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Synergistic Inhibition of Human Immunodeficiency Virus Type 1 Replication In Vitro by Two-Drug and Three-Drug Combinations of 3′-Azido-3′-Deoxythymidine, Phosphonoformate, and 2′,3′-Dideoxythymidine

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The effects of 3′-azido-3′-deoxythymidine (AZT), phosphonoformate (PFA), and 2′,3′-dideoxythymidine (ddT) and their combinations on human immunodeficiency type 1 (HIV-1) replication were studied by measuring the HIV-1 p24 antigen expression and reverse transcriptase (RT) release in HIV-1-infected MT4 cells in vitro. RT activity was also measured in a cell-free system by using poly(rA)-oligo(dT) as the primer-template, and cell growth inhibition was measured in noninfected MT4 cells. The interactions of these two- and three-drug combinations were evaluated by the combination index (CI) method and isobologram techniques. The 50% effective concentrations (EC_{50}s) of AZT, PFA, and ddT were 0.014 to 0.005, 9.4 to 8.8, and 8.4 to 2.5 μM, respectively, for p24 enzyme-linked immunosorbent assays (ELISAs) and 0.0055 to 0.0034, 1.43 to 1.37, and 2.87 to 2.83 μM, respectively, for RT activity in vitro; for RT activity in the cell-free system, the EC_{50}s were 0.00019 to 0.00024, 0.012 to 0.02, and 0.00074 to 0.0005 μM, for AZT-5′-triphosphate, PFA, and ddT-5′-triphosphate, respectively. AZT in combination with PFA (1:200) or ddT (1:5) as well as the combination of these three drugs (1:200:5) synergistically inhibited HIV-1 replication and RT activity in the cell-free system over a wide range of drug concentrations, with the CIs ranging from 0.5 to 0.9, in which CIs of <1, 1, and >1 indicate synergism, additive effect, and antagonism, respectively. Three- and two-drug combinations of AZT, PFA, and ddT showed similar degrees of synergism against HIV-1 replication in p24 assays and RT release assays, whereas the combination of AZT and ddT was found to be the most selective in terms of its anti-HIV-1 effect versus cytotoxicity. Dose reduction indices calculated from both HIV-1 replication inhibition, as measured by p24 ELISA and by RT activity in the cell-free system, indicated that two- and three-drug combinations at high effect levels and the selected combination ratios allow 2- to 240-fold dose reduction over the single drug alone in terms of their anti-HIV-1 effects. The three-drug combination showed the highest dose reduction index. These findings suggest that increased efficacy and reduced toxicity may be achieved in AIDS therapy by using AZT, PFA, and ddT in two- or three-drug combinations.

Human immunodeficiency virus type 1 (HIV-1) has been identified as the etiological virus of AIDS and its related diseases (1, 2). There is no cure available at present. The successful treatment of patients with AIDS may require multiple-drug-combination therapy. The ideal multiple drug combination will have synergistic effects against HIV-1 but will have reduced toxicity toward the host. Previous studies have shown that 3′-azido-3′-deoxythymidine (AZT), phosphonoformate (PFA), and 2′,3′-dideoxythymidine (ddT) each inhibit HIV-1 replication in vitro (18–20). PFA has also been shown to be effective in treating cytomegalovirus retinitis in patients with AIDS (17). It is generally assumed that reverse transcriptase (RT) is the common target of AZT, PFA, and ddT. AZT and ddT act on RT through phosphorylation of their respective 5′-triphosphates by nucleoside and nucleotide kinases (18, 19), whereas PFA acts directly on RT (20). Usually, synergistic interactions are more likely to be obtained with anti-HIV-1 combinations of drugs that affect different molecular targets or different stages of the HIV-1 life cycle (5, 11–14, 21). For example, AZT, dideoxycytodine, or PFA in combination with alpha-A interferon or some other anti-HIV-1 compounds showed high degrees of synergistic antiviral interactions as determined by the combination index (CI) method and isobologram techniques (9–15, 25). Individually, AZT, PFA, and ddT may have their own antiviral actions that are spatially or temporally related, although they may eventually act on the same target. Since these agents may have different metabolic or mechanistic pathways before they act on the target(s) (28), and since PFA and ddT have no apparent cross-resistance toward HIV-1 strains that are resistant to AZT (16), such combinations deserve quantitative exploration. In this study, we evaluated the effects of AZT, PFA, and ddT and two- and three-drug combinations of these drugs on replication of a standard laboratory strain of HIV-1 in vitro and the inhibition of RT activity in a cell-free system. To our surprise, all of these drugs in different pairs of combinations showed an impressive synergistic interaction against HIV-1 replication without increased cytotoxicity.

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MATERIALS AND METHODS

Compounds and chemical agents. AZT was obtained from Burroughs-Wellcome Co. (Research Triangle Park, N.C.). PFA was from Astra Alab AB (Sodertalje, Sweden), ddT and its 5′-triphosphate (ddTTP) were purchased from Sigma (St. Louis, Mo.). AZTTP was synthesized by the Laboratory of Organic Chemistry of the Memorial Sloan-Kettering Cancer Center. Poly(rA)$_n$-oligo(dT)$_n$, (n = 12 to 18) was purchased from Pharmacia (Piscataway, N.J.); [^{3}H]dTTP (19.8 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.). The p24 enzyme-linked immunosorbent assay (ELISA) kit was the product of DuPont-NEN Research Products (Boston, Mass.).

Cells and virus. Human cutaneous T-cell lymphoma (H9) and human T-cell leukemia (MT4) cell lines were cultured at 37°C in 5% CO_2 and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. HIV-1 (HTLV-IIIB; from R. Gallo, National Cancer Institute, Bethesda, Md.) was multiplied in H9 cells. Concentrated stocks of HIV-1 were obtained from cell-free supernatants of HIV-infected H9 cells and were frozen at −80°C until use. The 50% tissue culture infective doses of the cell-free virus stocks per ml were determined on day 7 by endpoint titration with MT4 cells in 96-well microculture plates. The number of cells per well was 3×10^4.

Assay of HIV-1 replication. The effects of the compounds on HIV-1 replication in vitro were examined by means of p24 core antigen capture ELISA and RT release assay. MT4 cells were infected with HIV-1 at 200 50% tissue culture infective doses of virus per 10^6 cells. After an adsorption period of 1 h at 37°C, unadsorbed virus was removed by washing once with fresh medium followed by a centrifugation at 150 × g for 10 min. The cells were resuspended in fresh medium and were mixed with various concentrations of compounds in a 12-well plate at a density of 10^6/3 ml per well. After 5 days of incubation at 37°C in 5% CO_2, the HIV-1 p24 core antigen was determined by following the procedure of the p24 ELISA kit (DuPont-NEN Research Products). In the control infected MT4 cells without drug treatment, the maximal HIV titer was found on day 5. The presence of HIV-1 RT in the supernatants of infected, drug-treated and infected, untreated MT4 cell cultures was assayed by a modified macromethod of Spira et al. (23). The radioactivity of the DNA on the filters was determined with a scintillation counter (Tri-carb 1900 CA liquid scintillation analyzer; Packard Instrument Co., Inc.). Uninfected, untreated and infected, and untreated samples were included as controls in each experiment.

Assay of RT in the cell-free system. The enzyme preparation was made from MT4 cells infected with HIV-1, and RT was determined as described previously (27). The reaction mixture in 100 μl contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM dithiothreitol, 6 mM $MgCl_2$, 50 μg of nuclease-free bovine serum albumin, 1 μg of poly(rA)$_n$-oligo(dT)$_n$, (n = 12 to 18), 0.5 μM [^{3}H]dTTP (specific activity, 19.8 Ci/mmol), 10 to 20 μl of crude RT preparation, and five to six concentrations of the compounds tested. The enzyme reaction in this study was linear with time up to 60 min.

Assay of cytotoxicity. The cytotoxicities of the compounds in uninfected MT4 cells were evaluated by the 2′,3′-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) tetrazolium-formazan microculture assay as described by Scudiero et al. (22). Uninfected MT4 cells were incubated with the indicated concentrations of compounds tested for 72 h. A total of 50 μl of fresh XTT (1 mg/ml) and phenazine methosulfate (PMS; 0.025 mM) (XTT and PMS are products of Sigma) were then added to each well of the cell culture. After incubation at 37°C for 6 h, the samples were read by using a microplate reader (EL-340; Bio-Tek) at wavelengths of 450 and 630 nm. Day 3 was selected for the cytotoxicity assay so that the logarithmic phase of cell growth was maintained in the untreated control experiments.

Experimental design and data analysis. The dose-effect relationships for HIV-1 replication, RT activity inhibition, and cytotoxicity were determined for five or six concentrations of AZT, PFA, and ddT alone and for their paired and three-drug mixtures that were serially diluted from the stock concentrations of each drug. The combination molar ratios for AZT/PFA/ddT, AZT/PFA, AZT/ddT, and PFA/ddT were 1:200:5, 1:200, 1:5, and 40:1, respectively. In designing the experiments, the molar ratios for three drugs were selected first, and then the ratios for each pair of combinations were selected accordingly. The initial selection of molar ratios took into account the potency of each drug alone in the p24 ELISA and RT activity in the cell culture assay as well as RT activity in cell-free system assays. The details of the examples are given in the footnotes of Tables 1 through 8. Synergistic or antagonistic interactions for the two- or three-drug combinations were analyzed by the median-effect principle derived by Chou (5) and the CI and dose reduction index (DRI) formulated by Chou and colleagues (4–6) by using computer software (4). CIs of <1, 1, and >1 indicate synergism, additive effect, and antagonism, respectively. DRIs represent how many folds of dose (or concentration) reduction are possible in the combination for a given degree of effect when compared with the degree of effect of each drug alone if the combination proves to be synergistic (3, 5).

RESULTS

Effects of AZT, PFA, and ddT alone on HIV replication, RT activity, and cell growth inhibition. A dose-dependent inhibition of HIV-1 replication based on p24 ELISA and RT activity in HIV-1-infected MT4 cells and RT activity in the cell-free system was observed for all three compounds (Tables 1 and 2). Their 50% effective concentrations (EC_{50}s) (D_m) are presented in Tables 3 through 5. In general, there was a good correlation between viral yields determined by measuring RT and p24 levels. It should be noted that neither system can distinguish the difference between infectious and noninfectious (defective) virus particles. For the cell-free system RT assay, although a crude enzyme preparation was used, the quantitative dose-effect relationships were excellent, as shown by the r values given in Table 5.

The differences in the EC_{50}s of these compounds for inhibiting HIV-1 replication and for inhibiting RT activity in cell-free systems may be due to multiple factors, including differences in assay conditions. In this study, the p24 core antigen expression ELISA and assay of the RT activity released from HIV-1-infected MT4 cells were used to measure the effects of compounds on HIV-1 replication in HIV-1-infected MT4 cells. The active 5′-triphosphate forms of the compounds, made through phosphorylation by nucleotide kinases, are required in the process. On the other hand, the RT assay with HIV-1-infected cell supernatants as the enzyme source is used to detect the direct effects of the compounds on RT activity in a cell-free system. The triphosphate forms of the compounds are used directly, and this

TABLE 1. Effects of AZT, PFA, and ddT, alone or in combination, on HIV-1 replication measured by p24 ELISA and RT activity in HIV-1-infected MT4 cells

Drug regimen	Drug concn (μM)[a]			Fractional inhibition by:			
				p24 ELISA[b]		RT assay[c]	
	AZTTP	PFA	ddT	Expt 1	Expt 2	Expt 1	Expt 2
Each drug alone	0						
	0.08			0.9976	0.9957		
	0.04			0.9210	0.9795	0.9850	0.975
	0.02			0.660	0.9298	0.9023	0.932
	0.01			0.360	0.7729	0.7451	0.834
	0.005				0.5179	0.4986	0.644
	0.002						
		0					
		50 (25)		0.9988	0.936		0.997
		20 (12.5)		0.971	0.697	0.9960	0.981
		10 (6.25)		0.51	0.237	0.9580	0.949
		5 (3.12)			0.0946	0.8342	0.827
		(1.56)				0.6296	0.589
			0				
			10 (25)	0.69	0.9795	0.9756	0.949
			5 (12.5)	0.11	0.9603	0.9429	0.941
			2 (6.25)	0.012	0.8788	0.8270	0.814
			1 (3.12)		0.263	0.4665	0.477
			(1.56)			0.2678	0.270
AZT + PFA (1:200)[d]	0	0					
	0.04	8		0.992			
	0.02 (0.02)	4 (4)		0.893	0.9658		0.990
	0.01 (0.01)	2 (2)		0.366	0.8512	0.9720	0.912
	0.004 (0.005)	0.8 (1)		0.34	0.7064	0.7708	0.768
	0.002 (0.0025)	0.4 (0.5)			0.6187	0.5697	0.587
	0.001 (0.00125)	0.2 (0.25)		0.234	0.2621	0.2811	0.360
AZT + ddT (1:5)	0.16 (0.04)		0.8 (0.2)	0.998	0.9899	0.9875	0.982
	0.08 (0.02)		0.4 (0.1)	0.997	0.956	0.9313	0.942
	0.04 (0.01)		0.2 (0.05)	0.987	0.8261	0.8310	0.802
	0.02 (0.005)		0.1 (0.025)	0.941	0.534	0.5601	0.516
	0.01 (0.0025)		0.05 (0.0125)	0.729	0.4306	0.4630	0.360
	0.004 (0.00125)		0.02 (0.00625)	0.557	0.2103	0.1645	0.190
PFA + ddT (200:5)		32 (20)	0.8 (0.5)	0.992	0.9404		0.987
		16 (10)	0.4 (0.25)	0.962	0.7473		0.981
		8 (5)	0.2 (0.125)	0.839	0.4389	0.9665	0.941
		4 (2.5)	0.1 (0.0625)	0.407	0.0296	0.8300	0.839
		2 (1.25)	0.05 (0.0312)	0.341	0.0196	0.6571	0.680
		1 (0.625)	0.025 (0.0156)	0.164		0.3876	0.310
AZT + PFA + ddT (1:200:5)	0.04	8	0.2	0.9975			
	0.02	4	0.1	0.919	0.9649		0.988
	0.01	2	0.05	0.738	0.8015	0.9908	0.930
	0.005	1	0.025	0.601	0.5596	0.8512	0.776
	0.002 (0.0025)	0.4 (0.5)	0.01 (0.0125)		0.3303	0.7611	0.411
	0.001 (0.00125)	0.2 (0.25)	0.005 (0.00625)	0.407		0.5036	0.190

[a] The numbers in parentheses represent the different concentrations used in experiment 2 for the p24 ELISA and in experiments 1 and 2 for the RT activity assay.

[b] The p24 antigens in controls without drug were 1,483 ± 90 and 1,178 ± 34 ng/ml (±standard error) for experiments 1 and 2, respectively. These values were used as control values to calculate the fractional inhibition for the treated wells.

[c] The RT activities in controls without drug were 0.1672 ± 0.0065 and 0.1629 ± 0.0087 pmol/ml (±standard error) for experiments 1 and 2, respectively. These average values were used as control values to calculate the fractional inhibition in the treated tubes.

[d] Molar ratio of drug combinations.

TABLE 2. Effects of AZTTP, PFA, and ddTTP alone or in combination on RT activity in the cell-free system

Drug regimen	Drug concn (μM)			pmol/ml[a]		Fractional inhibition	
	AZTTP	PFA	ddTTP	Expt 1	Expt 2	Expt 1	Expt 2
Each drug alone	0			0.097[b]	0.1610[b]		
	0.01			0.0022	0.0107	0.9775	0.940
	0.005			0.0100	0.0256	0.8970	0.854
	0.002			0.0113	0.0347	0.8840	0.800
	0.001			0.0169	0.0410	0.8258	0.770
	0.0005			0.0360	0.0804	0.6300	0.540
	0.0002			0.0436	0.0896	0.5506	0.510
		0		0.0980[b]	0.1920[b]		
		5		0.0007		0.9925	
		2		0.0011	0.0047	0.9888	0.970
		1		0.0018	0.0085	0.9813	0.950
		0.5		0.0027	0.0098	0.9720	0.944
		0.2		0.0053	0.0164	0.946	0.906
		0.1		0.0213	0.0550	0.781	0.690
			0	0.0960[b]	0.1725[b]		
			0.05	0.0015	0.0062	0.9850	0.965
			0.01	0.0113	0.0142	0.8840	0.920
			0.005	0.0180	0.0396	0.8146	0.774
			0.002	0.0271	0.0425	0.7210	0.757
			0.001	0.0402	0.0720	0.5860	0.590
			0.0005	0.0529	0.0840	0.4550	0.520
AZTTP + PFA (1:200)[c]	0.005	1		0.0007	0.0027	0.9925	0.9800
	0.002	0.4		0.0013	0.0073	0.9869	0.9600
	0.001	0.2		0.0047	0.0175	0.9514	0.9000
AZTTP + ddTTP (1:5)	0.01		0.05		0.0040		0.980
	0.005		0.025	0.0009	0.0058	0.9906	0.970
	0.002		0.01	0.0036		0.9626	
	0.001		0.005	0.0151	0.0307	0.8446	0.830
PFA + ddTTP (40:1)		1	0.025	0.0009	0.0060	0.9910	0.970
		0.4	0.01	0.0024	0.0087	0.9757	0.950
		0.2	0.005	0.0051	0.0175	0.9476	0.900
AZTTP + PFA + ddTTP (1:200:5)	0.005	1	0.025	0.0006	0.0035	0.9944	0.980
	0.002	0.4	0.01	0.0015	0.0060	0.9851	0.970
	0.001	0.2	0.005	0.0031	0.0156	0.9690	0.910

[a] The specific activity was 5,500 cpm/pmol.
[b] The RT activities in controls without drug were 0.097 ± 0.00057 and 0.1753 ± 0.0091 pmol/ml (±standard error) for experiments 1 and 2, respectively. These average values were used as control to calculate the fractional inhibition in the treated tubes.
[c] Molar ratio of drug combinations.

assay is primer-template dependent (27). In addition, the rates of transport of these compounds through the cell membrane could account for the quantitative differences of the results that we observed. These two assays supplement each other, and more information can be obtained about the modes of action of the compounds tested. At the similar dose ranges which showed potent anti-HIV-1 effects, no cytotoxicity was observed by any of these three compounds.

Effects of the AZT and PFA combination. As shown in Table 3, the combined inhibitory effect of AZT and PFA at a molar ratio of 1:200 on HIV-1 replication was synergistic at EC_{50}, EC_{75}, and EC_{95} effect levels, as indicated by their CIs. At the EC_{95} level, this regimen showed an additive effect against HIV-1 replication, as measured by p24 ELISA (CI = 0.891 to 1.13). However, the synergism at EC_{90} and EC_{95} was evident by the RT activity assay in both the cell and the cell-free systems. We found that the CIs from EC_{50} to EC_{95} increased for HIV-1 replication, as determined by both p24 ELISA and the RT activity assay in MT4 cells, and the CIs from EC_{50} to EC_{95} decreased for RT activity inhibition in the cell-free system. The overall tendency at EC_{75} to EC_{95} for both systems fell into the synergistic or additive region, with CIs ranging from 1.0 to 0.59. The DRIs indicate that when AZT and PFA are combined, the doses of AZT and PFA can be reduced 1.4- and 6.1-fold, respectively, to inhibit 95% HIV replication in vitro (Table 6).

Effect of the AZT and ddT combination regimen. As shown in Tables 3 and 4, a strong synergistic interaction on the inhibition of HIV-1 replication was observed at all effect levels for the combination of AZT and ddT at a molar ratio

TABLE 3. Combination parameters calculated by the median-effect analysis on the basis of the p24 ELISA data given in Table 1[a]

Drug regimen	Expt no.	CIs[b] at inhibitions of:				D_m (μM)	m	r
		50%	75%	90%	95%			
AZT	1					0.0142	3.02	0.98
	2					0.0051	1.91	0.99
PFA	1					9.38	4.12	0.99
	2					8.75	2.43	0.99
ddT	1					8.41	3.20	0.99
	2					2.51	1.83	0.99
AZT + PFA (1:200)	1	0.401	0.600	0.869	1.130	0.870	1.51	0.90
	2	0.521	0.636	0.777	0.891	0.475	1.44	0.98
AZT + ddT (1:5)	1	0.296	0.378	0.483	0.571	0.025	1.80	0.99
	2	0.658	0.708	0.761	0.799	0.020	1.69	0.99
PFA + ddT (40:1)	1	0.333	0.456	0.622	0.769	3.12	1.89	0.98
	2	0.833	0.802	0.772	0.754	6.87	2.58	0.98
AZT + PFA + ddT[c] (1:200:5)	1	0.221	0.323	0.473	0.614	0.492	1.52	0.90
	2	0.903	0.914	0.92	0.937	0.835	1.91	0.99
(AZT + PFA)[d] + ddT (40.2:1)	1	0.548	0.546	0.543	0.542			
	2	0.898	0.911	0.926	0.937			
AZT + (PFA + ddT) (1:205)	1	0.325	0.421	0.552	0.667			
	2	0.768	0.755	0.745	0.740			
(AZT + ddT) + PFA (0.03:1)	1	0.625	0.722	0.844	0.946			
	2	0.871	0.884	0.891	0.912			

[a] For details of analysis, see text. D_m and m are the antilogarithm of the x intercept and the slope of the median-effect plot, respectively.
[b] CIs were calculated by the classical isobologram equation for two-drug combinations, CI = $(D)_1/(D_x)_1 + (D)_2/(D_x)_2$ (see Table 6, footnote *a*), by using computer software for simulation (4–6).
[c] For calculation of the CI for the three-drug combination, see footnote *a* of Table 7.
[d] The two drugs in parentheses are considered a single drug, and thus, the three-drug combination is dissected into three pairs of two-drug combinations.

of 1:5, with CIs ranging from 0.29 to 0.80. A synergistic interaction on RT activity inhibition in the cell-free system was observed at the EC_{95} level, with a CI of 0.80. Similar to the results for AZT plus PFA, an upward pattern of CIs for HIV-1 replication and a downward pattern of CIs for RT activity inhibition in cell-free system were obtained for AZT and ddT. The DRI for the combination suggests that 1.6-fold less AZT at EC_{95} can produce the same effect on HIV-1 replication as AZT alone does and 224-fold less ddT at EC_{95} can produce the same effect on RT inhibition when these two drugs are used in combination in a cell-free system (Table 6).

Effect of the PFA and ddT combination regimen. Inhibition of HIV-1 replication for the PFA plus ddT combination was synergistic at all effect levels, with CIs ranging from 0.33 to 0.98 (Tables 3 and 4). The inhibition of RT activity in the cell-free system for this regimen was in a transition pattern, from antagonism at the EC_{50} level (CIs = 1.83 to 1.1) to synergism or an additive effect at the EC_{95} level (CIs = 0.96 to 0.86) (Table 4). A similar upward CI pattern for HIV-1 replication and a downward CI pattern for RT activity in the cell-free system were observed. The 95% inhibition of HIV-1 replication also benefited from this two-drug combination by a 1.5-fold reduction of the EC_{95} for PFA and a 54-fold reduction of the EC_{95} for ddT (Table 6).

Effect of the AZT, PFA, and ddT three-drug combination. The CIs and DRIs calculated from an extension of the median-effect equation revealed that when AZT, PFA, and ddT were combined, they were synergistic at most effect levels for HIV-1 replication and RT activity inhibition in the cell-free system. The synergistic interaction of these three drugs was stronger than those of any other two-drug combinations in this study at the overall effect levels tested for HIV-1 replication, as measured by RT activity inhibition, with CIs ranging from 0.50 to 0.79 (Table 4). For RT activity inhibition in the cell-free system, the CIs ranged from 1.1 to 0.74 (Table 5), and at the higher dose level, the effects were more synergistic (lower CIs) (Table 7). In a comparison of the DRI of the combination of these three drugs with the DRI of the two-drug combination regimens, the concentration required to produce the same effect (e.g., EC_{50}) in the two-drug combination could be further reduced in the three-drug combination for both HIV-1 replication and RT activity inhibition in the cell-free system. These results suggest that the three-drug combination has a more beneficial effect than the two-drug combinations tested in this study.

Cytotoxicities of AZT, PFA, and ddT and their combinations. No increase in cytotoxicity was observed for the three drugs when they were given singly or in combination at the same dose range used in anti-HIV-1 experiments (data not shown). Much higher doses (385- to 22,696-fold) were required for all three drugs to produce 50% cell growth inhibitory cytotoxicity when they were used alone and in combination. A comparison of the effects of AZT, PFA, and ddT alone or in combination on the inhibition of HIV-1 replication and cell growth in vitro is summarized in Table 8. AZT was among the most selective drugs, with a selectivity

TABLE 4. Combination parameters calculated by median-effect analysis on the basis of the RT assay data given in Table 1[a]

Drug regimen	Expt no.	CIs[b] at inhibitions of:				D_m (μM)	m	r
		50%	75%	90%	95%			
AZT	1					0.0055	1.98	0.99
	2					0.0034	1.48	0.99
PFA	1					1.43	2.40	0.99
	2					1.37	1.93	0.99
ddT	1					2.87	1.78	0.99
	2					2.83	1.54	0.98
AZT + PFA (1:200)	1	0.693	0.707	0.723	0.735	0.433	2.07	0.99
	2	0.914	0.854	0.805	0.776	0.413	1.78	0.98
AZT + ddT (1:5)	1	0.632	0.711	0.800	0.867	0.021	1.63	0.99
	2	1.107	1.046	0.989	0.953	0.022	1.59	0.99
PFA + ddT (40:1)	1	0.618	0.707	0.809	0.887	0.894	1.85	0.99
	2	0.641	0.753	0.885	0.988	0.889	1.50	0.99
AZT + PFA + ddT[c] (1:200:5)	1	0.501	0.498	0.497	0.496	0.319	2.17	0.94
	2	1.215	1.029	0.878	0.792	0.561	2.12	0.99
(AZT + PFA)[d] + ddT (40.2:1)	1	0.722	0.704	0.687	0.675			
	2	1.329	1.204	1.091	1.020			
AZT + (PFA + ddT) (1:205)	1	0.637	0.594	0.554	0.529			
	2	1.441	1.154	0.926	0.797			
(AZT + ddT) + PFA (0.03:1)	1	0.666	0.609	0.562	0.535			
	2	1.135	1.000	0.884	0.814			

[a] For details of analysis, see text. D_m and m are the antilogarithm of the x intercept and the slope of the median-effect plot, respectively.
[b] CIs were calculataed by the classical isobologram equation for two-drug combinations, $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$, (see Table 6, footnote *a*), by using a computer software for simulation (4–6).
[c] For calculation of the CI for the three-drug combination, see footnote *a* of Table 7.
[d] The two drugs in parentheses are considered a single drug, and thus, the three-drug combination is dissected into three pairs of two-drug combinations.

index of 22,165 (50% inhibitory concentration [IC_{50}] of cytotoxicity/the EC_{50} of the anti-HIV-1 effect) when the drug was used alone. However, the selectivity of AZT in combination with ddT was similar to that of AZT alone, but it was more selective than ddT alone (selectivity index, 22,696). AZT in combination with PFA was much more selective than PFA alone, but it was less selective than AZT alone. PFA in combination with ddT was more selective than PFA alone but not ddT alone. In the case of the three-drug combination of AZT, PFA, and ddT, the regimen showed more selectivity than PFA alone, but it was less selective than AZT or ddT alone.

DISCUSSION

Examination of multiple antiviral drug combinations against HIV-1 is an important approach to improving therapeutic efficacy of drugs by maximizing the antiviral effect, minimizing the toxicity toward the host, and likely reducing the potential for drug resistance (5, 21). In this study, we found that AZT, PFA, and ddT in two- and three-drug combinations interacted synergistically over a wide range of dose and effect levels. By comparing the degree of synergism for these combination regimens on the basis of their CIs and DRIs calculated from p24 antigen expression and RT activity assays in the cell-free system, although the three-drug combination regimen had a degree of synergism against HIV-1 replication similar to that of the two-drug combinations under the same experimental conditions, the three-drug combinations showed the highest DRIs. The AZT and ddT combination turned out to be the most selective combination regimen on the basis of the selectivity index of the combination calculated from the ratio of anti-HIV EC_{50}/cytotoxic IC_{50} for the combinations. The dose of ddT can be reduced 54- to 240-fold to have a similar anti-HIV-1 effect at the EC_{95} effect level when ddT is used in combination with AZT, PFA, or both AZT and PFA. These drug combinations were shown to have synergistic activities, but they had no detectable cytotoxicity. These results suggest that some of these drug combination regimens would be of improved therapeutic value in the treatment of patients with AIDS, although in vitro data may not be directly extrapolatable to a clinical setting.

ddT is a relatively weak anti-HIV-1 nucleoside because of the relatively low phosphorylation of ddT into its active triphosphate form intracellularly (18, 24, 28). This can be confirmed by comparing the substantial difference between the EC_{50} for the inhibition of HIV-1 replication in the cell system used in this study and that for the inhibition of RT activity in the cell-free system used in this study. However, the anti-HIV-1 effect of ddT was very much synergistic when ddT was combined with AZT, PFA, or both, with DRIs ranging from 50 to 240 at the EC_{95} effect level. Furthermore, the AZT and ddT combination was among the most selective of the combinations against HIV-1. When they were combined, the concentrations of AZT and ddT at the EC_{95} effect

TABLE 5. Combination parameters calculated by the median-effect analysis based on the basis of the cell-free RT activity data given in Table 2[a]

Drug regimen	Expt no.	CIs[b] at inhibitions of:				D_m (μM)	m	r
		50%	75%	90%	95%			
AZTTP	1					0.00019	0.846	0.96
	2					0.00024	0.678	0.97
PFA	1					0.0117	0.858	0.96
	2					0.0205	0.793	0.93
ddTTP	1					0.00074	0.912	0.99
	2					0.0005	0.726	0.98
AZTTP + PFA (1:200)	1	1.44	1.03	0.74	0.59	0.013	1.157	0.95
	2	1.49	1.00	0.69	0.54	0.021	1.036	0.99
AZTTP + ddTTP (1:5)	1	4.46	2.35	1.23	0.80	0.0022	1.826	0.99
	2	2.82	1.75	1.09	0.79	0.0012	1.032	0.99
PFA + ddTTP (40:1)	1	1.80	1.36	1.04	0.86	0.0154	1.121	0.99
	2	1.10	1.02	0.98	0.96	0.0113	0.783	0.98
AZTTP + PFA + ddTTP[c] (1:200:5)	1	1.10	0.88	0.69	0.59	0.0078	1.059	0.99
	2	1.72	1.24	0.91	0.74	0.0150	0.953	0.94
(AZTTP + PFA)[d] + ddTTP (40.2:1)	1	0.88	0.86	0.90	0.91			
	2	1.38	1.24	1.16	1.1			
AZTTP + (PFA + ddTTP) (1:205)	1	0.70	0.70	0.68	0.68			
	2	1.62	1.22	0.92	0.76			
(AZTTP + ddTTP) + PFA (0.03:1)	1	0.80	0.70	0.64	0.67			
	2	1.07	0.95	0.87	0.83			

[a] For details of the analysis see text. D_m and m are the antilogarithm of the x intercept and the slope of the median-effect plot, respectively.
[b] CIs were calculated by the classical isobologram equation for two-drug combinations, CI = $(D)_1/(D_x)_1 + (D)_2/(D_x)_2$, (see Table 6, footnote *a*), by using a computer software for simulation (4–6).
[c] For calculation of the CI for the three-drug combination, see footnote *a* of Table 6.
[d] The two drugs in parentheses are considered a single drug, and thus, the three-drug combination is dissected into three pairs of two-drug combinations.

level could be reduced 1.6- and 224-fold, respectively. This dose reduction makes the concentration that can be used in patients with AIDS easily achievable, with the possibilities that the drugs will be less toxic and better tolerated over prolonged periods of treatment.

Both AZT and ddT are thymidine analogs and are phosphorylated intracellularly to their respective 5′-triphosphate forms, which inhibit HIV-1 RT (28). Previous studies showed that the efficacy of nucleoside analogs against HIV-1 replication correlates highly with their relative ability to generate their 5′-triphosphates intracellularly (24, 28). Interestingly, the AZT and ddT combination interacted synergistically against HIV-1, even though the two drugs may have similar mechanisms of action. The precise mechanism of

TABLE 6. DRI[a] for two- and three-drug combinations against HIV-1 replication in MT4 cells determined by p24 ELISA[b] and RT activity assay in the cell-free system by using poly(rA)-oligo(dT) as the template-primer[c]

Drug combination	DRI at EC_{95} by:					
	p24 ELISA for:			RT activity assay for:		
	AZT	PFA	ddT	AZT	PFA	ddT
AZT + PFA (1:200)	1.4 ± 0.1	6.1 ± 2.0		8.9 ± 1.2	2.2 ± 0.1	
PFA + ddT (40:1)		1.5 ± 0.1	54 ± 30		1.7 ± 0.1	3.1 ± 0.6
AZT + ddT (1:5)	1.6 ± 0.4		224 ± 90	5.4 ± 0.2		1.8 ± 0.2
AZT + PFA + ddT (1:200:5)	1.8 ± 0.2	6.7 ± 1.2	240 ± 104	11.0 ± 0.6	9.5 ± 7.4	4.9 ± 1.2

[a] DRI represents how many folds of dose reduction are possible in combination for a given degree of effect when compared with each drug alone, whereas the CI is determined by the median-effect plot and the multiple-drug-effect equation, as follows: $D_x = D_m[f_a/(1-f_a)]^{1/m}$, when $D_x = EC_{95}$, and the fractional inhibition $(f_a) = 0.95$; therefore, $CI_{95} = [(D)_1/(EC_{95})_1] + [(D)_2/(EC_{95})_2] + [(D)_3/(EC_{95})_3] = [1/(DRI_{95})_1] + [1/(DRI_{95})_2] + [1/(DRI_{95})_3]$, where drug 1 is AZT, drug 2 is PFA, and drug 3 is ddT. The numerator $(D)_1$ is the dose of AZT required in combination to achieve 95% inhibition of HIV-1 replication, whereas the denominator $(EC_{95})_1$ represents the dose of AZT alone to achieve the same effect. D_m and m are the antilogarithm of the x intercept and the slope of the median-effect plot, respectively. The $(DRI_{95})_1$ represents $(EC_{95})_1/(D)_1$. The values are the means and actual variations of two separate experiments, the results of which are given in Tables 3 and 5.
[b] Data and parameters from Tables 1 and 3 were used for automated determination of DRI by using computer software (4, 5).
[c] Data and parameters from Table 2 and 5 were used for automated determination of DRI by using a computer software (4, 5).

TABLE 7. Interaction of three drugs in inhibiting RT by using poly(rA)-oligo(dT) as the template-primer

Compound concns (μM)			Fractional inhibition	CI[a]
AZTTP	PFA	ddTTP		
0.001	0.2	0.005	0.9690	0.513
0.002	0.4	0.01	0.9851	0.399
0.005	1	0.025	0.9944	0.339

[a] The classical isobologram equation for the three-drug combination, CI = $(D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_3/(D_x)_3$ (see footnote *a* of Table 6 for definitions) was used (4–6) to calculate the CIs. The CIs for actual combination points were determined from m and D_m values for each drug alone.

synergism for the AZT and ddT combination remains to be determined. Vogt et al. (26) reported that ribavirin antagonizes the effect of AZT on HIV-1 replication through the inhibition of AZT phosphorylation by a metabolic alteration induced by ribavirin. This is probably due to the well-established ribavirin-induced increase in dTTP levels that result in a negative feedback regulation of thymidine kinase by dTTP (7, 8). Zhang et al. (28) recently found that AZT and ddT, at their respective antiretroviral concentrations, did not change the four nucleotide pool sizes in H9, MT4, CEM, and ATH8 cell lines. In phytohemagglutinin-stimulated human lymphoblasts, however, a slight but significant decrease (30 to 40%) in the dTTP pool size was observed after 24 and 48 h of exposure to 50 μM AZT (28). Given this observation, the observed synergistic interaction on HIV-1 replication inhibition by the combination of AZT and ddT in this study may be related to the effects of AZT and ddT on the regulation of physiological nucleotide pools.

The disparity between upward synergistic CIs for HIV-1 replication inhibition, as determined by p24 ELISA and the RT assay in MT4 cells, and the downward antagonistic, additive, and synergistic CIs for RT activity in the cell-free system may be due in part to the fact that the p24 ELISA and the RT activity assay in MT4 cells involved a long-term exposure of nucleoside analogs in cells, during which metabolic activation of the drug was processed. On the other hand, inhibition of RT activity in the cell-free system involved an initial, direct effect of the active forms (5′-triphosphate) of the nucleoside analogs on RT activity in the presence of a particular primer-template. Starnes and Cheng (24) reported that, in the purified HIV-1 RT inhibition assay system, the inhibition of HIV RT by PFA plus AZTTP and PFA plus ddTTP was mutually exclusive, suggesting similar or overlapping binding sites. The difference of fractional inhibition-CI plots in intact cells and the cell-free system observed in this study may be a hint that the synergistic interactions of these two- and three-drug combinations are more relevant to cell metabolism than they are to the virus target. Similar to the antiviral effectiveness of these nucleoside analogs, the impact of the two- or three-nucleoside analog combination on the formation of 5′-triphosphate and membrane transport may be a determining factor for their synergistic or antagonistic interactions. Since the RT assay was conducted in a cell-free system under artificial conditions, it is thought that cellular RT activity in MT4 cells would have more relevance to the clinical situation. As expected, data from two separate experiments showed some quantitative variations, as shown in Tables 3 through 5. Depending on whether molecular, cellular, or animal systems are being used, there is no absolute range of CIs that is considered to be an additive effect. Taking into account the experimental and biological variabilities, we consider that CIs of 0.9 to 1.1 indicate an additive effect in a cellular system, CIs of 0.95 to 1.05 indicate an additive effect in a molecular system, and CIs of 0.85 to 1.15 indicate an additive effect in an animal system. The parametric method used for determining synergism and antagonism in terms of CIs on the basis of multiple datum points yielded consistent conclusions, as shown in Tables 3 through 5, especially at the 90 and 95% effect levels.

We are aware of the complexity of drug combination studies and the limitation of extrapolation of results of those studies to clinical investigation (5). For example, the cell line and the HIV strain selected and the variations among the standard laboratory and clinical isolates before and after various therapies deserve further exploration. However, the methodology used in the present study represents an attempt to quantitatively assess drug interactions in a simple system to the fullest extent. It is worth noting that the dissections of three drug interactions described in Tables 3 through 5 are the first time that such data have been reported in the literature.

TABLE 8. Selectivity of effects of AZT, PFA, and ddT alone or in combination on HIV-1 replication[a] and on uninfected MT4 cell growth in vitro[b]

Drug regimen	EC_{50} for inhibition of HIV-1 repliation (μM)[c]	IC_{50} for inhibition of cell growth (μM)	Selectivity index (IC_{50}/EC_{50})
AZT	0.0097 ± 0.0046	215	22,165
PFA	9.07 ± 0.32	3,493	385
ddT	5.46 ± 2.95	39,525	7,239
AZT + PFA (1:200)	0.676 ± 0.201	3,729	5,516
AZT + ddT (1:5)	0.023 ± 0.002	522	22,696
PFA + ddT (40:1)	5.00 ± 0.19	4,988	998
AZT + PFA + ddT (1:200:5)	0.664 ± 0.172	3,199	4,818

[a] Inhibition of HIV-1 replication was determined by p24 ELISA in MT4 cells.

[b] Inhibition of cell growth was evaluated by the XTT microculture tetrazolium assay in uninfected MT4 cells.

[c] The values are means of two experiments, thea results of which are given in Table 3.

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