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Infectious Amplification of Wild-Type Human Immunodeficiency Virus from Patients' Lymphocytes and Modulation by Reverse Transcriptase Inhibitors In Vitro

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The relative in vitro potency of nine human immunodeficiency virus (HIV) type 1 reverse transcriptase inhibitors was evaluated in a coculture assay which measures the frequencies of infectious primary cells from HIV-positive patients by the limiting dilution technique and measures their apparent reduction under increasing concentrations of drugs. An advantage of this assay is that it utilizes a variety of wild-type viruses not selected by in vitro propagation. Potency ranking placed the (−)-L-enantiomer of 2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC], an oxathiolane pyrimidine nucleoside analog (90% effective concentration = 55 nM), before 2′,3′-dideoxycytidine (DDC) (74 nM), (−)-2′,3′-dideoxy-3′-thiacytidine (3TC) (300 nM), 3′-azido-3′-deoxythymidine (AZT) (530 nM), TIBO R82913 (670 nM), and 2′,3′-dideoxyinosine (DDI) (6,400 nM). HIV from AZT-naive patients' lymphocytes was more sensitive to the inhibitory effect of (−)-FTC, 3TC, or DDC than was highly AZT-resistant HIV obtained from AZT-treated patients' cells, indicating partial cross-resistance between thymidine and cytidine analogs. Combined inhibitory concentrations of AZT with (−)-FTC, 3TC, DDC, and DDI produced synergistic interactions as determined by the multiple-drug effect analysis. Synergistic interactions were demonstrable with AZT plus (−)-FTC or with AZT plus DDC with cells bearing AZT-resistant HIV. The inhibitory concentrations of AZT established by this cell-to-cell virus transmission assay are closer than those determined by the conventional assay system to the extracellular AZT concentrations required in patients' plasmas to achieve comparable levels of HIV inhibition in vivo.

Nucleoside antiviral agents continue to be the most successful class of compounds for the treatment of human immunodeficiency virus type 1 (HIV-1) infection in humans (15, 19). Recently, we reported on the in vitro anti-HIV-1 and anti-hepatitis B virus activities of 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) and its enantiomers (2, 5, 6, 18). The (−)-β-L-enantiomer of FTC [(−)-FTC] was found to be more potent than its (+)-β-D-counterpart. Since (−)-FTC is a leading candidate for clinical trials in individuals infected with HIV, its antiviral activity, along with those of a series of nucleoside and nonnucleoside reverse transcriptase (RT) inhibitors, was tested in a primary lymphocyte culture system which models in vivo infection. Single compound and combination studies with (−)-FTC and other inhibitors of HIV RT were performed with cells from 3′-azido-3′-deoxythymidine (AZT)-naive and AZT-treated patients.

MATERIALS AND METHODS

Compounds. The oxathiolane pyrimidine nucleosides were synthesized as previously reported (1, 3, 8, 16, 18). AZT was provided by Burroughs Wellcome Co., Research Triangle Park, N.C. 2′,3′-Dideoxyinosine (DDI) and 2′,3′-dideoxycytidine (DDC) were obtained from Sigma, St. Louis, Mo. TIBO R82913 [(+)-(5*S*)-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-thione] was obtained from G. Pialloux (Pasteur Hospital, Paris, France).

Infected PBMC. The naturally infected cells used as sources of HIV-1 for the cocultures had been drawn between 1986 and 1991 from the peripheral blood of HIV-1-infected individuals for the purpose of future virological studies. Informed consent had been obtained from each patient. In preliminary studies, aliquots of the cell specimens contained between 500 and 20,000 infectious cells per 10^7 peripheral blood mononuclear cells (PBMC) as determined by limiting dilution analysis (see below). The isolation of these PBMC using Ficoll-Hypaque gradients, their cytofluorometric characterization, and their freezing in dimethyl sulfoxide culture medium, storage in liquid nitrogen, thawing, washing, and counting at the time of culture were performed as described previously (13). Viability after the cells were thawed was ≥95% (13). As HIV amplifier cells for these studies, lymphocytes of four regular HIV-seronegative donors were selected because of the ability of their lymphocytes to support the replication of most HIV isolates to high titers within 12 days of coculture.

Quantitation of infectious cells of patients. In order to establish titrated standards of infectious cells, 61 reference cell specimens, obtained from patients infected with HIV for 1 to 7 years, were assayed for the frequencies of infectious cells by semiconservative limiting dilutions using up to 14 replicates of serial 2.24-fold cell dilutions. The culture conditions were as follows. The freshly thawed PBMC of patients were resuspended in RPMI 1640 supplemented with 1% glutamine, 1% pyruvate, 50 IU of human recombinant interleukin 2 (Roussel-UCLAF, Paris, France), and 10% (vol/vol) fetal bovine serum. Duplicates (500 μl) of each cell dilution were seeded in 24-well plates (Nunc Inc., Naperville, Ill.). To the diluted cells, 3.5×10^5 (in 0.5 ml of culture

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medium) freshly thawed uninfected human lymphocytes were added as targets for de novo infections and subsequent biological amplification. These amplifier cells had been stimulated in prior cultures with phytohemagglutinin (PHA) and interleukin 2 for 3 days, after which they were washed, frozen, and stored in liquid nitrogen until use, weeks or months later. The mixed cell suspensions were cultivated in a humidified 5% CO_2–95% air atmosphere for 12 days. One milliliter of fresh medium was added on day 3 or 4. On day 12, clarified supernatants were treated with Triton X-100 and tested for HIV-1 antigen by immunocapture using either a commercial kit (Abbott Research Laboratories, Chicago, Ill.) or an enzyme-linked immunosorbent assay (ELISA) system. Previous studies had indicated that by day 12, cultures which were to be HIV positive by day 21 were already >90% positive (13). For the ELISA, 96-well plates (Nunc) were precoated with the DEAE immunoglobulin G fraction from a single pool of serum samples obtained from multiple asymptomatic HIV carriers with high p24 serum antibody titers. After overnight incubation with the detergent-treated culture supernatants, the immunoglobulin G-precoated wells were washed and a titrated peroxidase-labeled polyclonal rabbit p24 antibody (Abbott) was added. Cocultures in which supernatants gave signal/cutoff ratios greater than 10 were considered positive for HIV propagation, while the infectious material borne by patients' unamplified cells consistently gave undetectable signals. Cultures giving signal/cutoff ratios between 3 and 10 at day 12 were maintained until day 14, when their supernatants were retested for p24-reactive HIV antigen. The mathematics of the limiting dilution technique predicts that the logarithms of the percentages of virus-negative replicate cultures should fall linearly with the numbers of donor cells per well if the rare event that is measured (an infectious event) does follow a "single-hit" order. Our experimental results were in agreement with the Poisson equation inasmuch as they fitted linear, first-order kinetics, at least for the initial part of the limiting dilution curves. Accordingly, culture replicates yielding 37% virus-negative (or 63% positive) results statistically contain one infectious cell on the average (10). The numbers of infectious cells detected in the reference specimens ranged between 3 to 5 and 10^4 or higher per 10^7 PBMC.

Frequencies of infectious cells and virus scores. The method used for determining virus scores has been described previously (13). Briefly, the routine titration of infectious cells of patients uses conservative limiting dilutions with three dilutions per cell specimen, each of them fivefold apart, and six replicates for each cell concentration tested. To maximize the mathematical use of such limited dilutions, the virus-positive replicates from two contiguous dilutions were combined to yield virus scores, i.e., percentages of virus-positive wells from 6 + 6 proximate cell dilutions (13). Plotting virus scores for the 61 reference specimens against the frequencies of infectious cells provided a series of linear curves which allowed a direct readout of infectious-cell frequencies from a given virus score. For instance, one virus-positive culture out of 6 + 6 replicates of 500×10^3 and 100×10^3 cells of patients, i.e., a virus score of 8%, corresponds to 3 to 5 infectious cells per 10^7 PBMC on our standard reference curves (data not shown).

Interassay variability of the system. A total of 110 frozen specimens from different patients were selected to cover the entire range of our titration system, between 3 to 5 and 2×10^4 (or more) infectious units per 10^7 PBMC. The first and second runs were performed 1 to 22 months apart, and the frequencies of infectious cells obtained in each paired experiment were averaged. The differences between the calculated means and the percentage of virus-positive replicates within a given pair were in turn averaged.

Titration of infectious cells in the presence of various concentrations of antiviral compounds. Six replicates of freshly thawed infected-cell suspensions, serially diluted fivefold as described above, were cocultivated with 3×10^5 PHA-stimulated normal human PBMC which had been exposed either to medium (control) or to medium containing the antiviral compounds for at least 2 h before cocultivation. Antiviral compounds were maintained throughout the cocultivation period. Immunocapture assays (p24) of culture supernatants were performed on day 12. The apparent frequencies of infectious cells producing biologically amplifiable HIV in the presence of the drug were derived from virus scores. Up to 10 times more cells of patients were inoculated into the cultures exposed to the highest drug concentrations so as to eventually detect >99% inhibitions of HIV replication rates. The final results are given as percentages of inhibition in reference to the frequencies of infectious cells expected from the inoculated cells of patients from their frequencies in cultures concurrently evaluated without drug. In experiments in which a given patient's cell inoculum was used with different amplifier cells, the resulting mean frequencies were taken into account. The 50, 90, and 99% effective concentrations (EC_{50}s, EC_{90}s, and EC_{99}s, respectively) were derived from computer-generated median effect plots of the dose-response data (4). To explore whether synergistic, additive, or antagonistic antiviral interactions were obtained in cultures treated with combinations of AZT and other compounds, the multiple-drug effect analysis developed by Chou and Talalay (4) was used. The interaction of the drugs was determined by calculating the combination index (CI) with assumptions of mutually nonexclusive interactions since the dose response curves were not parallel. A CI of <1 indicates synergy, a CI of 1 indicates additive effects, and a CI of >1 indicates antagonism. The concentrations for each of the compounds tested were nontoxic to the lymphocytes, in agreement with previous data (1, 6, 16, 18). The type of interaction for AZT and FTC in experiments using the cells from AZT-treated patients was also determined by using the fractional effect method as described previously (20).

RESULTS

Reproducibility of the quantitation system. The mean variation ± standard deviation (SD) among 110 repeat specimens tested twice over 1- to 22-month periods was 41% ± 35% (data not shown). When each of the two runs was performed with a different donor of PHA-stimulated lymphocytes, the variation coefficient for 51 determinations was 48% ± 35%. Thus, two specimens which would differ by 2.5-fold would have 95 chances in 100 of actually carrying different infectious loads. Consequently, apparent reductions in HIV propagation rates smaller than 60% would not be statistically different from no inhibition. Nevertheless, EC_{50}s were computed from the median effect plots of the dose response curves (4). The effect of different amplifying cells and clinical cell specimens on the inhibitory potency of a given drug was also examined. The inhibitory efficacy of AZT on the replication of AZT-naive HIV obtained from four infected persons was tested on PHA-stimulated normal PBMC from our four regular donors; the variations of inhibition levels were well within the standard error of the titration system (data not shown).

TABLE 1. Inhibition of cell-to-cell infection by nine nucleoside and nonnucleoside RT inhibitors in experiments using human PBMC obtained from HIV-infected and uninfected healthy individuals

Concn (μM)	% Inhibition[a] (no. of donors[b])									
	(−)-FTC	(+)-FTC	(±)-FTC	3TC [(−)-BCH-189]	(+)-BCH-189	DDC	DDI	AZT	TIBO R82913	(−)-FTC (AZT^r)[c]
0.003	38 ± 25 (5)									
0.01						46 ± 29 (6)				
0.015	57 ± 13 (7)									
0.02	59 ± 24 (9)	58 (2)			49 (2)	71 ± 15 (4)		35 ± 31 (5)		
0.03				38 ± 25 (4)					8 ± 17 (5)	62 ± 25 (10)
0.045								44 ± 25 (6)		
0.075	88 ± 13 (11)		33 ± 40 (3)	34 ± 32 (7)						
0.1						88 ± 6 (3)		55 ± 25 (27)		
0.2	99 ± 0.5 (18)		95 ± 0.2 (3)	78 ± 12 (6)			20 ± 23 (6)			84 ± 19 (13)
0.3									57 ± 18 (8)	
0.5				96 ± 3 (3)		99 ± 0.4 (4)	45 ± 32 (10)	89 ± 9 (22)		
0.6	99 ± 0.3 (7)									96 ± 0.6 (9)
0.75				99 ± 0.2 (4)						
0.9									91 ± 4 (3)	
1.0							66 ± 10 (4)			
1.5							55 ± 33 (4)	95 ± 5 (20)		
1.8										99.6 ± 0.6 (7)
2	99 (2)	60 (2)	99 (2)	99 ± 0.1 (6)	55 (2)				99 ± 0.9 (8)	
4.5							83 ± 10 (14)	99 ± 1 (20)		
25							97 (2)			
50							99 ± 0.2 (5)			

[a] Mean ± SD.

[b] Number of different primary HIV$^+$ cell donors whose cells were cocultivated with activated normal human lymphocytes. The human donors had not previously been treated with AZT.

[c] Numbers in parentheses are numbers of cell specimens in which more than 10% of infectious cells express HIV that is resistant to 4.5 μM AZT. More than 80% of all recovered infectious HIV in these cells was resistant to 0.45 μM AZT.

Concentration-dependent antiviral potency of (−)-FTC and other RT inhibitors in activated normal human lymphocytes. The ranking of anti-HIV replication potency among eight nucleoside analogs and one nonnucleoside RT inhibitor was established (Tables 1 and 2). (−)-FTC and the racemic mixture [(±)-FTC, containing equimolar amounts of (+)-FTC and (−)-FTC] were the most active in the assay. The three cytidine analogs tested, (−)-FTC, DDC, and 3TC [(−)-BCH-189], were sufficiently active at submicromolar concentrations (≤0.85 μM) to inhibit 99% of the virus replication as measured by inhibition of replication rates. AZT required a significantly greater concentration (6.4 μM) to achieve similar potency. The (+)-enantiomers of both FTC and BCH-189 had no significant effect (<63% inhibition) on HIV replication at concentrations as high as 2 μM. DDI also displayed low antiviral potency in this system, reaching a significant level of inhibition (63%) at about 2 μM and reaching 90% efficacy at 6.4 μM (Tables 1 and 2). The nonnucleoside RT inhibitor TIBO R82913 exhibited potent activity with an EC_{90} of 0.7 μM.

TABLE 2. Effective concentrations of inhibitors in experiments using human PBMC obtained from HIV-infected and uninfected healthy individuals[a]

Inhibitor	EC_{50}	EC_{90}	EC_{99}
(−)-FTC	0.0085 (0.008)	0.055 (0.04)	0.43
(+)-FTC	NA (0.84)	>2 (2.8)	NA
(±)-FTC	0.065 (0.03)	0.3 (0.4)	1.6
3TC [(−)-BCH-189]	0.11 (0.002)	0.3 (0.024)	0.85
(+)-BCH-189	NA (0.2)	>2 (1.2)	NA
DDC	0.011 (0.02)	0.074 (0.07)	0.6
DDI	0.76 (0.12)	6.4 (1.8)	65.8
AZT	0.055 (0.004)	0.53 (0.02)	6.4
TIBO R82913	0.17 (0.05)	0.67 (0.13)	2.95
(−)-FTC (AZT^R)	0.029	0.18	1.4

[a] Values are micromolar concentrations, calculated by the median effect method (4) from data in Table 1. Values in parentheses are results of the standard acute infection assays in PBMC in experiments using cell-free virus (18).

Effect on cells from AZT-treated patients. PBMC from nine patients who had been treated with AZT for several months were selected because most of their infectious cells contained HIV that efficiently replicated in amplifier cells continuously exposed to 0.4 μM AZT. The mean inhibitions of replication events mediated by the presence of (−)-FTC with these cells were compared with those of specimens from 18 subjects never exposed to AZT. The replication of infectious material borne by cells from AZT-naive patients was inhibited by 99.6% ± 0.5% (mean ± SD) with 0.2 μM (−)-FTC (n = 18), whereas cells from AZT-treated individuals were inhibited by only 84.0% ± 19% (n = 13) ($P < 0.05$) at the same concentration of (−)-FTC. The EC_{90}s and EC_{99}s of (−)-FTC with the AZT-resistant viruses were 0.18 and 1.4 μM, respectively, whereas with the AZT-naive viruses these values were 0.055 and 0.43 μM, respectively. AZT-treated patients' cells demonstrated a threefold increase of resistance to (−)-FTC compared with cells obtained from AZT-naive individuals. Similar results were obtained with DDC and 3TC (see Discussion).

In vitro interaction between AZT and (−)-FTC or other nucleoside analogs. Infected cells from seven AZT-naive patients were cocultivated with uninfected cells exposed to increasing concentrations of AZT and (−)-FTC alone and in combination (Table 3). With 100 nM AZT or 15 nM (−)-FTC alone, marginal inhibition (≤60%) was noted. In contrast, the same cocultures simultaneously exposed to both AZT

TABLE 3. Effects of AZT and (−)-FTC alone and in combination on cell-to-cell infection from different patients' cells to activated normal lymphocytes

Cell source and inhibitor(s) (ratio)	Concn of each inhibitor (nM)	No. of donors	% Inhibition (SD)[a]	CI at an f_a of[b]:			
				0.5	0.75	0.90	0.95
AZT-naive patients							
AZT and (−)-FTC (6.7:1)	20 and 3	7	51.2 (27.7)	0.83	0.50	0.31	0.23
	45 and 6.75	4	70.9 (24.7)				
	100 and 15	7	99.4 (0.37)				
AZT and 3TC (1.3:1)	20 and 15	5	76.3 (13.9)	0.42	0.26	0.18	0.18
	45 and 33	5	82.9 (13.6)				
	100 and 75	3	99.5 (0.24)				
AZT and DDC (10:1)	20 and 2	4	42.2 (22.2)	0.82	0.58	0.41	0.32
	100 and 10	6	84.0 (8.7)				
	250 and 25	4	97.0 (2.2)				
AZT and DDI (1:2)	20 and 40	2	54.1 (NA[c])	0.50	0.29	0.17	0.12
	100 and 200	4	82.6 (6.5)				
	250 and 500	4	99.3 (0.22)				
AZT-treated patients							
AZT	500	9	15.0 (24.0)				
	1,500	12	23.0 (21.0)				
	4,500	8	31.0 (22.0)				
(−)-FTC	75	6	61.8 (13.0)				
	225	4	90.7 (7.0)				
	750	4	96.4 (6.2)				
DDC	50	4	52.9 (31.3)				
	150	4	69.8 (16.3)				
AZT and (−)-FTC (6.7:1)	100 and 15	3	35.2 (25.5)	0.54	0.43	0.36	0.32
	200 and 30	4	60.7 (22.7)				
	500 and 75	7	81.0 (16.0)				
	1,500 and 225	4	97.5 (2.8)				
AZT and DDC (10:1)	150 and 15	4	32.5 (25.5)	0.68	0.34	0.17	0.10
	500 and 50	4	67.7 (26.3)				
	1,500 and 150	4	86.3 (11.5)				

[a] The values for inhibition with each drug alone are shown in Table 1.
[b] Calculated by the multiple-drug effect equation for a mutually nonexclusive interaction (4). f_a is the fraction affected (percent inhibition divided by 100). A CI of less than 1 indicates synergy (see Materials and Methods).
[c] NA, not available.

and (−)-FTC resulted in 99.4% ± 0.37% (mean ± SD) inhibition ($P < 0.001$ compared with the single agents alone). Applying the multiple-drug effect analysis (4) to the complete data, CI values below 1 were obtained, suggesting synergism at inhibitory levels of 50% and above. At the 90 and 95% inhibitory levels, lower CI values were noted with this combination (Table 3). Inhibition of cell proliferation or viability was not apparent with this combination even at concentrations 100-fold greater (data not shown). Interactions were also evaluated for AZT combined with 3TC (1.3:1), DDC (10:1) or DDI (1:2). The results indicated that all these combinations of nucleosides with AZT consistently produced synergistic interactions at relevant effect levels (≥50% inhibition).

Effects of AZT combined with (−)-FTC or DDC in experiments using AZT-resistant inocula. Infectious cells from patients previously treated with AZT were selected. AZT at concentrations up to 4,500 nM produced no significant inhibition when these patients' PBMC were exposed to uninfected cells. Cocultures of such patients' PBMC with amplifier cells simultaneously exposed to both 100 nM AZT and 15 nM (−)-FTC also led to an insignificant inhibition of 35% ± 26%, compared with 99.4% inhibition for the same combination applied to AZT-naive patients' cells (see above). Likewise, cocultures of AZT-resistant patients' PBMC with amplifier cells exposed to a combination of 500 nM AZT and 50 nM DDC were inhibited by 68% ± 26% compared with a 97% inhibition with a combination of 250 nM AZT and 25 nM DDC applied to AZT-susceptible HIV. When the data for these two combinations were analyzed by the multiple-drug effect analysis method, synergistic interactions were noted since the CI values were clearly below 1. However, it should be noted that the slope of the dose-response curve for AZT alone was shallow (0.43), making the estimated EC_{50} high (28.2 μM). Since the multiple-drug effect analysis method developed by Chou and Talalay (4) is inappropriate for determining the interaction of a combination of an essentially ineffective agent with an active compound, the fractional product method (20) was applied to the data for the combination of AZT and (−)-FTC. The results

indicated a subadditive interaction, since the combined inhibitory effect found was less than the calculated value for additivity (67.5% for 500 nM AZT–75 nM FTC and 92.8% for 1,500 nM AZT–225 nM FTC) but greater than the inhibitory effect of the most effective agent alone.

DISCUSSION

The in vitro culture system presented here is quite distinct from other systems designed to evaluate the potency of antiviral agents. In this system, the naturally infected human PBMC contain a diversified source of virus inocula in their normal environment. This natural unpassaged infectious material is mitogenically activated for infection and amplification in contiguous permissive lymphocytes with kinetics inherent to cell-to-cell transmission (7, 11, 13, 14). The limiting dilution technique was used to specify the number of infectious cells brought into contact with amplifier lymphocytes and to measure their apparent reduction upon in vitro inhibition. The colinearity between virus-positive or virus-negative replicates and the donor cell concentrations conformed to the Poisson equation, which predicts a stochastic "one (infectious) event" in cultures yielding 63% positive replicates. From this observation, we derived the assumption that one infectious event was equivalent to one infectious cell. A similar application of the Poisson statistics on limiting dilutions of cellular DNA on the one hand and of infectious cells on the other led Simmonds et al. (22) to make similar assumptions.

We have examined a series of nucleoside and nonnucleoside RT inhibitors known to interfere with acute de novo HIV replication. The decreasing order of potency in our assay system was (−)-FTC > DDC > (±)-FTC ≥ 3TC > AZT ≥ TIBO ≫ DDI = (+)-FTC = (+)-BCH-189 (Table 2). Studies were also performed with infected cells obtained from patients who had undergone AZT therapy for a prolonged period. At least 10% of the virus carried by these cells could initiate virus replication in PHA-stimulated lymphocytes continuously exposed to a high concentration of AZT (4.2 μM). (−)-FTC was completely inhibitory at 0.2 μM with AZT-naive patients' cells; however, it was marginally less active with cells obtained from AZT-treated individuals. These results suggest a modest cross-resistance between the thymidine analog AZT and the cytidine analog (−)-FTC, which is consistent with previous work by our group with acutely infected PBMC cultures infected with well-characterized AZT- and (−)-FTC-resistant viruses (17, 18). Experiments with the two other cytidine analogs DDC and 3TC with AZT-resistant-infected PBMC have yielded results similar to those obtained with (−)-FTC, suggesting that the three cytidine analogs might share common modest cross-resistance with AZT (data not shown). It is interesting that DDI was marginally effective in our cell culture system when evaluated at concentrations up to 2 μM (Tables 1 and 2).

As shown by the lack of significant p24 signals in cocultures with high concentrations of RT inhibitor, the patients' cells themselves never contained or produced detectable viral protein without marked in vitro amplification (>10^5-fold). Other steps in the replication cycle of HIV within cells are also potentially rate limiting, and the assay can thus be used for any compound interfering with this cycle. This includes *Tat* inhibitors, protease inhibitors, and antisense oligonucleotides (13). However, in the case of the antiproliferative cytokine alpha interferon, doses up to 2,000 IU/ml did not alter rates of in vitro HIV replication initiated by multiple primary patients' cells in optimally preactivated human lymphocytes (data not shown). Whether this pertained to cell-to-cell HIV transmission or to abnormal interferon receptor-mediated signaling in the selected amplifier cells will require further investigations.

The cell-to-cell propagation design of our culture system may model some of the virological and cellular conditions pertinent for HIV propagation in activated lymphocytes within lymphoid tissues where donor and acceptor cells are closely packed. The main differences between our system and assays which utilize cell-free laboratory-passaged virus inocula reside with the direct cell-to-cell propagation of unselected multiple wild-type virus, with the multiplicities of infection of 3 to 4 (representing a 25- to 125-fold increment of infectious cell numbers added) tested for each donor cell specimen and each drug concentration, and with the mode of calculation inherent in the multiple replicate combinations used. For comparison, we tested the antiviral potency of (−)-FTC and that of AZT in a cell-free virus assay using a fresh clinical HIV isolate. In such a system, AZT is extremely potent (the EC_{90}s were 4 to 5 nM with 10 or 100 50% tissue culture infective doses at day 7), but this is not true with higher multiplicities of infection and prolonged cultivation (the EC_{90}s increased to 100 and >4,000 nM on days 12 and 28, respectively, for 100 50% tissue culture infective doses of cell-free virus). This was consistent with a previous report by Smith et al. (23) and suggested that AZT, although present in the culture supernatant throughout, had interfered temporarily with the HIV replication cycle. The results with (−)-FTC in a cell-free virus assay were not as dependent on time and dose of virus as were those with AZT (the EC_{90}s increased to 30 and 100 nM on days 12 and 28, respectively, with 100 50% tissue culture infective doses of cell-free virus), indicating a more stable interference of the cytidine analog with the process of viral replication. Similar results with (−)-FTC in experiments using well-characterized laboratory strains of HIV have been reported by Schinazi et al. (18).

Our assay is not exempt from a number of artifacts which also plague conventional cell-free virus assay systems, such as the large excess of optimally activated acceptor lymphocytes over donor infectious cells (1,000/1 to 10,000/1) and the lack of macrophages or of a large fraction of quiescent $CD4^+$ T cells, both well represented in patients' lymph nodes. Thus, EC_{90}s determined in in vitro assays could theoretically be far below or far above the actual extracellular drug concentrations needed to see a diminution of HIV replication rates of similar magnitudes in the patients. Of note, however, is that the EC_{90}s determined for AZT in our study (0.53 μM) come much closer than those of standard cell-free virus assays (<0.05 μM) to the maximum concentrations of drug in plasma of approximately 1 μM (24) attained with treatment regimens under which HIV replication rates have rapidly declined by 90% or more in some AZT responder patients (12).

If the EC_{90}s and EC_{99}s determined for (−)-FTC are pharmacologically attainable and toxicologically tolerated in patients, we would predict that the antiviral potency of (−)-FTC would surpass that of the three 2′,3′-dideoxynucleosides useful for the treatment of HIV-1 infections. The in vitro results with AZT combined with (−)-FTC, 3TC, DDC, or DDI indicated synergy at conveniently low extracellular concentrations (Table 3). The results for the combination of AZT and DDI are consistent with previous work by our group using cell-free virus inocula (21). The striking antiviral activity for the combination of AZT and (−)-FTC (or 3TC) is interesting. Synergistic interactions were also demonstrable

with AZT and (−)-FTC in experiments with cells bearing AZT-resistant HIV when the multiple-drug effect analysis method was used (Table 3). However, synergy seems essentially lost with cells bearing AZT-resistant viruses when the fractional product analysis method was used. Of note is that in cells of either type, no antagonism was noted irrespective of the method used to determine drug interactions. Viruses containing the Met-184-to-Val mutation in the RT region associated with (−)-FTC and 3TC resistance have been isolated under selective pressure in vitro, and those associated with 3TC resistance have also been isolated in vivo (17). Whether the combination of (−)-FTC and AZT can prevent or delay the emergence of resistance remains to be determined.

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