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ANTI-RETROVIRUS ACTIVITY OF 3′-FLUORO- AND 3′-AZIDO-SUBSTITUTED PYRIMIDINE 2′,3′-DIDEOXYNUCLEOSIDE ANALOGUES

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Abstract—The 3′-fluoro-and 3′-azido-substituted derivatives of 2′,3′-dideoxythymidine (ddThd), 2′,3′-dideoxyuridine (ddUrd), 2′,3′-dideoxy-5-ethyluridine (ddEtUrd) and 2′,3′-dideoxycytidine (ddCyd) have been synthesized and evaluated for their anti-retrovirus activity [against human immunodeficiency virus (HIV) and murine Moloney sarcoma virus (MSV)]. Based on their 50% effective doses the most potent inhibitors of HIV replication in human MT4 lymphocytes were: FddThd (0.001 μM), AzddThd (0.004 μM), FddUrd (0.04 μM) and AzddUrd (0.36 μM). Their selectivity indexes were 197, 5000, 500 and 677, respectively. In contrast, none of the 3′-substituted ddEtUrd derivatives had a marked antiviral effect. The 2′,3′-dideoxynucleoside analogues showed poor, if any, substrate affinity for (bacterial) dThd phosphorylase. AzddThd and FddThd inhibited human dThd kinase to a much greater extent (K_i/K_m: 0.66 and 3.4, respectively) than did AzddUrd or FddUrd (K_i/K_m: 71 and 171, respectively). The K_i/K_m values of FddCyd and AzddCyd for human dCyd kinase were about 60. Although phosphorylation is a prerequisite for the anti-retrovirus activity of the 2′,3′-dideoxynucleoside derivatives, there is no close correlation between the anti-retrovirus potency of the 3′-fluoro- and 3′-azido-substituted ddUrd, ddThd, ddEtUrd and ddCyd derivatives and their affinity for dThd kinase or dCyd kinase.

The first nucleoside analogue reported to have potent *in vitro* activity against human immunodeficiency virus (HIV) was 3′-azido-2′,3′-dideoxythymidine (AzddThd, AZT) [1]. This thymidine analogue is a 2′,3′-dideoxynucleoside analogue containing an azido (N_3) group at the 3′-C atom of the 2′,3′-dideoxyribose moiety. AzddThd proved effective *in vitro* against the replication of HIV in several human T4-lymphocyte cell lines including ATH8, H9 and MT4 [1–15], Moloney murine sarcoma virus (MSV) in murine C3H fibroblasts [6], feline leukemia virus (FeLV) in 81C sarcoma-positive/leukemia-negative cells [7], and equine infectious anemia virus (EIAV) in equine dermis cells [8]. These observations have triggered a world-wide effort to reinvestigate the anti-retrovirus properties of several 2′,3′-dideoxynucleosides that have been synthesized in the past for other purposes, and to synthesize novel derivatives of 2′,3′-dideoxynucleosides with modifications in the base and/or sugar moieties of the molecules.

In order to gain a better insight into the structural requirements that 2′,3′-dideoxynucleoside analogues have to fulfil for optimal anti-HIV activity, we synthesized the 3′-fluoro- and 3′-azido-substituted derivatives of 2′,3′-dideoxyuridine (FddUrd, AzddUrd), 2′,3′-dideoxythymidine (FddThd, AzddThd), 2′,3′-dideoxy-5-ethyluridine (FddEtUrd, AzddEtUrd) and 2′,3′-dideoxycytidine (FddCyd, AzddCyd). These compounds were evaluated for their anti-retrovirus effects (against HIV and MSV), their antimetabolic effects and their affinities for human pyrimidine nucleoside kinases and bacterial thymidine phosphorylase.

MATERIALS AND METHODS

Compounds. 2′,3′-Dideoxythymidine (ddThd) and 2′,3′-dideoxycytidine (ddCyd) were obtained from Pharmacia PL-Biochemicals (Piscataway, NJ). 3′-Azido-2′,3′-dideoxycytidine was a kind gift of Dr D. G. Johns (National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD, USA). Previously published methods were used to synthesize 2′,3′-dideoxyuridine (ddUrd) [9] and 3′-azido-2′,3′dideoxythymidine (AzddThd) [10]. The synthesis of 2′,3′-dideoxy-5-ethyluridine (ddEtUrd), 3′-azido-2′,3′-dideoxy-5-ethyluridine (AzddEtUrd), 3′-fluoro-2′,3′-dideoxy-5-ethyluridine (FddEtUrd), 3′-fluoro-2′,3′-dideoxythymidine (FddThd) and 3′-fluoro-2′,3′-dideoxycytidine (FddCyd) have recently been described by Herdewijn *et al.* [5]. The synthesis of 3′-fluoro-2′,3′-dideoxyuridine (FddUrd) and 3′-azido-2′,3′-dideoxyuridine (AzddUrd) will be described elsewhere.

The structural formulae of the test compounds are depicted in Fig. 1.

Radiochemicals. [2-^{14}C]dThd (specific radioactivity 50–60 mCi/mmol), [5-^{3}H]dCyd (specific radioactivity 20 Ci/mmol), [1′,2′-^{3}H]dUrd (specific radioactivity 27 Ci/mmol), [*methyl*-^{3}H]dThd (specific radioactivity 40 Ci/mmol) and [U-^{14}C]dCyd (specific radioactivity 480 mCi/mmol) were obtained from the Radiochemical Centre Amersham (Amersham, U.K.) whereas [5-^{3}H]dUrd (specific radioactivity 23 Ci/mmol) was from ICN Pharmaceuticals (Irvine, CA).

Cells. Raj/0 cells and Raji/TK$^-$ [a thymidine

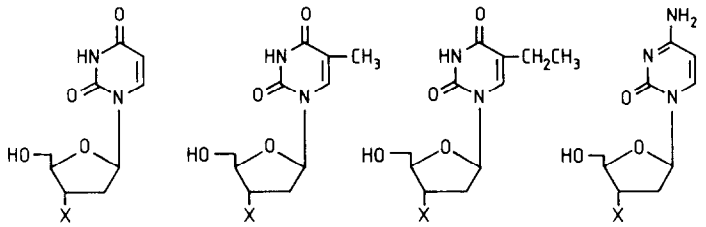

Fig. 1. Structural formulae of 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleosides.

(dThd) kinase-deficient mutant cell line derived from wild-type Raji/0 cells], were grown in 75 cm^2 plastic tissue culture flasks (International Medical, Brussels, Belgium), in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated foetal calf serum (Gibco, Glasgow, Scotland, U.K.), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland, U.K.) and 1% THM (5 mM Tes, 7.5 mM Hepes and 5 mM (MOPS) buffer (pH 5.3). Characterization of the Raji/0 and Raji/TK^- cells has been described previously [11]. Molt/4F, CEM and H9 cells were grown in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated foetal calf serum and 2 mM L-glutamine. MT4 cells were cultivated in RPMI 1640 medium (Gibco) containing 20 mM Hepes buffer, 10% (v/v) inactivated foetal calf serum (Gibco) and 2 mM L-glutamine (Flow Laboratories).

Viruses. HTLV-III_B (designated HIV) was derived from a pool of American patients with AIDS, and obtained from the supernatant of HIV-infected H9 cell cultures [12].

Moloney murine sarcoma virus (MSV) was prepared from tumors induced by *in vivo* infection of 10-day-old NMRI mice according to the procedure described by De Clercq and Merigan [13].

Anti-HIV assays. Human T-lymphocyte MT4 cells (5×10^5 cells/ml) were suspended in fresh RPMI-1640 culture medium (Gibco) containing 10% v/v fetal calf serum (Gibco), 2 mM L-glutamine (Flow Laboratories), 20 mM Hepes buffer, 0.075% (w/v) $NaHCO_3$ (Flow Laboratories), 2.5 μg/ml Fungizone (Squibb N.V., Brussels, Belgium) and 20 μg/ml Geomycine (Essex N.V., Heist-o/d-Berg, Belgium), and infected with 200 $CCID_{50}$ (Cell Culture Infective Dose-50) HIV per ml cell suspension. Then 100 μl of the infected cell suspension was added to 100 μl of an appropriate dilution of test compound in 200 μl microplate wells of a Flat Bottom Microtest III Plate (Falcon, Becton Dickinson, Oxnard, CA) (i.e. 20 $CCID_{50}$ HIV/200 μl well/5×10^4 cells), and further incubated at 37° in a CO_2-controlled humidified atmosphere. After incubation for 5 days, viable cell counts were determined for both virus-infected cell cultures and non-infected cell cultures (which had been incubated with the same concentration of compounds as the virus-infected cells). The 50% effective dose (ED_{50}) and 50% cytotoxic dose (CD_{50}) were defined as the compound concentrations required to reduce by 50% the number of viable cells in the virus-infected and non-infected cell cultures, respectively.

Transformation of C3H mouse embryo fibroblasts by Moloney murine sarcoma virus (MSV). C3H cells were seeded at 20,000 cells per ml into wells of Costar Tissue Culture Cluster plates (48 wells per plate). Twenty-four hours later, cell cultures were then infected by 80 foci-forming units of MSV over 90 min, whereafter the culture medium was replaced by 1 ml fresh medium containing different concentrations of the test compound. After 6 days, the transformation of the cell cultures was examined microscopically.

Inhibition of cell proliferation. All assays were performed in flat bottom Microtest III Plates (96 wells) (Falcon) as previously described [14]. Briefly, the cells were suspended in growth medium and added to the microplate wells at a density of 7.5×10^4 cells/well (200 μl) in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 68–72 hr at 37° in a humidified, CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter Counter (Coulter Electronics Ltd., Harpenden, Herts, U.K.). The 50% inhibitory dose (ID_{50}) was defined as the concentration of compound that reduced the number of cells by 50%,

Inhibition of [1′,2′-^{3}H]*dUrd,* [methyl-^{3}H]*dThd and* [5-^{3}H]*dCyd incorporation into Molt/4F cellular DNA.* The incorporation of radiolabelled precursors into Molt/4F cellular DNA was measured in microtest III plates (96 wells) (Falcon) as previously described [15]. Shortly, to each well of the microplate were added 10^5 Molt/4F cells and 0.25 μCi of [1′,2′-^{3}H]dUrd, [*methyl*-^{3}H]dThd or [5-^{3}H]dCyd. The cells were allowed to proliferate for 20–24 hr at 37° in a humidified CO_2-controlled atmosphere. At the end of this incubation period, the contents of the wells (200 μl) were brought onto 25-mm glass-fiber filters (type A/E, Gelman Instrument Company, Ann

Table 1. Anti-retrovirus effects of 3'-fluoro- and 3'-azido-substituted pyrimidine 2',3'-dideoxynucleoside analogues

Compound	HIV-induced cytopathogenicity			MSV-induced cytopathogenicity		
	ED_{50}* (μM)	CD_{50}† (μM)	S.I.‡	ED_{50}* (μM)	CD§ (μM)	S.I.‡
ddUrd	210	>625	>3	>400	>400	—
FddUrd	0.04	16	400	>400	>400	—
AzddUrd	0.36	244	677	15	>400	>27
ddThd	6	>625	104	48	>400	>8
FddThd	0.001	0.197	197	0.06	>400	>6666
AzddThd	0.004	20	5000	0.02	>400	>20,000
ddEtUrd	>625	>625	—	>400	>400	—
FddEtUrd	330	>625	>1.9	>400	>400	—
AzddEtUrd	64	418	6.5	>200	>200	—
ddCyd	0.3	40	120	25	>400	>16
FddCyd	16	26	1.6	284	>400	>1.2
AzddCyd	7.6	160	21	70	>400	>5.7

*50% antiviral effective dose, required to affect a 50% reduction in the cytopathic effect of HIV for MT4 cells or transformation of C3H cells by MSV.

†50% cytotoxic dose required to reduce the number of viable cells in the untreated MT4 cell cultures by 50%.

‡Selectivity index or ratio of CD to ED_{50}.

§Cytotoxic Dose. The parameter followed here was an alteration of normal cell morphology.

Arbor, MI) and washed twice with cold phosphate-buffered saline, twice with cold 10% trichloroacetic acid (TCA), twice with cold 5% TCA, once with ethanol and once with ether. The filters were then assayed for radioactivity in a toluene-based scintillant.

Inhibition of tritium release from [5-^{3}H]*dUrd or* [5-^{3}H]*dCyd in Molt/4F cells.* The procedure to measure tritium release from [5-^{3}H]dUrd or [5-^{3}H]dCyd in intact cells has been described previously [16]. Briefly, 10^7 Molt/4F cells/ml were preincubated with an appropriate amount of test compound for 15 min at 37°. After this incubation period, radiolabelled [5-^{3}H]dUrd or [5-^{3}H]dCyd (100 μCi/ml; 0.1 μM) were added, and at various times (0, 15, 30, 45, 60 min), 100 μl of the reaction mixture was withdrawn, and mixed with 500 μl of cold suspension of carbon black (100 mg/ml) in 5% TCA. After centrifugation at 1000 *g* for 10 min, the supernatants were analysed for radioactivity.

Enzyme assays. dThd kinase and dCyd kinase were prepared from exponentially growing Molt/4F cells, which were first washed (2×) with phosphate-buffered saline at 4° and then homogenized by sonication. The suspensions were clarified by centrifugation at 25,000–27,000 *g* for 30 min. The 30–70% $(NH_4)_2SO_4$ precipitate of the supernatant was resuspended in buffer containing 10 mM potassium phosphate, pH 7.5, 10 mM β-mercaptoethanol and 0.1 M KCl, dialyzed against the same buffer, and used for the dThd kinase and the dCyd kinase assays. In these experiments, [2-^{14}C]dThd and [U-^{14}C]dCyd served as the radiolabelled substrates. The apparent K_m and K_i values were derived from Lineweaver–Burk plots, using a linear regression analysis program. The assay procedures have been described in detail [15, 17].

Phosphorolytic cleavage of the test compounds was assessed using purified bacterial dThd phosphorylase (Sigma Chemical Co., St. Louis, MO) with the aid of a Beckman spectrophotometer UV/Vis 5200, the absorbance change during the enzyme reaction was continuously monitored at 25° at the wavelength where the difference between the nucleoside and its free base was maximal. The reaction mixtures for all assays consisted of enzyme and non-saturating concentrations of nucleoside in 0.1 M sodium phosphate buffer, pH 7.0. The initial velocity of phosphorolysis was expressed as nmol/min/enzyme unit.

RESULTS

Anti-retrovirus effects of 3'-substituted pyrimidine 2',3'-dideoxynucleoside analogues

The 2',3'-dideoxynucleosides ddUrd, ddThd, ddEtUrd and ddCyd and their 3'-fluoro- and 3'-azido-substituted counterparts were evaluated for their inhibitory effects on HIV-induced cytopathogenicity in MT4 cells (Table 1, Fig. 2). Marked differences were noted in the anti-HIV potencies of the 3'-substituted pyrimidine 2',3'-dideoxyribosides. With an ED_{50} of 0.001 and 0.004 μM, FddThd and AzddThd were the most potent, and with an ED_{50} of 64–>625 μM, ddUrd, ddEtUrd, FddEtUrd and AzddEtUrd were the least potent HIV inhibitors. ddThd showed an ED_{50} value of 6 μM. Although FddThd was almost as effective as AzddThd in inhibiting the cytopathogenicity of HIV in MT4 cells, it proved considerably more cytostatic than AzddThd for the uninfected lymphocytes. Consequently, the selectivity index (S.I.) of AzddThd was far greater than that of FddThd: 5000 vs 197 (Table 1). Introduction of a 3'-azido- or 3'-fluoro-group also increased the anti-HIV potency

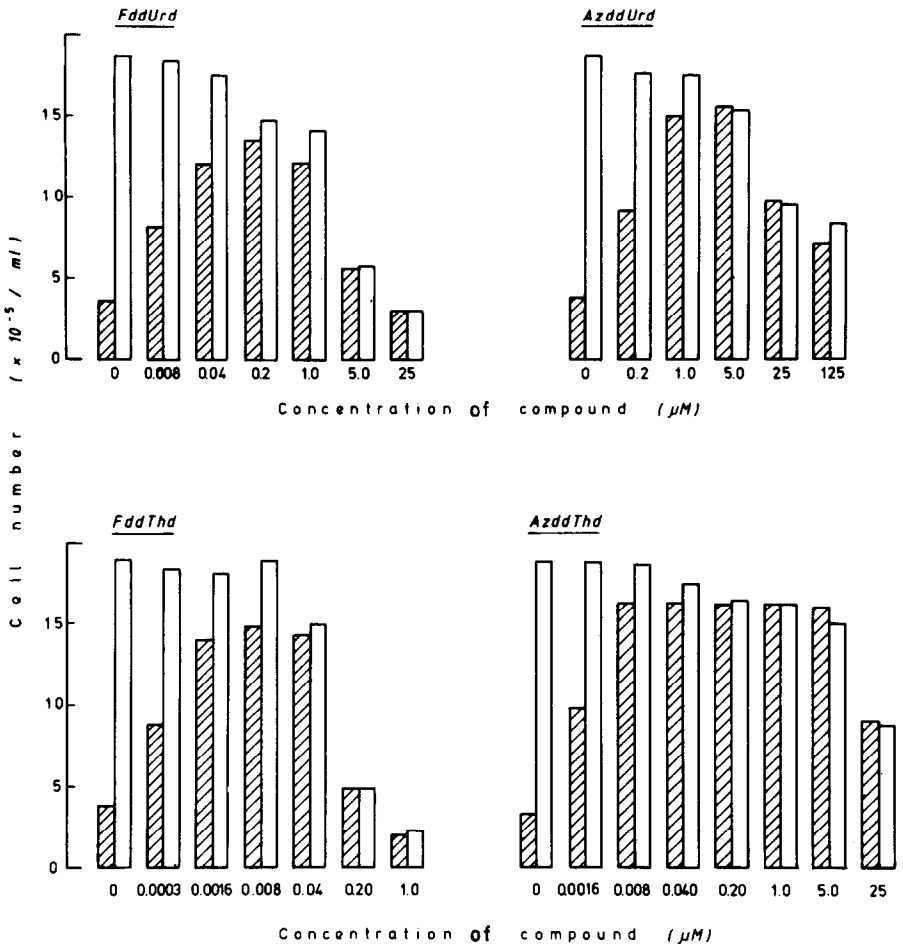

Fig. 2. Inhibition of the cytopathogenicity of HIV for MT4 cells by FddUrd, AzddUrd, FddThd and AzddThd. Viability of the cells was measured by trypan blue exclusion after an incubation period of 5 days. Mock-infected cells, incubated in the presence of different concentrations of the test compounds are indicated by open columns (□); HIV-infected cells, incubated in the presence of different concentrations of the test compounds are indicated by black columns (▨). Data represent average values for 3–5 separate experiments.

of ddUrd. With an ED_{50} of 0.04 μM FddUrd was 5000-fold and with an ED_{50} of 0.36 μM AzddUrd was 600-fold more active against HIV replication than the parent compound ddUrd (ED_{50}: 210 μM). Although FddUrd was 9-fold more effective against HIV than AzddUrd, it was also more cytostatic. This resulted in a selectivity index of 400 for FddUrd as compared to 677 for AzddUrd.

Among the ddCyd derivatives, 3′-fluoro-ddCyd and AzddCyd were substantially less potent as anti-HIV agents than ddCyd. When evaluated in another T-lymphocyte cell line (ATH8), AzddCyd proved also much less effective than ddCyd [18].

When examined for their inhibitory effect on the transformation of mouse embryo C3H fibroblasts by Moloney murine sarcoma virus (MSV), FddThd and AzddThd were considerably more effective than the other compounds tested (Table 1). Their ED_{50} values were 0.06 and 0.02 μM, respectively, and their selectivity indexes amounted up to >6666 and >20,000. In contrast, FddUrd was devoid of any anti-MSV activity (ED_{50} >400 μM), and AzddUrd showed modest activity (ED_{50}: 15 μM). None of the ddEtUrd derivatives had any inhibitory effect on C3H cell transformation by MSV. Among the ddCyd analogues, ddCyd had some activity (ED_{50}: 25 μM); the 3′-substituted ddCyd derivatives were less effective.

Cytostatic and antimetabolic effects of 3′-substituted pyrimidine 2′,3′-dideoxynucleoside analogues

When the 3′-fluoro- and 3′-azido-substituted derivatives of ddUrd, ddThd, ddEtUrd and ddCyd were examined for their cytostatic effects on B-lymphoblast (Raji/0), T-lymphoblast (Molt/4F) and T-lymphocyte (CEM, H9, MT4) cell lines, marked differences emerged, depending on the cell line (Table 2). CEM cells were 70-fold more susceptible to the cytostatic action of FddUrd (ID_{50}: 14 μM) than the other cell lines (ID_{50} > 1000 μM). The differences

Table 2. Cytostatic effect of 3'-substituted pyrimidine 2',3'-dideoxynucleoside analogues against human B- and T-cell lines

Compound	ID_{50}* (μM)					
	Raji/0	Raji/TK$^-$	Molt/4F	CEM	H9	MT4
ddUrd	>1000	>1000	≥1000	>1000	>1000	≥1000
FddUrd	>1000	>1000	≥1000	14	>1000	179
AzddUrd	≥1000	>1000	>1000	423	>1000	164
ddThd	>1000	>1000	>1000	>1000	>1000	>1000
FddThd	27	>1000	7	0.43	≥1000	8.5
AzddThd	130	>1000	>1000	228	632	58
ddEtUrd	>1000	>1000	>1000	>1000	>1000	326
FddEtUrd	>1000	>1000	>1000	>1000	>1000	>1000
AzddEtUrd	>1000	>1000	>1000	>1000	>1000	160
ddCyd	23	28	7	6	243	162
FddCyd	95	118	24	7	>1000	≥1000
AzddCyd	—	—	—	—	—	—

*50% inhibitory dose required to reduce the cell number by 50%.

in the sensitivity of the cell lines to the cytostatic action of FddThd was even more striking. In contrast to ddThd which did not inhibit the proliferation of any of the cell lines at 1000 μM, FddThd proved highly inhibitory to CEM cell proliferation (ID_{50}: 0.43 μM), modestly inhibitory to Molt/4F, MT4 and Raji/0 cells (ID_{50}: 7, 8.5 and 27 μM, respectively), and inert against Raji/TK$^-$ and H9 cells (ID_{50} > 1000 μM). The cytostatic activity of AzddThd against the different cell lines was less pronounced and less fluctuating than that of FddThd: AzddThd was most inhibitory to MT4 cells (ID_{50}: 58 μM); it was not cytostatic for Raji/TK$^-$ and Molt/4F cells (ID_{50} > 1000 μM). It is noteworthy that both FddThd and AzddThd were not cytostatic for the dThd kinase-deficient Raji/TK$^-$ cell line, although they proved cytostatic to the wild-type Raji/0 cells. None of the 3'-substituted ddEtUrd derivatives, including ddEtUrd itself, were potent inhibitors of cell proliferation. Among the 2',3'-dideoxycytidine derivatives, ddCyd and 3'-FddCyd were more inhibitory to the proliferation of CEM and Molt/4F cells (ID_{50}: 6–24 μM) than Raji (ID_{50}: 23–95 μM), H9 and MT4 cells (234–> 1000 μM).

The impact of the 3'-fluoro- and 3'-azido-substituted pyrimidine 2',3'-dideoxynucleoside analogues on cellular DNA biosynthesis was evaluated by monitoring the effect of these compounds on the incorporation of radiolabelled [1',2'-^{3}H]dUrd, [*methyl*-^{3}H]dThd and [5-^{3}H]dCyd into DNA of Molt/4F cells. ddUrd, ddThd and ddEtUrd had no effect on the incorporation of [1',2'-^{3}H]dUrd, [*methyl*-^{3}H]dThd and [5-^{3}H]dCyd into Molt/4F cell DNA (Table 3). In contrast, the 3'-fluoro- and 3'-azido-substituted derivatives of ddThd proved significantly inhibitory to [1',2'-^{3}H]dUrd incorporation (ID_{50}: ~ 1 μM), slightly inhibitory to [*methyl*-

Table 3. Inhibitory activity of 3'-fluoro- and 3'-azido-substituted pyrimidine 2',3'-dideoxynucleosides on the incorporation of [1',2'-^{3}H]dUrd, [*methyl*-^{3}H]dThd and [5-^{3}H]dCyd into MOLT/4F cell DNA

Compound	ID_{50}* (μM)		
	[1',2'-^{3}H]dUrd incorporation	[*methyl*-^{3}H]dThd incorporation	[5-^{3}H]dCyd incorporation
ddUrd	>1000	>1000	>1000
FddUrd	16	>1000	>1000
AzddUrd	172	>1000	725
ddThd	>1000	>1000	>1000
FddThd	1.1	483	>1000
AzddThd	1.3	57	>1000
ddEtUrd	>1000	>1000	>1000
FddEtUrd	>1000	>1000	>1000
AzddEtUrd	>1000	>1000	>1000
ddCyd	32	743	>1000 (stimulation)
FddCyd	27.3	>1000	>1000 (stimulation)
AzddCyd	—	—	—

*50% inhibitory dose required to reduce the incorporation of the radiolabelled precursor by 50%.

Table 4. Inhibitory activity of 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleosides on tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd in MOLT/4F cells

Compound	ID_{50}* (μM)	
	[5-^{3}H]dUrd	[5-^{3}H]dCyd
ddUrd	>250	>250
FddUrd	126	>250
AzddUrd	20	>250
ddThd	>250	>250
FddThd	1.5	>250
AzddThd	4.2	>250
ddEtUrd	>250	—
FddEtUrd	>250	—
AzddEtUrd	51	>250

*50% inhibitory dose required to reduce tritium release by 50%.

^{3}H]dThd incorporation (ID_{50}: 483 and 57 μM, respectively), and not inhibitory to [5-^{3}H]dCyd incorporation into DNA. 3′-Fluoro- and 3′-azido-ddUrd also inhibited [1′,2′-^{3}H]dUrd incorporation into DNA to a greater extent than [*methyl*-^{3}H]dThd or [5-^{3}H]dCyd incorporation. None of the ddEtUrd derivatives showed any effect on the incorporation of the radiolabelled DNA precursors (Table 3). When ddCyd and FddCyd were evaluated for their inhibitory effects on [1′,2′-^{3}H]dUrd, [*methyl*-^{3}H]dThd and [5-^{3}H]dCyd incorporation, both compounds proved 25- > 40-fold more inhibitory to [1′,2′-^{3}H]dUrd incorporation than [*methyl*-^{3}H]dThd incorporation. They significantly stimulated [5-^{3}H]dCyd incorporation into DNA at the highest concentrations tested (200 μM and 1000 μM). the stimulatory effect of both ddCyd and FddCyd on [5-^{3}H]dCyd incorporation into DNA amounted to 1.7-fold at 200 μM, and to 2-fold at 1000 μM, as compared to the control (data not shown).

Effect of 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleosides on tritium release from [5-^{3}H]dUrd and [5-^{3}H]*dCyd in Molt/4F cells*

None of the compounds evaluated affected tritium release from [5-^{3}H]dCyd even at a concentration of 250 μM (Table 4). In contrast, FddThd and AzddThd, but not ddThd, inhibited tritium release from [5-^{3}H]dUrd at 1.5 and 4.2 μM, respectively. Among the 3′-substituted ddUrd derivatives, AzddUrd, and to a lesser extent FddUrd inhibited tritium release from [5-^{3}H]dUrd (ID_{50}: 20 and 26 μM, respectively). AzddEtUrd was the only ddEtUrd derivative inhibiting tritium release from [5-^{3}H]dUrd (ID_{50}: 51 μM) (Table 4).

Affinity of Molt/4F dThd kinase and dCyd kinase for 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleosides

The K_i and K_i/K_m values of Molt/4F dThd kinase for ddUrd, ddThd, ddEtUrd, and their 3′-fluoro- and 3′-azido-substituted derivatives are presented in Table 5. The K_m value of dThd kinase for dThd ranged from 2.5 to 6.6 μM. The unsubstituted 2′,3′-dideoxynucleosides (i.e. ddUrd, ddThd and ddEtUrd) had a substantially lower affinity for dThd kinase than their 3′-fluoro- and 3′-azido-substituted counterparts. Moreover, the 3′-azido substituent conferred a greater increase in affinity of the 2′,3′-dideoxynucleoside for dThd kinase than did the 3′-fluoro substituent. The most potent inhibitors of dThd kinase were AzddThd and FddThd. Their K_i/K_m values were 0.66 and 3.4, respectively, that is 200- and 40-fold lower than the K_i/K_m value of ddThd. The 3′-azido- and 3′-fluoro-substituted ddUrd and ddEtUrd derivatives had a lower affinity for dThd kinase than AzddThd and FddThd, the K_i/K_m values for FddUrd and AzddUrd being 171 and 71, respectively. For ddUrd and ddEtUrd no inhibitory activity against dThd kinase could be detected, even when evaluated at a concentration as high as 750 μM or 1000 μM (Table 5). For those ddUrd, ddThd and ddEtUrd analogues that were active as

Table 5. Inhibition of MOLT/4F dThd kinase and dCyd kinase by 3′-substituted pyrimidine 2′,3′-dideoxynucleoside analogues

Compound	Enzyme	K_i	K_i/K_m*	Type of inhibition
ddUrd	dThd kinase	>1000	>250	—
FddUrd	dThd kinase	833	171	competitive
AzddUrd	dThd kinase	224	71	competitive
ddThd	dThd kinase	653	124	competitive
FddThd	dThd kinase	13	3.4	competitive
AzddThd	dThd kinase	2.9	0.66	competitive
ddEtUrd	dThd kinase	>750	>200	—
FddEtUrd	dThd kinase	266	72	competitive
AzddEtUrd	dThd kinase	175	28	competitive
ddCyd	dCyd kinase	1965	404	competitive
FddCyd	dCyd kinase	360	63	competitive
AzddCyd	dCyd kinase	354	58	competitive

*K_m values obtained in the individual experiments ranged from 2.5 to 6.6 μM for dThd kinase and 5.2 to 6.4 μM for dCyd kinase.

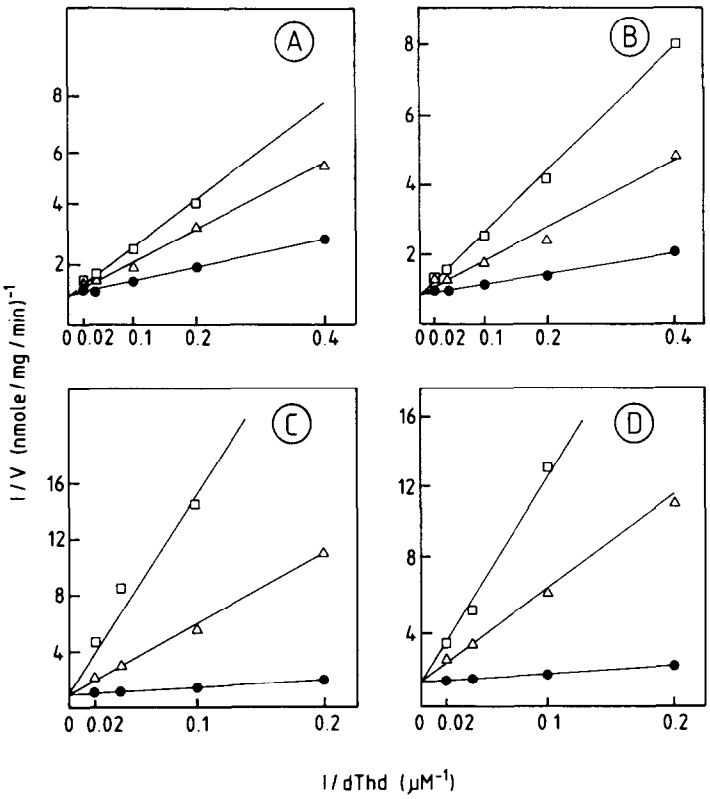

Fig. 3. Double-reciprocal plots for inhibition of Molt/4F dThd kinase by FddUrd (A), AzddUrd (B), FddThd (C) and AzddThd (D). Inhibitor concentrations: none (●), 1000 μM (△) and 2000 μM (□) for FddUrd; none (●), 500 μM (△) and 1000 μM (□) for AzddUrd; none (●), 25 μM (△) and 50 μM (□) for FddThd; none (●), 25 μM (△), and 50 μM (□) for AzddThd. Data represent average values for 3 separate experiments.

inhibitors of dThd kinase, the type of inhibition appeared to be competitive with respect to dThd. Lineweaver–Burk plots are shown for FddUrd, AzddUrd, FddThd and AzddThd (Fig. 3).

The K_i/K_m values of Molt/4F dCyd kinase for ddCyd, FddCyd and AzddCyd are also presented in Table 5. The K_m value of the enzyme for dCyd ranged from 5.2 μM to 6.4 μM. FddCyd and AzddCyd had a relatively low affinity for the enzyme ($K_i/K_m \sim 60$), and for the parent nucleoside ddCyd the K_i/K_m value was even higher (Table 5). In all cases, inhibition was competitive with respect to radiolabelled dCyd as the competing substrate. Lineweaver–Burk plots are shown for FddCyd and AzddCyd in Fig. 4.

Nucleoside phosphorolysis

The 3'-fluoro- and 3'-azido-substituted, as well as the unsubstituted, ddUrd, ddThd and ddEtUrd nucleoside analogues were evaluated for their susceptibility to phosphorolysis by purified bacterial dThd phosphorylase (Table 6). As compared to the 2'-deoxyribosides dUrd, dThd and dEtUrd, the 2',3'-dideoxyribosides showed a 1000- to 2000-fold decrease in the velocity of phosphorolysis by dThd phosphorylase (Table 5) (see also ref. 19). For ddEtUrd no phosphorolytic cleavage whatsoever was detectable, and this was also the case for FddEtUrd and AzddEtUrd. Introduction of a fluorine at C-3' of ddUrd and ddThd further decreased the affinity for dThd phosphorylase. Introduction of an azido group at C-3' of ddUrd or ddThd made the compounds even totally resistant to hydrolysis by dThd phosphorylase. Similarly, ddCyd, FddCyd and AzddCyd proved totally resistant to the action of dThd phosphorylase.

DISCUSSION

The fact that 3'-azido-2',3'-dideoxythymidine (AzddThd) showed potent and selective anti-HIV activity [1] prompted the synthesis of several 3'-substituted 2',3'-dideoxyribopyrimidine derivatives structurally related to AzddThd. We have demonstrated that FddThd is as potent and selective an anti-HIV agent as AzddThd, when evaluated in HIV-infected ATH8 cells [5]. These data were recently confirmed by Polsky *et al.* [20] who found that the anti-retrovirus activity of FddThd against HIV and feline leukemia virus (FLV) *in vitro* is comparable to that of AzddThd. The present findings indicate that FddThd and AzddThd also have comparable anti-HIV activity in MT4 cells and anti-MSV activity in C3H cells (Table 1). This is in agreement with our previous data for ATH8 cells. Surprisingly, FddThd

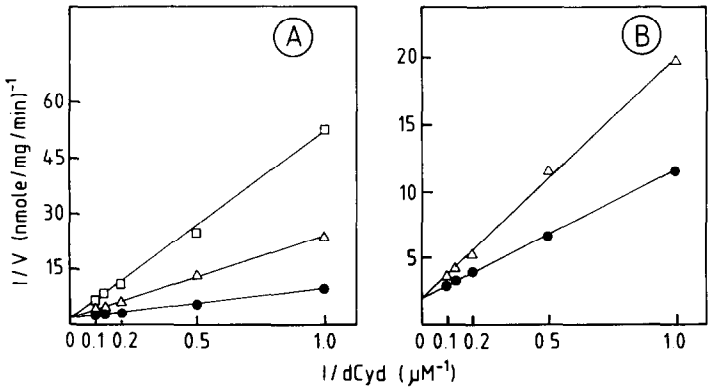

Fig. 4. Double-reciprocal plots for inhibition of Molt/4F dCyd kinase by FddCyd (A) and AzddCyd (B). Inhibitor concentrations: none (●), 1000 μM (□) and 200 μM (△) for FddCyd; none (●) and 250 μM (△) for AzddCyd. Data represent average values for 3 separate experiments.

was considerably more cytostatic for MT4 cells (CD_{50}: 0.2 μM) (Table 1 and 2) than for ATH8 (CD_{50}: 15 μM). Polsky and coworkers found FddThd cytostatic for HL-60 cells at an ID_{50} of 19 μM; and the *in vitro* proliferative responses of normal peripheral blood lymphocytes to mitogens was reduced by 50% at 1 μM FddThd [20]. Thus, the cytostatic and cytotoxic effects of FddThd seem to vary considerably from one cell line to another.

To investigate whether thymidylate synthase might be considered as a potential target for the cytostatic activity of these compounds, we examined the effect of FddThd and AzddThd on the incorporation of [1′,2′-^{3}H]dUrd and [*methyl*-^{3}H]dThd into DNA and tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd in intact cells. Both FddThd and AzddThd inhibited the incorporation of [1′,2′-^{3}H]dUrd into Molt/4F DNA to a considerable higher extent than [*methyl*-^{3}H]dThd (Table 3), and tritium release from [5-^{3}H]dUrd in Molt/4F cells was also much more affected than tritium release from [5-^{3}H]dCyd (Table 4). We have previously shown that compounds whose cytostatic action is due to an inhibition of thymidylate synthase [16] strongly inhibit tritium release from [5-^{3}H]dCyd. The fact that tritium release from [5-^{3}H]dCyd was not inhibited by any compound (Table 4) at a concentration as high as 250 μM (that is more than 100-fold the concentration that inhibits cell proliferation) suggests that thymidylate synthase is not a target for the cytostatic effect of these compounds. The observation that tritium release from [5-^{3}H]dUrd and the incorporation of [1′,2′-^{3}H]dUrd into DNA is substantially inhibited by FddThd and AzddThd is most probably related to the strong affinity both compounds have towards their activating enzyme, dThd kinase. We have made a similar observation for dThd and 5-iodo-dUrd [16], which also show a marked affinity for dThd kinase [17].

Since dUrd inhibits dThd kinase to a lesser extent than FddThd or AzddThd (K_i/K_m for dUrd: 36), both FddThd and AzddThd may be expected to be superior to dUrd in competing with dThd for phosphorylation by dThd kinase. Hence, they should inhibit intracellular tritium release from [5-^{3}H]dUrd and incorporation of [1′,2′-^{3}H]dUrd into cell DNA. Although phosphorylation of FddThd and AzddThd by dThd kinase is a prerequisite for their biological activities (i.e. FddThd and AzddThd are not cytostatic for dThd kinase-deficient Raji/TK$^-$ cells), the initial phosphorylation step is clearly not the sole parameter that governs the biological activity of FddThd and AzddThd. Indeed, FddThd is less efficient a substrate for Molt/4F dThd kinase than is AzddThd, yet FddThd is more cytostatic for Molt/4F cells than AzddThd. Moreover, while the affinities of AzddThd and FddThd for Molt/4F dThd kinase are almost identical to those for MT4 dThd kinase (K_i/K_m; 1.0 and 4.29, respectively), AzddThd is more than 20-fold more cytostatic for MT4 cells than for Molt/4F, FddThd being almost equally cytostatic in both cell lines.

In addition to AzddThd and FddThd, we also examined the 3′-fluoro- and 3′-azido-substituted derivatives of ddUrd. FddUrd is a novel compound whose synthesis and biological properties have not previously been reported. AzddUrd was also synthesized by Chu and coworkers [21, 22] (and given the code name: CS-87 or AVS-2353) and examined for anti-HIV activity. AzddUrd was found to selectively inhibit HIV replication in human peripheral blood mononuclear cells (ED_{50}: 0.18 μM), ATH8 cells (ED_{50}: 0.40 μM) and HeLa-T4 cells (ED_{50}: 1.24 μM). AzddUrd did not prove superior to AzddThd in PBM or HeLa-T4 cells, but was more potent than AzddThd in ATH8 cells [1, 21, 22]. In none of these cell lines AzddUrd was significantly toxic at 200 μM. The anti-HIV activity of AzddUrd has now been confirmed in MT4 cells. However, we found the novel FddUrd derivative to be 10-fold more potent against HIV than AzddUrd.

When AzddUrd and FddUrd were evaluated for their inhibitory effects on viral antigen expression in H9 cells infected with HIV at a high multiplicity of infection (M.O.I.: 0.1) FddUrd achieved a 50%-inhibition of HIV antigen expression at 5.6 μM, that is at a 3-fold lower dose than AzddUrd but a 100-fold higher dose than AzddThd (data not shown). Although the ED_{50}-values recorded with the compounds in the H9 cell system are higher than those

obtained in the MT4 system, the relative order of activity is similar.

Surprisingly, FddUrd was devoid of any anti-MSV activity (Table 1). The reason for this inactivity is unclear. The inhibitory effects of AzddUrd and FddUrd on the incorporation of [1',2'-^{3}H]dUrd and [*methyl*-^{3}H]dThd into DNA and tritium release from [5-^{3}H]dUrd are less pronounced than for FddThd and AzddThd. This is in agreement with our hypothesis that FddUrd and AzddUrd inhibit dUrd phosphorylation by dThd kinase to a lesser extent than FddThd and AzddThd. In fact, FddUrd and AzddUrd have a 60–80-fold lower affinity for dThd kinase than their ddThd counterparts (Table5).

Although the 3'-substituted ddEtUrd derivatives have a slightly better affinity for dThd kinase than the corresponding ddUrd derivatives, they slow little, if any, anti-retrovirus activity (Tables 1 and 5). Our observations with AzddEtUrd in HIV-infected MT4 cells confirm our previous findings with AzddEtUrd in HIV-infected ATH8 cells [5] but contrast sharply with claims made by Schinazi and co-workers [23] that AzddEtUrd would be equally active as AzddThd against HIV, and in adddition, markedly less toxic than AzddThd in a variety of cell lines (i.e. H9, PBM).

Although obligatory, the initial phosphorylation of the nucleoside analogues may not be sufficient to ensure potent anti-retrovirus activity. Thus, the inactivity of the 3'-substituted ddEtUrd derivatives may reside in their low affinity for the pyrimidine nucleotide kinases (i.e. dTMP-dUMP kinase, nucleoside 5'-diphosphate kinase) involved in the second or third phosphorylation step and/or a poor affinity of the 5'-triphosphates for the HIV reverse transcriptase.

While introduction of a fluorine or azido at C-3' of ddUrd or ddThd led to an increased anti-HIV activity, a 3'-fluoro or 3'-azido substituent decreased the anti-retrovirus activity of ddCyd. The differential effects of 3'-fluoro and 3'-azido substitution of the biological activity of ddUrd, ddThd and ddEtUrd on the one hand, and ddCyd on the other, may be related to differences in the affinity of these compounds for the kinases involved in their phosphorylation, as well as differences in the affinity of the resulting 5'-triphosphates for the reverse transcriptase.

Akin to ddUrd and ddThd [19], FddUrd and FddThd also proved far more resistant to phosphorolytic cleavage by dThd phosphorylase than did dThd and dUrd. In fact, none of the 3'-azido-substituted ddUrd, ddThd or ddEtUrd derivatives proved to be a substrate for dThd phosphorylase under experimental conditions where dUrd and dThd were easily hydrolysed to the corresponding bases. This property may be advantageous, as it would retard catabolism and thus permit a better conversion of the compounds to the 5'-triphosphate forms.

In conclusion, we found that 3'-fluoro- and 3'-azido-substitution significantly increased the anti-HIV activity of ddUrd and ddThd, while it decreased the anti-HIV activity of ddCyd. Thymidylate synthase does not appear to be a target enzyme for the biological activity of these compounds. Although phosphorylation is a prerequisite for their biological action, there is no correlation between the affinity of the compounds for the pyrimidine nucleoside (dThd or dCyd) kinases and their anti-retrovirus and/or cytostatic activity. We also demonstrated that FddUrd, whose synthesis and biological activity has not been previously revealed, is a potent and selective anti-HIV agent. FddUrd should therefore be considered as a novel candidate compound for further investigations for its efficacy in the treatment of retrovirus infections, i.e. AIDS.

Table 6. Kinetic properties of 3'-substituted pyrimidine 2',3'-dideoxynucleoside analogues for bacterial thymidine phosphorylase

Compound	Initial velocity of phosphorolysis (nmol/min/unit)
dUrd	361
ddUrd	0.270
AzddUrd	N.D.*
FddUrd	0.018
dThd	216
ddThd	0.120
AzddThd	N.D.
FddThd	0.008
dEtUrd	0.893
ddEtUrd	N.D.
AzddEtUrd	N.D.
FddEtUrd	N.D.
dCyd	N.D.
ddCyd	N.D.
AzddCyd	N.D.
FddCyd	N.D.

*Phosphorolysis not detectable with 25 units of enzyme.

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