ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1987, p. 168–172
0066-4804/87/020168-05$02.00/0

Vol. 31, No. 2

Synergistic Inhibition of Human Immunodeficiency Virus In Vitro by Azidothymidine and Recombinant Alpha A Interferon

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Received 8 August 1986/Accepted 28 October 1986

Both recombinant alpha A interferon and azidothymidine inhibit the replication of human immunodeficiency virus in peripheral blood mononuclear cells. Combinations of recombinant alpha A interferon and azidothymidine at concentrations that are easily achievable in patients synergistically inhibit human immunodeficiency virus in vitro with minimal toxicity. Combinations of antiretroviral compounds that act by different mechanisms may prove useful in the treatment of acquired immunodeficiency syndrome-related disorders.

Several compounds inhibit the replication of human immunodeficiency virus (HIV) in vitro. Many of these agents, including azidothymidine (AZT) (11) and phosphonoformate (16), appear to act by inhibiting viral reverse transcriptase (RT). In contrast, alpha interferon probably acts at stages of viral replication other than reverse transcription (10, 13). Phosphonoformate or nucleoside analogs and interferons may have synergistic antiviral action against herpesviruses (1, 12). We previously reported synergistic inhibition of HIV by recombinant alpha A interferon (rIFN-α_A) and phosphonoformate (9). In these studies, we evaluated the interactions of AZT and rIFN-α_A on HIV replication in vitro.

MATERIALS AND METHODS

rIFN-α_A was obtained from Hoffmann-La Roche Inc., Nutley, N.J., and assayed by a cytopathic effect reduction method with vesicular stomatitis virus and human foreskin fibroblasts. Interferon preparations were compared with a standard alpha interferon preparation obtained from the National Institutes of Health to determine their potency. AZT was obtained from Burroughs-Wellcome Co., Research Triangle Park, N.C.

Peripheral blood mononuclear cells from healthy HIV-seronegative volunteer donors were obtained by Ficoll-Hypaque sedimentation of heparinized blood. Cells were treated with 10 μg of phytohemagglutinin per ml and grown in RPMI 1640 medium supplemented with 20% fetal calf serum, antibiotics, L-glutamine, and 10% interleukin-2 (Electro-Nucleonics, Inc., Silver Spring, Md.). From 4 to 6 days after exposure to phytohemagglutinin, 4×10^5 cells per ml were placed in 25-cm^2 flasks containing 5 ml of medium and then exposed to drugs and virus as described below. The day of virus inoculation is referred to as day 0. On day 4, fresh medium was added. Every 3 to 4 days thereafter, a portion of the cell suspension was removed for analysis and replaced with cell-free medium. Experiments 2 and 4 were terminated after 14 days, and experiments 1 and 3 were ended after 16 days.

Virus stocks were supernatant fluids of H9 cells (obtained from R. C. Gallo) infected with human T-cell lymphotropic virus type III_B (HIV) and frozen in aliquots at −70°C. The 50% tissue culture infective dose ($TCID_{50}$) of the virus stock was 10^5/ml (as described below).

Experimental outline. Four separate experiments were performed on peripheral blood mononuclear cells from different donors (Table 1). In experiment 1, both drugs were added after cells were exposed to virus. In experiments 2 to 4, cells were incubated for 24 h in medium with or without rIFN-α_A and then exposed to AZT and virus. Virus was added directly to the cultures in a small volume of medium and not washed off. Drug concentrations were adjusted at each medium change to maintain the original concentrations.

In all experiments, serial twofold dilutions of a fixed combination of rIFN-α_A and AZT were studied. Each concentration of rIFN-α_A and AZT used in combination was also studied alone. Duplicate cultures were maintained for each drug concentration and for infected and uninfected controls.

Assays of viral replication. After approximately 1 week, cultures were evaluated every 3 to 4 days for presence of virus. Cells were evaluated for HIV antigens by indirect immunofluorescence; supernatant fluids were evaluated for RT activity, virus yield, and HIV p24 antigen by radioimmunoassay.

(i) Indirect immunofluorescence assay. The indirect immunofluorescence assay was performed as previously described (17) with serum from an HIV seropositive donor and fluorescein isothiocyanate-labeled sheep anti-human immunoglobulin G. Control cells were exposed to phosphate-buffered saline followed by the fluorescein-labeled conjugate.

(ii) RT assay. Cell-free virus was precipitated in polyethylene glycol, and particulate RT activity was measured as previously described (15). All RT results, corrected for viable cell counts in the culture, were expressed as counts per minute per 10^6 cells. This was done to control for possible antiproliferative drug effects.

(iii) Virus yield assay. Infectious virus was assessed by an endpoint titration method with H9 cells in 96-well plates. In well 1, 50 μl of culture supernatant fluid was added to 150 μl of medium which contained enough polyclonal rabbit anti-alpha interferon immunoglobulin (Meloy Laboratories, Springfield, Va.) to neutralize any residual interferon. All other wells contained 150 μl of medium. A 50-μl sample from well 1 was serially diluted, resulting in 9 fourfold dilutions; 50 μl containing 8×10^5 H9 cells per ml was then added to each well. One-half of the cell suspension was replaced with

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TABLE 1. Experimental outline

Expt	HIV inoculation method[a]	HIV added ($TCID_{50}$)	Time[b] (h) of drug addition		Initial concn of drug	
			rIFN-α_A	AZT	rIFN-α_A (U/ml)	AZT (μM)
1	A	10^5	0	0	128	3.2
2	B	4×10^3	−24	0	128	0.16
3	B	10^3	−24	0	128	0.16
4	B	2×10^3	−24	0	128	0.08

[a] A, Cells (40×10^6) were suspended in 20 ml of medium containing 10^5 $TCID_{50}$ of HIV for 1 h at 37°C, washed three times, and resuspended; B, indicated amount of virus was added to 2×10^6 cells in medium (the cells were not subsequently washed).

[b] From time of addition of virus inoculum. Zero indicates that the drug and virus were added simultaneously; negative values indicate that the drug was given before the addition of virus.

fresh medium on days 4 and 7. On day 11, cytopathic effects consisting of cell syncytia and ballooning cells (14) were evaluated. Titrations were done in quadruplicate for each culture; hence, eight separate titrations could be evaluated at each concentration level. The $TCID_{50}$ was determined from these eight values by the method of Reed and Muench (6).

(iv) Competition radioimmunoassay for HIV p24. The competition radioimmunoassay was developed by Du Pont Co. Wilmington, Del., and performed according to the directions of the manufacturer. Culture supernatant fluid to be assayed for HIV p24 was inactivated by adding Triton X to a final concentration of 0.5%. Standards were prepared by diluting an inactivated viral lysate containing a known quantity of HIV p24 in assay buffer. The standards and test samples (100 μl) were then added to 100 μl of a rabbit antiserum to HIV p24 and incubated at room temperature for 2 h. To this solution, 100 μl of ^{125}I-labeled, affinity-purified HIV p24 was added and incubated overnight at room temperature. A 500-μl volume of antibody reagent 2, which precipitates the primary antigen-antibody complex, was then added and incubated at 4°C for 15 min. This mixture was centrifuged, the supernatant was removed, and the precipitated radioactivity was counted on a gamma counter. The percent bound radioactivity was determined. A standard curve was plotted by using results from standard samples, and the number of nanograms of HIV p24 in test samples was determined by comparison with this curve. Values beyond those on the standard curve were approximated based on previous studies with a broader range of sample dilutions.

Synergy calculations. The multiple drug effect analysis of Chou and Talalay (5) was used to calculate combined drug effects. This method involves plotting of dose-effect curves for each agent and for multiply diluted, fixed-ratio combinations of the agents by using the median-effect equation: $f_a/f_u = (D/D_m)^m$. In this equation, D is dose, D_m is the dose required for 50% effect (e.g., 50% inhibition of RT), f_a is the fraction affected by dose D (e.g., 0.9 if RT activity is inhibited by 90%), f_u is the unaffected fraction, and m is a coefficient of the sigmoidicity of the dose-effect curve. The dose-effect curve is plotted by using a logarithmic conversion of this equation which determines the m and D_m values. Based on the slope of the dose-effect curves, it can be decided whether the agents have mutually exclusive effects (e.g., similar mode of action) or mutually nonexclusive effects (e.g., independent mode of action). A combination index (CI) is then determined with the equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{\alpha(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

where $(D_x)_1$ is the dose of agent 1 required to produce x percent effect alone, and $(D)_1$ is the dose of agent 1 required to produce the same x percent effect in combination with $(D)_2$. Similarly $(D_x)_2$ is the dose of agent 2 required to produce x percent effect alone, and $(D)_2$ is the dose required to produce the same effect in combination with $(D)_1$. If the agents are mutually exclusive, then α is 0 (i.e., CI is the sum of two terms); if the agents are mutually nonexclusive, α is 1 (i.e., CI is the sum of three terms). If it is uncertain whether the agents act in a similar or independent manner, the formula may be solved both ways. Different values of CI may be obtained by solving the equation for different values of f_a (e.g., different degrees of inhibition of RT). CI values of <1 indicate synergy, values >1 indicate antagonism, and values equal to 1 indicate additive effects. Computer programs (4) based on the median effect plot and the CI equation have been used for data analysis.

TABLE 2. Effect of rIFN-α_A and AZT on mean RT values

Expt (day)	AZT (μM)	Mean RT values[a] (virus yield[b]) at following concn of rIFN-α_A (U/ml):					
		0	8	16	32	64	128
2 (10)	0	205 ($10^{4.8}$)	173	150	193 (10^4)	169	151 ($10^{3.7}$)
	0.01	159	85				
	0.02	110		42			
	0.04	71 ($10^{3.4}$)			10 ($10^{2.3}$)		
	0.08	31				4	
	0.16	7 ($10^{2.4}$)					1 ($<10^{1.9}$)
3 (13)	0	157 ($10^{5.7}$)		143 ($10^{5.6}$)	117 ($10^{5.3}$)	117 ($10^{5.1}$)	130 ($10^{5.0}$)
	0.02	51 ($10^{4.8}$)		10 ($10^{1.9}$)			
	0.04	10 ($10^{3.0}$)			0 ($<10^{1.9}$)		
	0.08	2 ($10^{1.9}$)				0 ($<10^{1.9}$)	
	0.16	5 ($<10^{1.9}$)					0 ($<10^{1.9}$)
4 (11)	0	32			5	4	2
	0.02	8			1		
	0.04	4				0	
	0.08	1					0

[a] 10^3 cpm/10^6 cells.

[b] $TCID_{50}$ per milliliter.

TABLE 3. Mean cell counts, percent T4 cells, and T4/T8 ratios found in experiments 2 and 3[a]

Drug concn		Mean cell count (10^6 viable cells/ml)						% T4		T4/T8 ratio	
AZT (μM)	rIFN-α_A (U/ml)	Expt 2 on day:			Expt 3 on day:			Expt 2 on day 10	Expt 3 on day 9	Expt 2 on day 10	Expt 3 on day 9
		7	10	14	6	9	16				
0	0	4.1	1.6	2.4	5.0	4.0	1.9	11	25	0.13	0.29
0.04	0	4.0	1.7	2.2	4.5	3.3	2.3	ND[b]	ND	ND	ND
0.16	0	3.7	2.1	2.7	4.6	4.1	2.5	34	35	0.49	0.39
0	32	5.0	1.5	1.7	4.7	3.3	2.8	ND	ND	ND	ND
0	128	4.2	1.6	1.5	4.4	4.0	2.3	24	39	0.30	0.44
0.04	32	4.3	1.9	1.6	4.8	3.1	3.8	ND	ND	ND	ND
0.16	128	4.1	1.7	1.3	5.0	2.9	2.0	33	38	0.45	0.44

[a] In experiment 4, no antiproliferative effects were seen on days 7 and 11. On day 14, antiproliferative effects of AZT and rIFN-α_A were noted, but the combined effects were less than expected if the drugs had an additive effect.
[b] ND, Not determined.

Data were also evaluated by the isobologram technique, a dose-oriented geometric method of assessing drug interactions which yields the same results as the effect-oriented CI method described above. In the isobologram method, the concentration of one agent producing a desired (e.g., 50% inhibitory) effect is plotted on the horizontal axis, and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a line is drawn connecting these points. The concentration of the agents in combination which produces the same effect is plotted. If this point falls below the line, the combination is considered synergistic for mutually exclusive drugs (3, 5). Computer programs (4) based on the median-effect plot and the CI equation for automated construction of mutually exclusive (conventional) isobolograms and mutually nonexclusive (conservative) isobolograms were used with an IBM-PC computer for the present data analysis.

Assessment of drug toxicity. Viable cell counts were determined by trypan blue exclusion every day that viral replication was assessed. The percentages of T4, T3, and T8 positive cells were measured with fluorescein-labeled monoclonal antibodies (obtained from Ortho Diagnostics, Inc., Raritan, N.J.) and flow cytometry as described previously (19). T4 cell numbers and T4/T8 ratios were then calculated.

RESULTS

AZT alone. A dose-dependent inhibition of HIV replication was observed in the range between 0.01 to 0.16 μM, based on RT activity (Table 2; data not shown), virus yield (Table 2; data not shown), radioimmunoassay positivity (Table 4), and indirect immunofluorescence assay (data not shown). Concentrations of ≥0.2 μM (experiment 1) were fully inhibitory. The degree of viral inhibition varied slightly among experiments. The higher viral inoculum (4×10^3 $TCID_{50}$) may account for the diminished effect of AZT seen in experiment 2 when compared with subsequent experiments. There was no consistent antiproliferative effect of AZT alone at the doses utilized (Table 3). T4 cell numbers and T4/T8 ratios were higher in AZT-treated infected cultures than in untreated infected controls (Table 3).

TABLE 4. Effects of rIFN-α_A and AZT on HIV p24[a]

AZT (μM)	HIV p24 level (ng of protein/ml) at following concn of rIFN-α_A (U/ml):			
	0	32	64	128
0	200–300	100–200	50–100	25–30
0.02	100–200	2.2		
0.04	25–30		1.0	
0.08	11.2			0.8

[a] From day 14 of experiment 4. Similar findings were obtained on day 8.

Interferon alone. Greater variability was seen in the antiviral effect of rIFN-α_A than in that of AZT from experiment to experiment. As in our previous experiments (9, 10), dose-related effects were generally observed early in the course of experiments but became attenuated at later times. No concentration used was fully inhibitory. Our previous results indicated an inverse relationship between HIV inoculum size and rIFN-α_A antiviral effect, and results from days 6 and 10 in experiments 2 (Table 2) and 3 (data not shown) support this concept. On day 10 in experiment 2, 128 U/ml induced only a 30% inhibition of RT activity, while the same dose induced 85% inhibition on day 9 in experiment 3, in which the virus inoculum was fourfold less than it was in experiment 2. Variability in interferon response may also be related to the use of different donors of peripheral blood mononuclear cells.

Antiproliferative effects of rIFN-α_A were not seen until day 11, when the antiviral effects were marked. Thereafter, high interferon concentrations (128 U/ml) were variably associated with antiproliferative effects; antiviral effects did not parallel toxicity. For example, although rIFN-α_A had a greater antiviral effect in experiment 4, a greater antiproliferative effect was not noted (Table 3). T4 numbers and T4/T8 ratios were higher in cultures treated with 128 U of rIFN-α_A per ml than in infected untreated controls (Table 3).

Combination of AZT and interferon. In experiment 1, all concentrations of AZT were fully inhibitory, making it impossible to judge the combined effects. In experiments 2 to 4, a synergistic interaction of the two agents was consistently observed (Tables 2, 4, and 5). The synergism was evident by all measures of viral replication employed and persisted even when the effect of rIFN-α_A alone was negligible (Fig. 1 and 2). Synergy calculations were performed by applying the multiple drug effect analysis to RT data from experiments 2 to 4, virus yield data from experiments 2 and 3, and radioimmunoassay data from experiment 4. Synergy was found whether the mutually exclusive or nonexclusive assumption was used in calculations. The isobologram method also indicated the same degrees of synergism as those obtained from the median-effect and CI equations.

Antiviral effects were not related to effects on cell proliferation. In most instances in which antiviral effects were noted, no antiproliferative activity was observed. Antiproliferative effects of the combination were not seen in situations where single agents had no effect. When a single

TABLE 5. CIs for AZT and rIFN-α_A from RT data in experiments 2 to 4

Expt	No. of days in culture	CI[a] at following % of RT inhibition:		
		50	90	95
2	7	2.14	0.34	0.23
	10	0.37	0.30	0.28
3	6	0.26	0.73	1.39
	13	0.12	0.15	0.17
	16	<0.01	0.02	0.05
4	8	0.01	0.03	0.04
	14	0.02	0.07	0.12

[a] CI values are determined by solving the equation for different degrees of RT inhibition. CI values of <1 indicate synergism. The CI values given were obtained by assuming the agents had mutually nonexclusive effects; values obtained by assuming mutually exclusive effects were always slightly lower.

agent (usually rIFN-α_A) did inhibit cell growth, the antiproliferative effects of the combination with the same dose were less than additive on four occasions, additive on three, and greater than additive on one. (Table 3; data not shown). In experiment 1, in which substantially higher doses of AZT were used, combined antiproliferative effects were not greater. RT values were corrected for cell counts to control for possible antiproliferative effects. T4 cell numbers and T4/T8 ratios increased in cultures treated with the combination compared with infected controls, but the values were not higher than those of cultures treated with 0.16 μM AZT alone (Table 3).

DISCUSSION

The search for treatment of HIV infections poses many problems. The virus infects lymphocytes and macrophages and can induce both latency and persistent productive infections (7). Considerable genetic variability exists among clinical isolates (8), and drug resistance may become a problem. One way to reduce potential resistance, maximize antiviral effects, improve the volume of distribution, and minimize toxicity may be to utilize antiviral combinations against HIV. Similar combination chemotherapy is widely used in other infections, such as tuberculosis, and has been demonstrated in viral infections as well (1, 12, 18).

Several agents have shown anti-HIV activity in vitro, including RT inhibitors and rIFN-α_A. Whereas RT inhibitors act at an early stage of virus replication, interferons are believed to act later, perhaps against retrovirus assembly or release (13). We previously showed that phosphonoformate, an RT inhibitor, and rIFN-α_A are synergistic against HIV in vitro (9). Our current studies demonstrate that over a broad range of drug concentrations easily achievable in patients, AZT and rIFN-α_A are also synergistic, whether one measures HIV replication by RT activity, antigen production, or virus yield. Synergism was shown without toxicity to the peripheral blood mononuclear cells utilized in our studies.

Both AZT and rIFN-α_A are currently undergoing clinical trials in patients with acquired immunodeficiency syndrome (AIDS). In preliminary studies, AZT showed encouraging results in partially restoring certain immune functions (21). It is orally bioavailable and crosses the blood-brain barrier. Large-scale placebo-controlled trials recently showed reduced motality among patients treated with AZT within 120 days of their first episode of *Pneumocystis carinii* pneumonia (2). rIFN-α_A repeatedly demonstrated a modest beneficial effect on Kaposi's sarcoma, a major manifestation of AIDS (20). Moreover, recent studies suggest an anti-HIV effect of rIFN-α_A in patients with AIDS (S. E. Krown, Proc. Int. Conf. AIDS **88**:s14f, p. 35, 1986.). Placebo-controlled trials have been conducted with rIFN-α_A in patients with AIDS, and these are currently under analysis. Based on our observations of a synergistic anti-HIV interaction between AZT and rIFN-α_A in vitro, a clinical trial of this combination would appear warranted, perhaps in patients with early Kaposi's sarcoma, as both clinical and antiviral effects could be most easily demonstrated in these patients. Phase I trials of this combination are planned by the AIDS Treatment

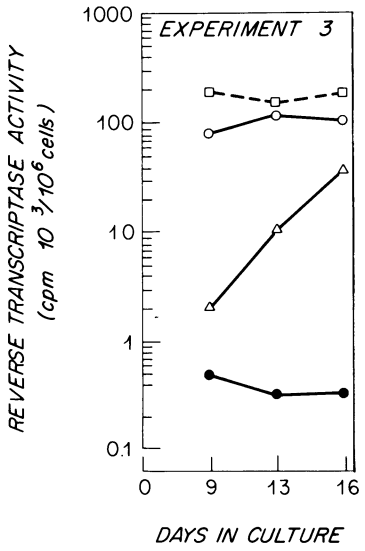

FIG. 1. Effect of rIFN-α_A (32 U/ml) and AZT (0.04 μM), alone and combined, on RT activity. Infected-control values shown for comparison. Symbols: □, control; ○, rIFN-α_A (32 U/ml); △, AZT (0.04 μM); ●, IFN (32 U/ml) and AZT (0.04 μM).

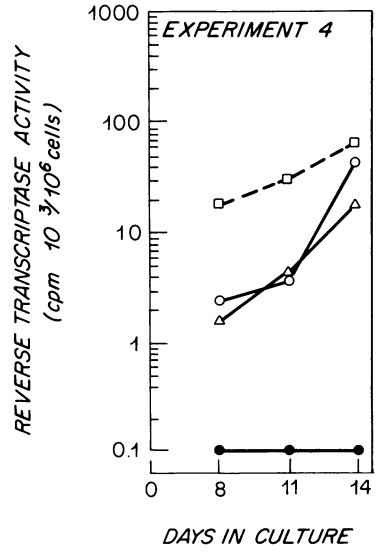

FIG. 2. Effect of rIFN-α_A (64 U/ml) and AZT (0.04 μM), alone and combined, on RT activity. Infected-control values shown for comparison. Symbols: □, control; ○, IFN (64 U/ml); △, AZT (0.04 μM); ●, IFN (64 U/ml) and AZT (0.04 μM).

Evaluation Unit program of the National Institutes of Health.

ACKNOWLEDGMENTS

We thank Timothy Paradis and Manuel Banham for valuable technical assistance and Laura Surman and Janet Steele for manuscript preparation.

This study was supported by Public Health Service grants CA09382, CA27569, and CA12464 from the National Cancer Institute, a Massachusetts Department of Public Health contract, and the Mashud A. Mezerhane B. Fund. M.W.V. is supported by the Swiss National Science Foundation.

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