThd) resulted in a dramatic decrease of the levels of phosphorylated metabolites of AZT and D4T. Under our experimental conditions, the AZT 5′-mono- and 5′-triphosphate levels decreased by 80- and 35-fold, respectively; the D4T 5′-monophosphate levels decreased by 30-fold; and the D4T-TP levels even fell below the detection limit (0.005 nmol/10⁹ cells) (Table VI). Addition of a lower concentration of exogenous dThd (25 µM) or addition of 1000 µM dCyd also resulted in a significant but less marked diminution of the phosphorylated AZT and D4T pools than that observed with 250 µM dThd.

Effect of Preincubation of MT-4 Cells with AZT and D4T on the Intracellular Phosphorylation of 0.04 µM [methyl-³H]dThd—MT-4 cells were incubated for 24 h with different concentrations of AZT or D4T prior to a pulse labeling of the cells with 0.04 µM [*methyl-*³H]dThd (10 µCi/4 × 10⁶ cells/culture) for 2 h. Then, the intracellular ratios of [*methyl-*³H]dTMP/([*methyl-*³H]dTDP + [*methyl-*³H]dTTP) were determined. Without inhibitor, the ratio of radiolabeled dTMP/(dTDP + dTTP) was 0.71 (Table VII). This corresponds to 160, 32, and 137 pmol of [*methyl-*³H]dTTP, [*methyl-*³H]dTDP, and [*methyl-*³H]dTMP per 10⁹ MT-4 cells, respectively. However, increasing initial concentrations of AZT resulted in a dramatic increase of the [*methyl-*³H]dTMP/([*methyl-*³H]dTDP + [*methyl-*³H]dTTP) ratio (17 in the presence of 500 µM AZT). In contrast, 500 µM D4T afforded a slight increase of the ratios of radiolabeled dTMP/(dTDP + dTTP) (Table VII).

Inhibitory Effects of AZT-TP and D4T-TP on HIV-associated Reverse Transcriptase and DNA Polymerase α—AZT-TP and D4T-TP were evaluated for their inhibitory effects on the reverse transcriptases of MLV and HIV and DNA polymerase α. The initial concentration of [*methyl-*³H]dTTP in the reaction mixture was 1 µM. AZT-TP and D4T-TP were strongly inhibitory to the reverse transcriptases. Their 50% inhibitory concentrations were in the range of 0.5–1.5 µM for the MLV reverse transcriptase and in the range of 0.02–0.1 µM range for HIV reverse transcriptase (Table VIII). AZT-MP had no effect on the enzyme, even at a concentration of 1000 µM (27). AZT-TP and D4T-TP were considerably less inhibitory to DNA polymerase α than MLV and HIV reverse transcriptase. Their 50% inhibitory concentrations were >500 and 175 µM, respectively.

Lipophilicity of AZT and D4T—To estimate the lipid solubility of AZT, D4T, dThd, and ddThd, their R_F values were determined by thin layer chromatography in a mixture of

chloroform:methanol (Table IX). The R_F values of AZT, D4T, dThd, and ddThd were 0.43, 0.33, 0.12, and 0.34, respectively. The partition of the test compounds between 1-octanol and potassium phosphate buffer was also measured (Table IX). AZT was far more lipophilic than D4T (partition coefficient (P):0.964 and 0.154, respectively). The lipophilicity of D4T was higher than that of dThd but lower than that of ddThd (Table IX).

DISCUSSION

The 2',3'-dideoxythymidine analogues AZT and D4T are potent inhibitors of HIV and MSV replication *in vitro* and show a marked "therapeutic index" in MT-4 cells (ratio of the compound concentration required to inhibit cell growth by 50% to the concentration required to inhibit virus replication by 50%). Although structurally related, AZT and D4T show a totally different pattern of intracellular phosphorylation. In contrast to AZT that accumulates mainly as its 5'-monophosphate resulting in relatively small levels of the 5'-di- and 5'-triphosphate derivatives, D4T does not accumulate as it 5'-monophosphate. Due to the low extent by which D4T is initially phosphorylated to its 5'-monophosphate, the D4T-TP levels that are eventually achieved are lower than the AZT-TP levels, at least when starting with an input nucleoside concentration of 1 μM or less. However, when the initial dose of AZT and D4T was increased, anabolism of D4T to D4T-TP was facilitated to a relatively greater extent over AZT-TP formation from AZT, so that at input nucleoside concentrations greater than 10 μM the levels obtained for D4T-TP exceeded those of AZT-TP.

Furman *et al.* (15) demonstrated that AZT-MP accumulation causes a severe inhibition of dTMP kinase resulting in an efficient blockage of its further phosphorylation to AZT-DP. From our findings, one may assume that when the initial AZT concentrations are higher than 1 μM, dTMP kinase of MT-4 cells must be blocked to a significant extent. Consequently, increase of the initial AZT concentration from 1 to 50 μM would not lead to a proportional increase in the formation of AZT-DP and AZT-TP. D4T is apparently unable to block its own phosphorylation, and, hence, increase in the initial D4T concentration results in a fairly proportional increase in the levels of all phosphorylated products of D4T (Table IV). Additional evidence that D4T-MP unlike AZT-MP does not significantly inhibit dTMP kinase is obtained from our pulse-labeling experiments in which MT-4 cells were preincubated with AZT or D4T and then labeled with [*methyl*-³H]dThd. A block at the level of dTMP kinase should result in an increase of intracellular radiolabeled dTMP and a concomitant decrease of the radiolabeled dTTP pools compared to control. In contrast to AZT, D4T did not lead to a significant accumulation of [*methyl*-³H]dTMP and depletion of [*methyl*-³H]dTTP pools. The fact that D4T, unlike AZT, does not interfere with dTMP kinase and thus fails to affect the salvage pathway of dThd, may be clinically advantageous in that a shut-off of this pathway, because of the ensuing reduction in dTTP pool levels, may lead to substantial cytotoxicity.

It has been established that AZT and dThd are phosphorylated equally well by the cytosol dThd kinase (15, 16). The K_m and V_{max} values of dThd kinase are very similar for both compounds. We now find that D4T is phosphorylated by MT-4 cell extracts at a much higher K_m and with a significantly lower V_{max} than AZT and dThd. The phosphorylation of D4T in MT-4 cells is sensitive to inhibition by dThd, and so is the phosphorylation of AZT. dTTP, a well known allosteric inhibitor of dThd phosphorylation by dThd kinase, also inhibits

D4T and AZT phosphorylation by MT-4 cell extracts. This suggests that dThd kinase is responsible for the phosphorylation of AZT and D4T. However, the phosphorylation patterns obtained for D4T and AZT in Raji/0 and Raji/TK⁻ cells implicate dThd kinase in the activation of AZT but not D4T. Furthermore, D4T is equally cytostatic for Raji/0 and Raji/TK⁻ cells and inhibit Simian AIDS-related retrovirus (SRV)-induced syncytium formation almost to a similar extent in Raji/0 and Raji/TK⁻ cells (data not shown). These observations, again, argue against the role of dThd kinase in the phosphorylation of D4T. Thus, it is unclear which enzyme(s) are responsible for the phosphorylation of D4T. Whatever enzyme is responsible, it is highly susceptible to inhibition by dThd as well as dTTP.

The phosphorylation data obtained in MT-4 extracts are in agreement with our observation that (i) phosphorylation of D4T and AZT in intact MT-4 cells is severely suppressed by dThd, and (ii) both the cytostatic and antiretroviral activity of D4T and AZT in MT-4 cells are reversed by the addition of dThd. The latter findings also suggest that a phosphorylated product of D4T and AZT, presumably their 5'-triphosphate derivatives, accounts for the biological activity of the compounds. One of the factors that may contribute to the decreased intracellular formation of phosphorylated D4T products in the presence of dThd (and dCyd) may be the competition of dThd with D4T for the catalytic site of D4T kinase. Additional inhibition may occur after the conversion of dThd to dTTP, because dTTP could act as a potent (allosteric) inhibitor of the putative D4T kinase. Also, interference with the cellular transport (uptake) of D4T may be taken in consideration to explain the decreased anabolism of D4T in the presence of dThd. Should this be the case, however, it is surprising that 25 μM dThd would afford a similar inhibitory effect on the D4T transport system as 1000 μM dCyd.

AZT is active as an inhibitor of HIV replication in MT-4 cells and of C3H cell transformation by MSV at lower concentrations than is D4T. Since AZT-TP and D4T-TP are equally inhibitory to the HIV- and MLV-associated reverse transcriptases, this greater potency of AZT against HIV replication can most probably be attributed to its more efficient phosphorylation within the cells to the 5'-triphosphate. Indeed, higher 5'-triphosphate levels are achieved within MT-4 cells for AZT than for D4T if the nucleosides are added to the cells at a concentration of 0.25–0.37 μM. Due to the relatively low specific radioactivity of both compounds, phosphorylation of the compounds at lower (antivirally active) concentrations could not be accurately determined.

The observation that HIV reverse transcriptase has a very high affinity for AZT-TP and D4T-TP, this affinity being several orders of magnitude higher than the affinity for cellular DNA polymerase α, may explain the selectivity and potency of both AZT and D4T as an antiretroviral chemotherapeutic agent *in vitro*. In this respect, D4T-TP proved 3-fold more inhibitory to DNA polymerase α than AZT-TP.

In view of the propensity of HIV to infect and damage the central nervous system, potential treatment schedules for AIDS patients should address the ability of candidate anti-AIDS drugs to cross the blood brain barrier. Preliminary evidence suggests that AZT favorably influences the course of neurological disorders associated with AIDS (32). Compounds with high lipophilicity may be expected to cross the blood brain barrier more easily than polar compounds. AZT proved clearly more lipophilic than dThd (P, 0.964 and 0.067, respectively). D4T showed a higher partition coefficient than dThd, but its lipophilicity was inferior to that of AZT. Also,

D4T had a higher partition coefficient value than ddCyd (P, 0.050). Since it has been established that dThd as well as ddCyd cross the blood brain barrier (33, 34), one may speculate from our lipophilicity data that D4T also does so.

Finally, we found that D4T is considerably less active than AZT as an antiretroviral agent in mice. Treatment of the MSV-infected newborn mice for 5 subsequent days with AZT at 125 mg/kg/day increased the mean tumor initiation time by 2-fold, and resulted in a 71% survival of the mice at day 25. In contrast, D4T treatment at 125 and 25 mg/kg/day only resulted in a modest delay of tumor formation, without a dramatic effect on the increase of the survival rate of the mice (data not shown). This decreased efficiency may, at least in part, be related to the low efficiency by which D4T is converted by the cellular phosphorylating enzymes to its 5'-monophosphate and eventually 5'-triphosphate in the murine model. The *in vivo* data are consistent with the *in vitro* data obtained in MSV-infected murine C3H cells. D4T was 100-fold less efficient in the latter system than AZT.

In conclusion, this is the first report in which the cellular metabolism and kinetic properties of D4T were investigated and compared to those of AZT. A close correlation was found between the antiviral and cytostatic activity of the compounds and their metabolism to their 5'-triphosphate form. D4T has unique metabolic features in that it does not accumulate as its 5'-monophosphate and generates similar levels of 5'-mono-, 5'-di-, and 5'-triphosphate. The fact that D4T does not seem to block dTMP kinase may give D4T a potential edge over AZT.

Note Added in Proof—After this paper was submitted, a communication with regard to the cellular pharmacology of D4T appeared in *Biochemical Pharmacology* (35).

REFERENCES

1. Mitsuya, H., and Broder, S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1911–1915
2. Balzarini, J., Pauwels, R., Herdewijn, P., De Clercq, E., Cooney, D. A., Kang, G.-J., Dalal, M., Johns, D. G., and Broder, S. (1986) *Biochem. Biophys. Res. Commun.* **140,** 735–742
3. Balzarini, J., Kang, G.-J., Dalal, M., Herdewijn, P., De Clercq, E., Broder, S., and Johns, D. G (1987) *Mol. Pharmacol.* **32,** 162–167
4. Lin, T.-S., Schinazi, R. F., Chen, M. S., Kinney-Thomas, E., and Prusoff, W. H. (1987) *Biochem. Pharmacol.* **36,** 311–316
5. Mitsuya, H., Weinhold, K. J., Furman, P. A., St. Clair, M. H., Nusinoff Lehrman, S., Gallo, R. C., Bolognesi, D., Barry, D. W., and Broder, S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7096–7100
6. Herdewijn, P., Balzarini, J., De Clercq, E., Pauwels, R., Baba, M., Broder, S., and Vanderhaeghe, H. (1987) *J. Med. Chem.* **30,** 1270–1278
7. Balzarini, J., and Broder, S. (1988) in *Clinical Use of Antiviral Drugs* (De Clercq, E., ed) pp. 361–385, Martinus Nijhoff Publishing, Norwell, MA
8. Nakashima, H., Matsui, T., Harada, S., Kobayashi, N., Matsuda, A., Ueda, T., and Yamamoto, N. (1986) *Antimicrob. Agents Chemother.* **30,** 933–937
9. Baba, M., Pauwels, R., Herdewijn, P., De Clercq, E., Desmyter, J., and Vandeputte, M. (1987) *Biochem. Biophys. Res. Commun.* **142,** 128–134
10. Lin, T.-S., Schinazi, R. F., and Prusoff, W. H. (1987) *Biochem. Pharmacol.* **36,** 2713–2718
11. Schinazi, R. F., Chu, C. K., Ahn, M.-K., and Sommadossi, J.-P. (1987) *J. Cell. Biochem.* Suppl. 11D, 74
12. Balzarini, J., Baba, M., Pauwels, R., Herdewijn, P., and De Clercq, E. (1988) *Biochem. Pharmacol.* **37,** 2847–2856
13. Matthes, E., Lehmann, Ch., Scholz, D., Rosenthal, H. A., and Langen, P. (1988) *Biochim. Biophys. Res. Commun.* **153,** 825–831
14. Balzarini, J., Matthes, E., Meeus, P., Johns, D. G., and De Clercq, E. (1988) *Proceedings of the VI International Symposium on Human Purine & Pyrimidine Metabolism, Hakone, Japan, July 17–21, 1988,* in press
15. Furman, P. A., Fyfe, J. A., St. Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Nusinoff Lehrman, S., Bolognesi, D. P., Broder, S., Mitsuya, H., and Barry, D. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8333–8337
16. Balzarini, J., Pauwels, R., Baba, M., Herdewijn, P., De Clercq, E., Broder, S., and Johns, D. G. (1988) *Biochem. Pharmacol.* **37,** 897–903
17. Cooney, D. A., Dalal, M., Mitsuya, H., McMahon, J. B., Nadkarni, M., Balzarini, J., Broder, S., and Johns, D. G. (1986) *Biochem. Pharmacol.* **35,** 2065–2068
18. Balzarini, J., Cooney, D. A., Dalal, M., Kang, G-J., Cupp, J. E., De Clercq, E., Broder, S., and Johns, D. G. (1987) *Mol. Pharmacol.* **32,** 798–806
19. Starnes, M. C., and Cheng, Y. (1987) *J. Biol. Chem.* **262,** 988–991
20. Langen, P., Kowollik, G., Etzold, G., Venner, H., and Reinert, H. (1972) *Acta Biol. Med. Ger.* **29,** 483–494
21. Mitsuya, H., Guo, H.-E., Cossman, L., Megson, M., Reitz, M. S., Jr., and Broder, S. (1984) *Science (Wash. D.C.)* **225,** 1484–1486
22. Pauwels, R., De Clercq, E., Desmyter, J., Balzarini, J., Goubau, P., Herdewijn, P., Vanderhaeghe, H., and Vandeputte, M. (1987) *J. Virol. Methods* **16,** 171–185
23. Harada, S., Koyanagi, Y., and Yamamoto, N. (1985) *Science* **229,** 563–566
24. Balzarini, J., De Clercq, E., Torrence, P. F., Mertes, M. P., Park, J. S., Schmidt, C. L., Shugar, D., Barr, P. J., Jones, A. S., Verhelst, G., and Walker, R. T. (1982) *Biochem. Pharmacol.* **31,** 1089–1095
25. Popovic, M., Sarngadharan, M. G., Read, E., and Gallo, R. C. (1984) *Science (Wash. D.C.)* **224,** 497–500
26. De Clercq, E., and Merigan, T. C. (1971) *Proc. Soc. Exp. Biol. Med.* **137,** 590–594
27. Balzarini, J., Herdewijn, P., Pauwels, R., Broder, S., and De Clercq, E. (1988) *Biochem. Pharmacol.* **37,** 2395–2403
28. Hoard, D. E., and Ott, D. G. (1965) *J. Am. Chem. Soc.* **87,** 1785–1788
29. Balzarini, J., Pauwels, R., Baba, M., Robins, M. J., Zou, R., Herdewijn, P., and De Clercq, E. (1987) *Biochem. Biophys. Res. Commun.* **145,** 269–276
30. Balzarini, J., Baba, M., Pauwels, R., Herdewijn, P., Wood, S. G., Robins, M. J., and De Clercq, E. (1988) *Mol. Pharmacol.* **33,** 243–249
31. Balzarini, J., De Clercq, E., Mertes, M. P., Shugar, D., and Torrence, P. F. (1982) *Biochem. Pharmacol.* **31,** 3673–3682
32. Yarchoan, R., Klecker, R. W., Weinhold, K. J., Markham, P. D., Lyerly, H. K., Durack, D. T., Gelmann, E., Nusinoff Lehrman, S., Blum, R. M., Barry, D. W., Shearer, G. M., Fischl, M. A., Mitsuya, H., Gallo, R. C., Collins, J. M., Bolognesi, D. P., Myers, C. E., and Broder, S. (1986) *Lancet* **1,** 575–580
33. Zaharko, D. S., Bolten, B. J., Chiuten, D., and Wiernik, P. H. (1979) *Cancer Res.* **39,** 4777–4781
34. Yarchoan, R., Perno, C. F., Thomas, R. V., Klecker, R. W., Allain, J.-P., Wills, R. J., McAtee, N., Fischl, M. A., Dubinsky, R., McNeely, M. C., Mitsuya, H., Pluda, J. M., Lawley, T. J., Leuther, M., Safai, B., Collins, J. M., Myers, C. E., and Broder, S. (1988) *Lancet* **1,** 76–81
35. August, E. M., Marongiu, M. E., Lin, T.-S., and Prusoff, W. H. (1988) *Biochem. Pharmacol.* **37,** 4419–4422