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ACCELERATED COMMUNICATION

5-Halogeno-3′-fluoro-2′,3′-dideoxyuridines as Inhibitors of Human Immunodeficiency Virus (HIV): Potent and Selective Anti-HIV Activity of 3′-Fluoro-2′,3′-dideoxy-5-chlorouridine

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SUMMARY

The novel 5-chloro-, 5-bromo-, and 5-iodo-derivatives of 3′-fluoro-2′,3′-dideoxyuridine (FddUrd), designated FddClUrd, FddBrUrd, and FddIUrd, respectively, have been synthesized and evaluated for their antiretrovirus activity against human immunodeficiency virus (HIV) and murine Moloney sarcoma virus. All three 5-halogeno-FddUrd analogues inhibited HIV-1 replication in MT4 cells with an effective dose (ED_{50}) of about 0.2–0.4 μM. However, FddClUrd was markedly more selective in its anti-HIV-1 activity than FddBrUrd or FddIUrd. The selectivity index of FddClUrd was similar to that of 3′-azido-2′,3′-dideoxythymidine (AZT) when evaluated in parallel (1408 and 1603, respectively). The FddUrd derivatives also had a marked inhibitory effect on HIV-2 replication in MT4 cells and HIV-1-induced antigen expression in HUT-78 cells. However, neither FddUrd nor its 5-halogeno derivatives were inhibitory to Moloney sarcoma virus-induced transformation of murine C3H cells. The anti-HIV-1 activity of FddUrd, FddClUrd, FddBrUrd, and FddIUrd was reversed by the addition of thymidine and 2′-deoxycytidine. The 5-halogeno-FddUrd analogues had a markedly higher affinity for MT4 thymidine kinase than FddUrd (K_i/K_m, 4.0–4.7, as compared with 302 for FddUrd).

Since the advent of AIDS, many efforts have been conducted world-wide to develop novel and selective inhibitors of HIV replication *in vitro*. 2′,3′-Dideoxynucleoside analogues have attracted considerable attention as anti-HIV compounds, and several congeners of this class have been or are the subject of clinical trials [i.e., AzddThd, (AZT, retrovir), 2′,3′-dideoxycytidine, 2′,3′-dideoxyadenosine, and 2′,3′-dideoxyinosine] (for an overview, see Refs. 1 and 2). Recently, we synthesized the 3′-fluoro derivatives of 2′,3′-dideoxyuridine (FddUrd), 2′,3′-dideoxythymidine (FddThd), 5-ethyl-2′,3′-dideoxyuridine, and 2′,3′-dideoxycytidine and investigated their antiretroviral activities and antimetabolic properties (3). FddThd and FddUrd rank among the most potent anti-HIV compounds within the group of the pyrimidine 2′,3′-dideoxynucleoside analogues (3). From our studies, it also emerged that FddUrd had a 150- to 600-fold lower affinity for dThd kinase, the enzyme that is required for its activation, than had either FddThd or AzddThd (3). It was reasoned that compounds that are structurally related to FddUrd, but endowed with a higher affinity for dThd kinase than FddUrd, may possibly surpass FddUrd in either antiretroviral potency or selectivity, or both. To this end, a series of novel FddUrd derivatives was synthesized whereby an halogen atom (chlorine, bromine, or iodine) was introduced at the uracil C-5 position of FddUrd. This strategy was based on our previous knowledge that introduction of a chloro, bromo, or iodo group at C-5 of 2′-deoxyuridine markedly increased the substrate affinity for cytosol dThd kinase (4). The premise was borne out, in that the 5-halogeno-FddUrd analogues proved to be much better substrates for dThd kinase than FddUrd. FddClUrd, FddBrUrd, and FddIUrd also proved to be potent inhibitors of HIV-1 and HIV-2 replication in MT4 and HUT-78 cells. Of the 5-halogeno-FddUrd analogues, FddClUrd showed the greatest selectivity as an anti-HIV agent [selectivity index (50% cytotoxic dose/50% antiviral effective dose), 1408] and, therefore, FddClUrd deserves further consideration as a novel candidate drug for the treatment of AIDS.

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ABBREVIATIONS: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus, FddUrd; 3′-fluoro-2′,3′-dideoxyuridine; AzddThd, 3′-azido-2′,3′-dideoxythymidine; FddThd, 2′,3′-dideoxythymidine; dThd, thymidine; DMSO, dimethylsulfoxide; $CCID_{50}$, cell culture 50% infective dose; ED_{50}, 50% effective dose; CD_{50}, 50% cytotoxic dose; dCyd, 2′-deoxycytidine; MSV, murine sarcoma virus; ID_{50}, 50% inhibitory dose; dUrd, 2′-deoxyuridine.

Materials and Methods

Compounds. The detailed synthesis of FddClUrd, FddBrUrd, and FddIUrd will be described elsewhere.

Briefly, chlorination of the C-5 position of FddUrd was performed with *N*-chlorosuccinimide. Therefore, the 5′-hydroxyl group was first protected by acetylation with acetic anhydride in pyridine. Reaction of 5′-acylated FddUrd with *N*-chlorosuccinimide in anhydrous pyridine at 100° for 30 min gave a single reaction product on thin layer chromatography ($CHCl_3$/methanol 9:1) and deacylation yielded 59% crystalline FddClUrd. Experimental analysis for FddClUrd ($C_9H_{10}N_2O_4FCl$): purity—calculated, C (40.84), H (3.81), N (10.59); found, C (40.79), H (3.96), N (10.38); identification—UV (methanol) λ_{max} 276.5 nm (ϵ = 8650); ^{1}H NMR (DMSO-d_6) δ; 2.01–2.63 (m, H-2′, H-2″), 3.65 (m, H-5′, H-5″), 4.21 (dt, H-4′, J = 3.5 Hz, $J_{4',F}$ = 27.3 Hz), 5.30 (t, 5′-OH, exchangeable D_2O), 5.31 (dd, H-3′, J = 4 Hz, $J_{3',F}$ = 54.4 Hz), 6.18 (t, H-1′, J = 7.2 Hz), 8.24 (s, H-6), 11.80 (br s, N-H, exchangeable D_2O).

Reaction of FddUrd with *N*-bromosuccinimide in glacial acetic acid for 30 min at reflux temperature gave a mixture of FddBrUrd and its 5′-acylated analogue. FddBrUrd was isolated in 45% yield. Experimental analysis for FddBrUrd ($C_9H_{10}N_2O_4FBr$): purity—calculated; C (34.97), H (3.26), N (9.06); found, C (34.89), H (3.33), N (8.92); identification—UV (methanol) λ_{max} 278 nm (ϵ = 8650); ^{1}H NMR (DMSO-d_6) δ, 2.27–2.60 (m, H-2′, H-2″), 3.64 (m, H-5′, H-5″), 4.20 (dt, H-4′, $J_{4',F}$ = 26.3 Hz), 5.30 (t, 5′-OH), 5.31 (dm, H-3′, $J_{3',F}$ = 53.6 Hz), 6.17 (dt, H-1′), 8.32 (s, H-6), 11.81 (br s, NH).

Iodination of FddUrd with 1.5 eq of iodine monochloride in methanol for 3 hr at reflux temperature gave 66% FddIUrd. Experimental analysis for FddIUrd ($C_9H_{10}N_2O_4FI$): purity—calculated, C (30.35), H (2.83), N (7.87); found, C (30.35), H (2.93), N (7.67); identification—UV (methanol) λ_{max} 284 nm (broad max) (ϵ = 7200); ^{1}H NMR (DMSO-d_6) δ, 2.06–2.63 (m, H-2′, H-2″), 3.65 (m, H-5′, H-5″), 4.19 (dt, H-4′, $J_{4',F}$ = 27.3 Hz), 5.27 (t, 5′-OH), 5.29 (dm, H-3′, $J_{3',F}$ = 53.9 Hz), 6.16 (dt, H-1′), 8.34 (s, H-6), 11.69 (br s, NH).

The structural formulae of FddClUrd, FddBrUrd, FddIUrd, and FddUrd are depicted in Fig. 1.

FddUrd, FddThd, and AzddThd were prepared according to previously published methods (5–7).

Radiochemicals. [*methyl*-^{3}H]dThd (specific radioactivity, 40 Ci/mmol) and [5-^{3}H] dCyd (specific radioactivity, 20 Ci/mmol) were obtained from the Radiochemical Centre Amersham (Amersham, U.K.), whereas [5-^{3}H]dUrd was from ICN Pharmaceuticals (Irvine, CA).

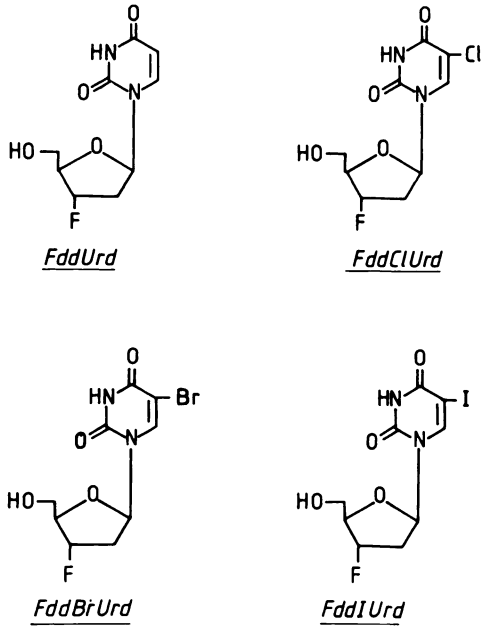

Fig. 1. Structural formulae of the 5-halogeno-FddUrd analogues.

Cells. MT4, CEM, H9, HUT-78, Raji/0, and Raji/TK$^-$ cells (the latter being a dThd kinase-deficient mutant cell line derived from wild-type Raji/0 cells) were grown as described previously (3). The Raji/0 and Raji/TK$^-$ cells were characterized as described earlier (8). Molt/8 cells were cultured in the same medium as described for MT4.

Viruses. HTLV-III$_B$ (designated HIV-1) was derived from a pool of American patients with AIDS and was obtained from the supernatant of HIV-1-infected H9 cell cultures (9). LAV-2 (designated HIV-2) was obtained from Dr. L. Montagnier (Paris, France). Moloney MSV was prepared from tumors induced *in vivo* (3-day-old NMRI mice) according to the procedure described by De Clercq and Merigan (10).

Anti-HIV assays. In a first set of experiments, human T lymphocyte MT4 cells (5×10^5 cells/ml) were suspended in fresh RPMI 1640 culture medium (GIBCO, Grand Island, NY) that contained 10% (v/v) fetal calf serum (GIBCO), 2 mM L-glumamine (Flow Laboratories, McLean, VA), 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 were infected with HIV-1 or HIV-2 at 200 $CCID_{50}$ (1 $CCID_{50}$ being the dose infective for 50% of the cell cultures) per ml of cell suspension. Then, 100 μl of the infected cell suspension were added to 100 μl of an appropriate dilution of test compound in 200-μl microplate wells of a flat-bottomed 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 mock-infected cell cultures (which had been exposed to the same concentrations of the compounds as the virus-infected cells). The ED_{50} and CD_{50} were defined as the compound concentrations required to reduce by 50% the number of viable cells in the virus-infected and mock-infected cell cultures, respectively.

In a second experiment, MT4 cells (10^6 cells/ml) were suspended in culture medium, as described above, and infected with 200 $CCID_{50}$ HIV-1/ml of cell suspension. Then, 50 μl of the infected cell suspension was added to 100 μl of an appropriate dilution of test compound and 50 μl of medium, containing 25 μM dThd, 1000 μM dCyd, or 250 μM dThd plus 1000 μM dCyd, in 200-μl microplate wells. After incubation for 5 days, viable cell counts were determined as described above.

In a third set of experiments, anti-HIV-1 activity of the test compounds was determined by monitoring viral antigen expression on the membrane of HUT-78 cells on the 12th day after HIV-1 infection. Indirect immunofluorescence, using a polyclonal antibody as probe, and laser flow cytofluorographic analysis (FACSTAR; Becton Dickinson) were used for the determination of antigen-positive cells. The polyclonal antibody was derived from a serum from a Zairian patient with high titer of antibodies against HIV-1. The serum was found to be positive by ELISA-HTLV-III (Abbott, North-Chicago, IL) (ratio > 13.5), HTLV-III confirmatory EIA (Abbott) (positive for both envelope and core proteins), and Western blot analysis (Biotech Research Laboratories, Rockville, MD). HUT-78 cells were infected with HIV-1 at 1000 $CCID_{50}$/ml and three quarters of the culture medium were replenished every 4th day. The test compounds were added to the HUT-78 cells immediately after infection and after the first replenishment of the culture medium.

In a fourth set of experiments, the effect of the test compounds on HIV-1-induced cell fusion was examined. One hundred microliters of the test compounds were diluted in 200-μl microplate wells of a flat-bottomed Microtest III Plate (Falcon). Then 5×10^4 HIV-1-infected HUT-78 cells were added to the wells, immediately followed by the addition of 5×10^4 Molt/8 cells, to yield a final volume of 200 μl. The mixed cell culture was then incubated at 37° in a CO_2-controlled humidified atmosphere. Under these experimental conditions, the first visible syncytia appear as soon as 2–5 hr after cocultivation, and 20 hr later an abundant number of syncytia are present in the cell culture.

Transformation of C3H mouse embryo fibroblasts by Molo-

ney MSV. C3H cells were seeded at 20,000 cells/ml into wells of Costar Tissue Culture Cluster plates (48 wells/plate). Twenty-four hours later, cell cultures were infected by 80 foci-forming units of MSV over 120 min, after which the culture medium was replaced by 1 ml of fresh medium containing different concentrations of the test compound. After 6 days, transformation of the cell cultures was examined microscopically.

Inhibition of L1210/0, L1210/TK⁻, Raji/0, Raji/TK⁻, CEM, HUT-78, H9, and MT4 cell proliferation. All assays were performed in flat-bottomed Microtests III Plates (96 wells) (Falcon) as previously described. Briefly, the cells were suspended in growth medium and added to the microplate wells at a density of 5×10^4 L1210 cells/well (200 μl), 6.25×10^4 CEM, HUT-78, H9, and MT4 cells/well, or 7.5×10^4 Raji cells/well in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 48 and 96 hr (L1210 cells), or 72 and 120 hr (other cell lines) 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 ID_{50} was defined as the concentration of compound that reduced the number of cells by 50%.

Inhibition of tritium release from [5-^{3}H]dUrd or [5-^{3}H]dCyd in MT4 cells. The procedure to measure tritium release from [5-^{3}H]dUrd or [5-^{3}H]dCyd in intact cells has been described previously (11). Briefly, 10^7 MT4 cells/ml were preincubated with an appropriate amount of test compound for 15 min at 37°. After this incubation period, radiolabeled [5-^{3}H]dUrd or [5-^{3}H]dCyd (100 μCi/ml; 0.1 μM) was added and, at various times (0, 15, 30, 45, 60 min), 100 μl of the reaction mixture were 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 analyzed for radioactivity.

Enzyme assay. dThd kinase was prepared from exponentially growing MT4 cells, which were first washed (twice) with phosphate-buffered saline at 4°, suspended in buffer containing 10 mM potassium phosphate, pH 7.5, 10 mM β-mercaptoethanol, and 0.1 M KCl, and then homogenized by sonication. The suspensions were clarified by centrifugation at 100,000 × g for 30 min. The enzyme fraction precipitated between 30% and 70% $(NH_4)_2SO_4$ was used in our experiments. [*methyl*-^{3}H]dThd served as the radiolabeled substrate. 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 previously (4).

Results

Antiretrovirus effects of 5-halogeno-FddUrd analogues. FddUrd and its 5-chloro-, 5-bromo-, and 5-iodo-substituted derivatives were evaluated for their inhibitory effects on HIV-1-induced cytopathogenicity in MT4 cells (Table 1; Fig. 2). As a rule, FddClUrd, FddBrUrd, and FddIUrd were 3- to 7-fold less active as antiviral agents than the parent compound FddUrd. Their ED_{50} values ranged from 0.16 to 0.41 μM. The 5-halogeno-FddUrd analogues were also very effective against HIV-2 replication in MT4 cells. In fact, no marked differences were found in the activities of the compounds against HIV-1 and HIV-2. This was also the case for AzddThd. However, marked differences were noted in the cytotoxic properties of the compounds. With CD_{50} values of 1.1 and 2.2 μM, FddUrd and FddIUrd were the most cytotoxic and, with a CD_{50} of 535 μM, FddClUrd was the least cytotoxic agent for the host (MT4) cells. FddBrUrd showed an intermediary cytotoxicity (CD_{50}, 24 μM). Consequently, the selectivity index (CD_{50}/ED_{50}) of the 5-halogeno-FddUrd analogues varied markedly from one compound to another. FddClUrd had the greatest selectivity index (1408) and FddIUrd had the lowest (14). Thus, FddClUrd proved superior to the other 5-halogeno-FddUrd analogues in its anti-HIV selectivity. Although 100-fold more potent than FddClUrd in inhibiting the cytopathogenicity of HIV in MT4 cells, AzddThd also proved about 100-fold more cytotoxic, so that the selectivity index of FddClUrd was quite comparable to that of AzddThd. On the other hand, FddThd, which is an extremely potent agent against HIV replication in MT4 cells, was about 2500-fold more cytotoxic than FddClUrd, so that the selectivity index of FddThd was 7-fold lower than that obtained for FddClUrd.

We also evaluated the 5-halogeno-FddUrd analogues for their inhibitory effects on viral antigen expression in HIV-1-infected HUT-78 cells (Table 1). FddUrd, FddClUrd, FddBrUrd, and FddIUrd were equally effective in inhibiting HIV-1 antigen expression (ED_{50}, 1.4–4.4 μM). None of the 5-halogeno-FddUrd analogues were markedly cytostatic to H9, CEM, or HUT-78 cells at concentrations up to 500 μM (Table 2). Also, the proliferation of murine L1210/0 and L1210/TK⁻ and human Raji/0 and Raji/TK⁻ cells was not affected by any of the test compounds at 500 μM (data not shown). Only with MT4 cells was cytotoxicity observed (Table 2), and this cytotoxicity markedly increased even up to 100-fold if the cells were incubated with the compounds (FddBrUrd, FddIUrd, FddUrd, FddThd, or AzddThd) for 5 instead of 3 days. The most cytotoxic was FddThd (Table 2); it was inhibitory to MT4 cell growth at a concentration of 0.1 μM (after 5 days). However, H9 cell growth was not impaired by FddThd even at a concentration of 500 μM.

To determine whether the test compounds were also inhibitory to HIV-1-induced cell fusion (syncytium formation), we cocultivated persistently infected HUT-78 cells with uninfected Molt/8 cells in the presence of the compounds. In this system, uninfected cells are able to interact with viral proteins, expressed on the surface of the HIV-1-infected HUT-78 cells, leading to syncytium formation within a few hours of cocultivation. None of the FddUrd derivatives, including FddThd, FddUrd itself, and AzddThd, showed any inhibition of syncytium formation at a concentration as high as 50 μM, whereas under the same conditions sulfated polysaccharides such as dextran sulfate are highly active in suppressing syncytium formation (data not shown).

When examined for their inhibitory effect on MSV-induced transformation of murine C3H fibroblasts, none of the 5-halogen-substituted FddUrd derivatives, including the parent FddUrd, showed a marked anti-MSV activity (ED_{50}, $\gg$100 μM), whereas, AzddThd and FddThd were extremely effective in inhibiting the retrovirus-induced transformation of the murine cells (ED_{50}, 0.02 and 0.06 μM, respectively) (Table 1.)

Effect of dThd, dCyd, and uridine on the anti-HIV-1 activity of 5-halogeno-FddUrd analogues in MT4 cells. If intracellular phosphorylation of the FddUrd derivatives by dThd kinase and other kinases involved in dThd metabolism is required for their antiretrovirus activity, addition of dThd should reverse the biological activity of the FddUrd derivatives. Indeed, addition of 250 μM dThd (in the presence of 1000 μM dCyd to avoid cytotoxicity of dThd) resulted in a decrease of the anti-HIV-1 activity of FddUrd and its congeners by more than 2 to 3 orders of magnitude (Table 3). Also, a dramatic decrease in selectivity index was observed. Addition of 25 μM dThd resulted in a 2- to 7-fold decrease of the anti-HIV-1 activity of the FddUrd analogues. Addition of uridine (1000 μM) decreased the activity and toxicity of FddUrd, FddClUrd, FddBrUrd, and FddIUrd to a similar extent, so that their

TABLE 1

Antiretroviral effects of 5-halogeno-FddUrd analogues

Compound	HIV-1-induced cytopathogenicity in MT4 cells			Viral antigen expression in HIV-1-infected HUT-78 cells, ED_{50}	HIV-2-induced cytopathogenicity in MT4 cells ED_{50}	MSV-induced transformation of C3H cells		
	ED_{50}[a]	CD_{50}[b]	SI[c]			ED_{50}	MCC[d]	SI
	μM			μM	μM	μM		
FddClUrd	0.38 ± 0.06	535 ± 41	1408	1.4 ± 0.7	0.69 ± 0.15	457 ± 7	>500	>1.1
FddBrUrd	0.41 ± 0.16	24 ± 18	59	3.4 ± 1.2	0.54 ± 0.12	313 ± 13	>500	>1.4
FddIUrd	0.16 ± 0.1	2.2 ± 2.0	14	4.4 ± 2.3	0.23 ± 0.03	>100	±500	<5
FddUrd	0.06 ± 0.02	1.1 ± 0.2	18	3.8 ± 1.7	0.16 ± 0.03	>500	>500	
FddThd	0.001[e]	0.197[e]	197[e]		0.015 ± 0.003	0.06[e]	>500	>8333
AzddThd	0.003 ± 0.001	4.81 ± 2.53	1603	0.031 ± 0.01	0.004 ± 0.0003	0.02[e]	>500	>25000

[a] 50% antiviral effective dose, or concentration required to cause a 50% reduction (i) in the number of viable cells in virus-infected MT4 cell cultures, (ii) in HIV-1-induced cell surface antigen expression in HUT-78 cells, or (iii) in MSV-induced transformation of C3H cells.
[b] 50% cytotoxic dose, or concentration required to reduce the number of viable cells in the uninfected MT4 cell cultures.
[c] Selectivity index or ratio CD_{50}/ED_{50} or MCC/ED_{50}.
[d] Minimum cytotoxic concentration. The parameter followed here was an alteration of normal cell morphology.
[e] Data taken from reference 3.

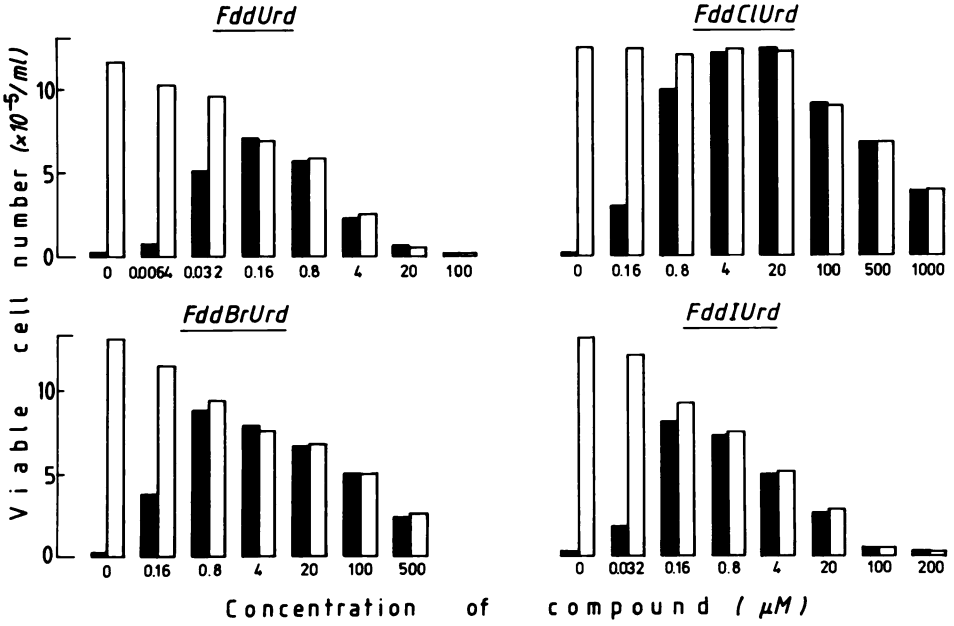

Fig. 2. Inhibition of HIV cytopathogenicity in MT4 cells by various 5-halogeno-FddUrd analogues. Viability of the cells was measured by the trypan blue exclusion method on the 5th day after infection. ■, HIV-infected cells; □, mock-infected cells.

TABLE 2

Cytostatic effect of 5-halogeno-FddUrd derivatives against human T lymphocyte CEM, HUT-78, H9, and MT4 cells

Compound	ID_{50}[a]							
	CEM		HUT-78		H9		MT4	
	3 day	5 day	3 day	5 day	3 day	5 day	3 day	5 day
	μM							
FddClUrd	>500	>500	>500	>500	>500	>500	>500	>500
FddBrUrd	>500	>500	>500	>500	>500	>500	>500	95
FddIUrd	383	>500	>500	434	>500	>500	38	3.3
FddUrd	420	>500	>500	>500	>500	>500	273	2.4
FddThd	8.4	117	102	38	>500	>500	2.8	0.1
AzddThd	>500	>500	>500	171	>500	>500	33	4.6

[a] 50% inhibitory dose, or concentration required to reduce the cell number by 50% following either a 3-day or a 5-day incubation period.

selectivity index remained essentially the same. For AzddThd, however, a 4-fold increase in selectivity was noted after combination with Urd.

Interaction of the 5-halogeno-FddUrd analogues with the phosphorylation of dThd. FddClUrd, FddBrUrd, FddIUrd, and FddUrd were evaluated for their potential to inhibit [*methyl*-^{3}H]dThd phosphorylation by crude enzyme extracts from MT4 cells (Table 4). all four compounds competitively inhibited the dThd kinase reaction (Fig. 3). The k_i/K_m ratios for FddClUrd, FddBrUrd, and FddIUrd were quite similar (4.7, 4.4, and 4.0, respectively). These low K_i/K_m values suggest that the compounds act as potent inhibitors of dThd phosphorylation and may serve as good substrates for the MT4 cell dThd kinase. The K_i/K_m of the MT4 cell dThd kinase for FddUrd was considerably higher than the K_i/K_m values obtained for the 5-halogeno-FddUrd derivatives. Yet, for FddThd and AzddThd, K_i/K_m values were found that were similar (FddThd) or only 4–5 times lower (AzddThd) than those of the 5-halogeno-FddUrd analogues (data not shown).

The differential affinities of the 5-halogeno-FddUrd deriva-

TABLE 3
Effect of the addition of dCyd and dThd on the anti-HIV-1 activity of 5-halogeno-FddUrd analogues

Compound	Addition	ED_{50}[a]	CD_{50}[b]	SI[c]
		μM		
FddClUrd	None	0.38	535	1408
	dThd (25 μM)	2.5	442	177
	dCyd (1000 μM)	11	789	72
	dThd (250 μM) + dCyd (1000 μM)	52	500	10
	Uridine (1000 μM)	5.2	>1000	>192
FddBrUrd	None	0.41	24	59
	dThd (25 μM)	0.76	79	104
	dCyd (1000 μM)	23	370	16
	dThd (250 μM) + dCyd (1000 μM)	140	135	0.96
	Uridine (1000 μM)	4.7	468	100
FddIUrd	None	0.16	2.2	14
	dThd (25 μM)	0.26	1.7	7
	dCyd (1000 μM)	8.0	71	9
	dThd (250 μM) + dCyd (1000 μM)	360	>500	1.4
	Uridine (1000 μM)	10	135	14
FddUrd	None	0.04	0.95	24
	dThd (25 μM)	0.09	5.9	66
	dCyd (1000 μM)	7.6	79	10
	dThd (250 μM) + dCyd (1000 μM)	>500	>500	
	Uridine (1000 μM)	19	440	23
AzddThd	None	0.003	4.8	1603
	dThd (25 μM)	0.006	7.0	1167
	dCyd (1000 μM)	0.035	66	1886
	dThd (250 μM) + dCyd (500 μM)	29	>400	>14
	Uridine (1000 μM)	0.02	134	6700

[a] 50% effective dose.
[b] 50% cytotoxic dose.
[c] Selectivity index or ratio of CD_{50}/ED_{50}.

TABLE 4
Inhibition of MT4 cell dThd kinase by 5-halogeno-FddUrd analogues

Compound	K_i	K_i/K_m[a]	Type of inhibition
	μM		
FddClUrd	6.1	4.7	Competitive
FddBrUrd	5.7	4.4	Competitive
FddIUrd	5.2	4.0	Competitive
FddUrd	392	302	Competitive

[a] K_m values obtained in the individual experiments ranged from 1.1 to 1.5 μM.

tives, as well as FddUrd, FddThd, and AzddThd, for dThd kinase closely correlated with their inhibitory effects on intracellular tritium release from [5-^{3}H]dUrd (Table 5). This ^{3}H release occurs during the dTMP synthase step and requires that [5-^{3}H]dUrd is phosphorylated to [5-^{3}H]dUMP. Hence, inhibitors of this phosphorylation may be expected to interfere with the ^{3}H release from [5-^{3}H]dUrd. AzddThd and FddThd, which are both good substrates and competitive inhibitors of dThd kinase (3, 12), also proved to be potent inhibitors of tritium release from [5-^{3}H]dUrd (ID_{50}, 2.7 and 29 μM, respectively). FddClUrd, FddBrUrd, and FddIUrd, which inhibit (*methyl*-^{3}H]dThd phosphorylation less efficiently than AzddThd and FddThd, also proved less inhibitory to tritium release from [5-^{3}H]dUrd (ID_{50}, 147–287 μM). FddUrd, a very weak inhibitor of dThd kinase, did not affect tritium release from [5-^{3}H]dUrd at 500 μM. None of the test compounds affected tritium release from [5-^{3}H]dCyd even at a concentration as high as 500 μM (Table 5).

Discussion

Our present findings indicate that the 5-halogen-substituted derivatives of FddUrd are potent inhibitors of both HIV-1 and HIV-2 replication *in vitro*. Their ED_{50} values for HIV replication in MT4 cells ranged from 0.2 to 0.8 μM. Striking differences were noted in the toxicity of the compounds for the host cells. FddClUrd proved much less cytotoxic to MT4 cells than FddUrd, FddBrUrd, or FddIUrd. Consequently, the selectivity index of FddClUrd markedly exceeded that of FddBrUrd, FddIUrd, or FddUrd. FddClUrd also proved more selective as an anti-HIV agent than FddThd. Its selectivity was similar to that AzddThd.

Recently, Mitsuya and co-workers (14) reported that AzddThd and 2′,3′-dideoxyadenosine, in contrast to dextran sulfate [a compound shown to inhibit HIV-1 binding to the cells (13, 14)], were ineffective against syncytium formation in cocultures of ATH8 cells and persistently HIV-1-infected H9 cells. We also investigated whether the 5-halogeno-FddUrd analogues were able to suppress syncytium formation in a coculture of persistently HIV-1-infected HUT-78 cells and uninfected Molt/8 cells and we found that none of the compounds prevented HIV-1-mediated cell fusion. In this respect the test compounds behaved like AzddThd, whose mechanism of antiretroviral action is assumed to be due to a selective inhibition of reverse transcriptase (15). Indeed, in the cocultivation assay, viral antigens present in the membrane of the persistently infected HUT-78 cells (presumably gp120) combine with the CD4 receptor present in the membrane of uninfected Molt/8 cells. This process results in giant cell formation. It is obvious that compounds that exert their antiviral action against a step in the replication cycle of the virus (i.e., reverse transcriptase) do not have any effect on the binding of gp120-expressing cells with CD4-expressing cells. Thus, most probably, 5-halogeno-FddUrd derivatives are, like AzddThd, targeted at the reverse transcriptase, which implies that they are previously phosphorylated to the 5′-mono-, 5′-di-, and 5′-triphosphate form.

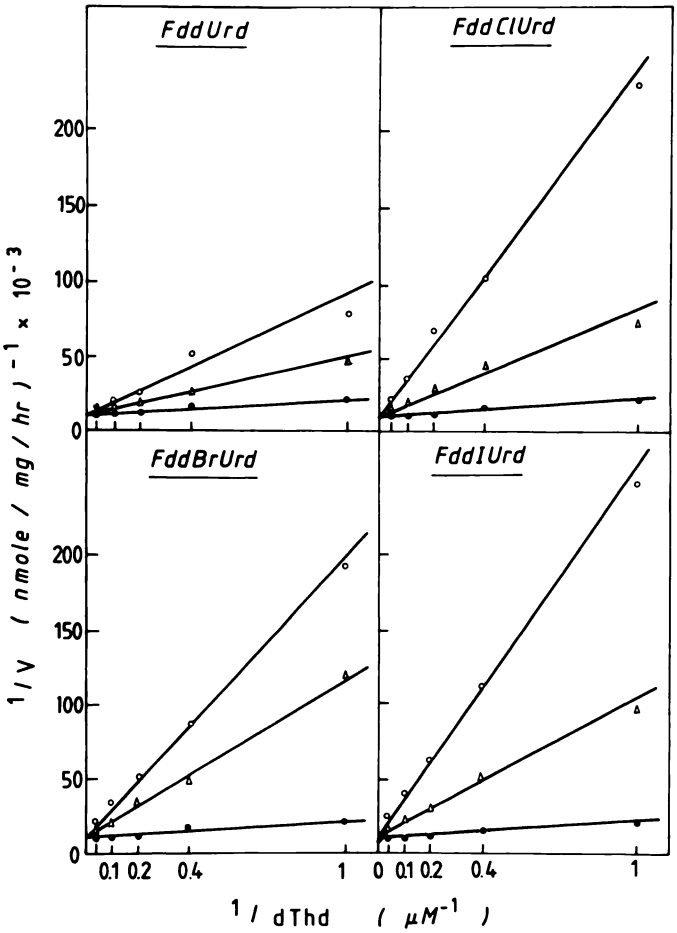

Fig. 3. Double-reciprocal plots for inhibition of MT4 cell dThd kinase by FddUrd (A), FddClUrd (B), FddBrUrd (C), and FddIUrd (D). Inhibitory concentrations: 0 mM ;(●), and 1 mM (△) for FddUrd; 0 mM (●), 100 μM (○), and 40 μM (△) for FddClUrd, FddBrUrd, and FddIUrd.

TABLE 5

Inhibitory activity of 5-halogeno-FddUrd analogues on tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd in MT4 cells

Compound	ID_{50}*	
	[5-^{3}H]dUrd	[5-^{3}H]dCyd
	μM	
FddClUrd	287 ± 100	>500
FddBrUrd	157 ± 82	>500
FddIUrd	147 ± 62	>500
FddUrd	>500	>500
FddThd	29 ± 17	>500
AzddThd	2.7 ± 1.3	>500

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

It is noteworthy that the 5-halogeno-FddUrd analogues and FddUrd itself were virtually inactive as inhibitors of MSV-induced transformation of C3H cells. In contrast, FddThd and AzddThd are exquisitely active as antiretroviral agents in this murine cell system. Datema and co-workers (16) recently reported that the 5′-triphosphates of several ddUrd analogues have a much lower affinity for the murine retrovirus (Rauscher leukemia virus) reverse transcriptase than for HIV reverse transcriptase. If these findings can be extended to the 5-halogeno-FddUrd analogues, they could readily explain why the compounds fail to inhibit murine retrovirus replication in cell culture. Synthesis of the 5′-triphosphate derivatives of the 5-halogeno-FddUrd analogues and direct testing of these compounds against MSV-specified reverse transcriptase will clarify this issue.

The 5-halogeno-FddUrd analogues seem to be better substrates for the MT4 cell dThd kinase than FddUrd. This assumption is based on the relatively low K_i/K_m values of MT4 cell dThd kinase for FddClUrd, FddBrUrd, and FddIUrd and their inhibitory effect on intracellular tritium release from [5-^{3}H]dUrd. None of the test compounds proved inhibitory to tritium release from [5-^{3}H]dCyd, which excludes thymidylate synthase as a possible target for the antimetabolic action of these compounds. Indeed, we have previously shown that compounds whose antimetabolic activity is mediated by an inhibition of thymidylate synthase strongly inhibit tritium release of both [5-^{3}H]dUrd and [5-^{3}H]dCyd (11).

As a rule, FddClUrd proved less cytotoxic to human cell lines than FddUrd and the other 5-halogeno-FddUrd analogues. However, marked differences were noted in the cytotoxic effects of the compounds depending the cell line. Also, the cytotoxic effects of the 3′-fluoro-ddUrd derivatives seemed to increase as a function of incubation time. The molecular basis of this phenomenon deserves further study.

Recently, we found AzddThd, AzddUrd, FddUrd, and FddClUrd highly dependent on dThd kinase to be inhibitory against simian AIDS-related virus-infected Raji cells (17). However, although phosphorylation of the test compounds is necessary for them to exert their antiviral (and cytostatic) action, there was no apparent correlation between the affinity (K_i/K_m) of the compounds for their activating enzyme dThd kinase and their eventual antiretroviral activity. For example, FddUrd had a 70-fold lower affinity for the MT4 cell dThd kinase than its 5-halogeno-substituted congeners and yet was 3- to 7-fold more potent against HIV-1 replication. Also, the cytotoxicity of the compounds did not directly correlate with their antiretroviral activity and/or their affinity for dThd kinase. It would seem imperative, therefore, to synthesize the 5′-triphosphate derivatives of the 5-halogeno-FddUrd analogues and to examine their affinities for HIV reverse transcriptase and cellular DNA polymerases α, β, and γ. From such investigations, we should be able to assess the selectivity of the 5-halogeno-FddUrd 5′-triphosphates towards the reverse transcriptase and to delineate a structure-activity relationship of these 5′-triphosphates for the reverse transcriptase and the cellular DNA polymerases. Also, radiolabeled 5-halogeno-FddUrd should be synthesised to determine the extent of incorporation of these compounds into viral and cellular DNA.

In conclusion, the findings that FddClUrd, FddBrUrd, and FddIUrd are inhibitory to HIV-1 and HIV-2 in several cell systems and that FddClUrd is endowed with a high selectivity against HIV *in vitro* make the 5-halogeno-FddUrd derivatives, and in particular FddClUrd, attractive candidates that should be further pursued for their potential in the treatment of AIDS.

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References

1. De Clercq, E. Perspectives for the chemotherapy of AIDS. *Anticancer Res.* **7**:1023–1038 (1987).
2. De Clercq, E., A. Van Aerschot, P. Herdewijn, M. Baba, R. Pauwels, and J. Balzarini. Anti-HIV-1 activity of 2′,3′-dideoxynucleoside analogues: structure-activity relationship. *Nucleosides Nucleotides,* in press.
3. Balzarini, J., M. Baba, R. Pauwels, P. Herdewijn, and E. De Clercq. Antiretrovirus activity of 3′-fluoro- and 3′-azido-substituted pyrimidine 2′,3′-dideoxynucleoside analogues. *Biochem. Pharmacol.* **37**:2847–2856 (1988).
4. Balzarini, J., E. De Clercq, M. P. Mertes, D. Shugar, and P. F. Torrence, 5-Substituted 2′-deoxyuridines: correlation between inhibition of tumor cell growth and inhibition of thymidine kinase and thymidylate synthetase. *Biochem. Pharmacol.* **31**:3673–3682 (1982).
5. Herdewijn, P., J. Balzarini, E. De Clercq, R. Pauwels, M. Baba, S. Broder, and H. Vanderhaeghe. 3′-Substituted 2′,3′-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. *J. Med. Chem.* **30**:1270–1278 (1987).
6. Horwitz, J. P., J. Chua, and M. Noel. Nucleosides. V. The monomesylates of 1-(2′-deoxy-β-D-lyxofuranosyl)thymine. *J. Org. Chem.* **29**:2076–2078 (1964).
7. Herdewijn, P., A. Van Aerschot, and L. Kerremans. Synthesis of nucleosides fluorinated in the sugar moiety: the application of diethylaminosulfur trifluoride to the synthesis of fluorinated nucleosides. *Nucleosides Nucleotides,* **8**:65–96 (1989).
8. Balzarini, J., E. De Clercq, P. F. Torrence, M. P. Mertes, J. S. Park, C. L. Schmidt, D. Shugar, P. J. Barr, A. S. Jones, G. Verhelst, and R. T. Walker. Role of thymidine kinase in the inhibitory activity of 5-substituted-2′-deoxyuridines on the growth of human and murine tumor cell lines. *Biochem. Pharmacol.* **31**:1089–1095 (1982).
9. Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science (Wash., D. C.)* **224**:497–500 (1984).
10. De Clercq, E., and T. C. Merigan. Moloney sarcoma virus-induced tumors in mice: inhibition or stimulation by (polyrI)·(polyrC). *Proc. Soc. Exp. Biol. Med.* **137**:590–594 (1971).
11. Balzarini, J., and E. De Clercq. Strategies for the measurement of the inhibitory effect of thymidine analogs on the activity of thymidylate synthase in intact murine leukemia L1210 cells. *Biochim. Biophys. Acta* **785**:36–45 (1984).
12. Balzarini, J., E. Matthes, P. Meeus, D. G. Johns, and E. De Clercq. The antiretroviral and cytostatic activity, and metabolism of 3′-azido-2′,3′-dideoxythymidine, 3′-fluoro-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine are highly cell type-dependent. *Proc. VI Int. Symp. Human Purine Pyrimidine Metabolism,* in press.
13. Baba, M., R. Pauwels, J. Balzarini, J. Arnout, J. Desmyter, and E. De Clercq. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**:6132–6136 (1988).
14. Mitsuya, H., D. J. Looney, S. Kuno, R. Ueno, F. Wong-Staal, and S. Broder. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4$^+$ cells. *Science, Wash., D. C.* **240**:646–649 (1988).
15. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. Nusinoff-Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333–8337 (1986).
16. Bazin, H., J. Chattopadhyaya, R. Datema, G. Gilljam, N. G. Johansson, R. Koshida, B. Oberg, G. Stening, L. Vrang, B. Wahren, and J.-C. Wu. Inhibition of replication of HIV and MULV by some 3′-blocked pyrimidine analogs. *Int. Symp. Basic Clin. Approaches Virus Chemother. Abstr.* 48 (1988).
17. Balzarini, J., A. Van Aerschot, P. Herdewijn, and E. De Clercq. 5-Chloro-substituted derivatives of 2′,3′-didehydro-2′,3′-dideoxyuridine, 3′-fluoro-2′,3′-dideoxyuridine and 3′-azido-2′,3′-dideoxyuridine as anti-HIV agents. *Biochem. Pharmacol.* **38**:869–874 (1989).

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