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Synergistic Inhibition of Human Immunodeficiency Virus Type 1 and Type 2 Replication In Vitro by Castanospermine and 3′-Azido-3′-Deoxythymidine

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Castanospermine and 3′-azido-3′-deoxythymidine (zidovudine) were evaluated in combination against human immunodeficiency virus (HIV) replication in vitro. Castanospermine and 3′-azido-3′-deoxythymidine inhibited HIV type 1 synergistically in acutely infected H9 cells. In addition, they synergistically inhibited both HIV type 1 and HIV type 2 in peripheral blood mononuclear cells. There were no additional toxic effects of these agents in combination. Drug interactions were evaluated by the median-effect principle and the isobologram technique. Combinations of a glycosylation inhibitor, such as castanospermine, with 3′-azido-3′-deoxythymidine deserve consideration for HIV-related chemotherapeutic intervention.

Numerous agents have been evaluated for their ability to interrupt the in vitro replication of human immunodeficiency virus (HIV) type 1 (HIV-1), the causative agent of the acquired immunodeficiency syndrome and related disorders. The activity of 3′-azido-3′-deoxythymidine (AZT; zidovudine), a reverse transcriptase inhibitor, against HIV-1 replication is well established (21, 22, 31). Others have reported that AZT has inhibitory effects against HIV type 2 (HIV-2) replication as well (20, 26). AZT has also demonstrated clinical efficacy in a multicenter double-blind, placebo-controlled study for patients with acquired immunodeficiency syndrome or advanced acquired immunodeficiency syndrome-related complex (8), although not without toxic side effects (27).

Combining AZT with agents that act by different mechanisms may result in increased therapeutic efficacy, particularly if synergistic interactions occur. Combination therapies may also reduce the likelihood of resistance emerging as well as reduce toxicity if lower doses of each agent can be used in combination. Previously, combinations of either AZT or 2′,3′-dideoxycytidine and recombinant alpha-A interferon have been shown to inhibit HIV-1 synergistically in vitro (12, 33). Alpha interferon probably targets a later step in HIV replication than reverse transcription (9, 11, 14). Clinical trials evaluating AZT and recombinant alpha-A interferon are now in progress (13).

Other anti-HIV agents which may also target later steps in the HIV replicative cycle include inhibitors of envelope glycosylation, such as castanospermine and 1-deoxynojirimycin (10, 32, 34). Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) (CAS) is a plant alkaloid that is extracted from the seeds of the Australian chestnut tree *Castanospermum australe* (16). CAS interrupts glycoprotein processing by the inhibition of the endoplasmic reticulum enzyme α-glucosidase I, which affects the removal of glucose residues from precursor oligosaccharide side chains during the synthesis of mature N-linked complex glycoproteins (24, 28, 29). These altered glycoproteins are composed of inadequately cleaved carbohydrate chains, which may affect subsequent function and cell surface expression (7, 23). CAS treatment of chronically HIV-1-infected H9 cells results in a relative excess of the precursor envelope glycoprotein (gp160), when compared with the cleaved exterior glycoprotein (gp120), as well as a decreased amount of gp120 cell surface expression (34). This reduction in both the rate of cleavage of gp160 to gp120 and the decreased surface expression of gp120 may account for the marked inhibition of envelope-mediated syncytium formation and virion infectivity (34). CAS has been demonstrated to have potent anti-HIV-1 effects in vitro at concentrations that do not demonstrate cellular toxicity (10, 32, 34). In the present study, the interactions of CAS and AZT in combination against HIV replication in vitro were evaluated.

MATERIALS AND METHODS

Cells and cell lines. The H9 clone, a $CD4^+$ human lymphoblastoid line, was provided by R. C. Gallo, National Cancer Institute, Bethesda, Md. (25). H9 cells were propagated in R-20 medium (RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum [Sigma Chemical Co., St. Louis, Mo.], penicillin [250 U/ml], streptomycin [250 μg/ml], L-glutamine [2 mM], and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer [10 mM]). Peripheral blood mononuclear cells (PBMCs) were obtained from HIV-seronegative donors via Ficoll-Hypaque density gradient centrifugation of heparinized blood. Cells were treated with phytohemagglutinin P (Difco Laboratories, Detroit, Mich.) (10 μg/ml) and propagated in R-20 medium supplemented with 10% interleukin-2 (Electro-Nucleonics, Inc., Silver Spring, Md.).

Viruses. The HTLV-IIIB isolate of HIV-1 was obtained from R. C. Gallo. The LAV-2/ROD isolate of HIV-2 was obtained from L. Montagnier, Pasteur Institute, Paris, France. These virus stocks were propagated in H9 cells and stored at −70°C. The 50% tissue culture infective doses ($TCID_{50}$) per ml of the original cell-free virus pools were determined by endpoint titration with H9 cells in 96-well microdilution plates as previously described (12), with 2×10^4 cells in 0.2 ml per well. The titrations were done in

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TABLE 1. Experimental design

Expt no.	Cell type[a]	HIV type	HIV inoculum ($TCID_{50}/10^6$ cells)	Method of virus inoculation[b]	Timing of drug addition[c]	Concn range tested (μM)	
						AZT	CAS
1	H9	1	2,500	A	I	0–0.64	0–339
2	H9	1	2,500	A	I	0–0.64	0–169
3a	PBMC	1	1,500	A	I	0–0.64	0–339
3b	PBMC	2	1,500	A	I	0–0.64	0–339
4a	PBMC	1	1,500	A	I	0–0.16	0–339
4b	PBMC	2	1,500	A	I	0–0.16	0–339
5a	PBMC	1	150,000	B	II	0–0.16	0–339
5b	PBMC	2	150,000	B	II	0–0.16	0–339

[a] The PBMC experiments were conducted in parallel.
[b] In method A, the indicated amount of virus was added to cells in medium, and the cells were not washed. In method B, the virus inoculum was incubated with the cells for 1 h at 37°C; the cells were then washed three times and suspended in medium.
[c] The drug(s) and virus inoculum were added simultaneously to the cell suspensions (I), or the drug(s) was added after the cells were incubated with the virus for 1 h and washed (II).

sextuplicate, and the $TCID_{50}$ values were calculated by the method of Reed and Muench as described previously (6).

Compounds. AZT was obtained in powder form from P. A. Furman, Wellcome Research Laboratories, Research Triangle Park, N.C. It was dissolved in sterile phosphate-buffered saline and stored at −20°C until used. CAS was provided by L. Rohrschneider, Fred Hutchinson Cancer Research Center, Seattle, Wash. The drug was greater than 99% pure as judged by paper chromatography and 360-MHz nuclear magnetic resonance spectroscopy (34).

Virus replication assays. (i) p24 antigen production. Cell-free culture supernatant fluids were assayed by either a competitive HIV-1 p24 core antigen radioimmunoassay (DuPont, NEN Research Products, Boston, Mass.) as previously described (12) or a second-generation HIV-1 p24 core antigen enzyme-linked immunosorbent assay (DuPont). All values represent duplicate determinations for each data point represented.

(ii) RT assay. The reverse transcriptase (RT) activity of cell-free culture supernatant fluids was determined as described previously (15).

(iii) Virus yield assay. The $TCID_{50}$ values of cell-free culture supernatant fluids were determined in triplicate by the endpoint dilution method described above (12).

Experimental design. The experimental design for six separate experiments is outlined in Table 1. In each experiment, flasks were established with multiply diluted fixed-ratio combinations of the drugs or with each drug alone. In addition, uninfected drug-treated toxicity controls were maintained at the peak concentration of each agent studied (both alone and in combination). Cell proliferation and viability were assessed at each harvest by the trypan blue dye exclusion technique. All data points represent the results from single cultures.

In the first set of experiments (experiments 1 and 2), uninfected H9 cells (2×10^6) were suspended in 5 ml of R-20 medium in T-25 flasks (Falcon, Becton Dickinson Laboratory, Lincoln Park, N.J.). Cell-free HIV-1 (5,000 $TCID_{50}$) and each drug (either alone or in combination) were added simultaneously to each flask. Cell suspensions were incubated at 37°C in 5% CO_2 for 12 days. On days 3, 5, 7, and 10, 2 ml of the cell suspensions from each flask was suspended in 5 ml of medium containing the original drug concentrations. On days 7, 10, and 12, cell-free supernatant fluids were harvested for determination of p24 antigen production, RT activity, and infectious virus yield.

In the second set of experiments (experiments 3 to 5), 4-day phytohemagglutinin-stimulated PBMCs from a single HIV-seronegative donor were grown in R-20 culture medium with 10% interleukin-2 and challenged with either HIV-1 or HIV-2. In experiments 3 and 4, drugs were added simultaneously with 7,500 $TCID_{50}$ of HIV-1 or HIV-2 per 5×10^6 PBMCs in a 5-ml final volume in T-25 flasks. In experiment 5, an initial virus inoculum of 1.5×10^5 $TCID_{50}$ of HIV-1 or HIV-2 per 10^6 PBMCs was incubated with the PBMCs in 5 ml of medium at 37°C for 1 h. Cells were then washed three times with phosphate-buffered saline and suspended at 10^6 cells per ml in 5 ml containing the respective drug(s). Identical drug treatment and culture conditions for the HIV-1 and HIV-2 experiments were maintained throughout the experiments. On days 4, 7, and 10, 2-ml samples of cell suspensions were suspended in 5 ml of R-20 medium with 10% interleukin-2 containing the original drug concentrations. On days 7, 10, and 14, cell-free supernatant fluids were harvested for determination of p24 antigen production and RT activity.

Synergy calculations. Drug interactions were analyzed by the median-effect principle and the isobologram technique (3–5, 12). The multiple drug effect analysis of Chou and Talalay was used to calculate combined drug effects. This method involves the plotting of dose-effect curves for each agent and for multiply diluted fixed-ratio combinations of the agents using the median-effect equation (3–5, 12). The slope of the plot, which signifies the shape of the dose-effect curve, and the x intercept of the plot, which signifies the potency of each compound and each combination, were then used for a computerized calculation of the combination index. Combination indices of <1, $=1$, and >1, indicate synergism, additive effects, and antagonism, respectively. In addition, the data were also analyzed by the isobologram technique, which evaluates drug interactions by a dose-oriented geometric method. Full details of these methods and the computer data analysis have been described previously (3–5, 12).

RESULTS

Antiviral effects of each agent tested alone. In acutely infected H9 cells (experiments 1 and 2), a dose-dependent inhibition of HIV-1 replication was seen with AZT at concentrations of ≥ 0.04 μM. In PBMCs infected with HIV-1 (experiments 3 through 5), AZT inhibited HIV-1 replication in the range of 0.01 to 0.16 μM; concentrations of ≥ 0.16 μM were fully inhibitory. These dose-related effects persisted throughout the 14-day PBMC HIV-1 experiments. In PBMCs infected with HIV-2 (experiments 3 through 5), inhibition with AZT was seen at concentrations of 0.01 to

TABLE 2. Inhibition of HIV-1 p24 antigen production by CAS and AZT in acutely infected H9 cells[a]

AZT (μM)	HIV-1 p24 antigen level at the following concn of CAS (μM):				
	0	5	21	85	339
0	355	180	33	9	2
0.01	300	175			
0.04	223		19		
0.16	198			3	
0.64	102				ND

[a] Results from day 10 of experiment 1 are shown. HIV-1 p24 core antigen radioimmunoassay determinations are given in nanograms of protein per milliliter. ND, Below the lower limit of detection (<0.625 ng/ml). Similar results were obtained when the data were adjusted for viable cell numbers.

0.16 μM. In experiments 3 and 4, where the virus inoculum was added simultaneously with AZT, dose-related inhibition of HIV-2 occurred at concentrations of 0.01 to 0.16 μM on day 7. However, by day 10 in culture, AZT at 0.16 μM was no longer fully inhibitory. In experiment 5, in which a high virus inoculum was preincubated with the PBMCs before drug addition, AZT at 0.16 μM was only 50% inhibitory on day 7. AZT at ≥0.64 μM was fully inhibitory against HIV-2 replication in experiment 3 throughout 14 days in culture. No cellular toxicity was seen in the H9 and PBMC experiments at any of the concentrations tested (data not shown).

In acutely infected H9 cells, CAS inhibited HIV-1 replication in the range of 42 to 339 μM; concentrations of 169 to 339 μM were more than 90% inhibitory in multiple experiments. These CAS effects were particularly evident when high levels of p24 antigen production and RT activity were achieved in the infected controls and persisted throughout the 12 days of the experiments. In contrast, the CAS effects in PBMCs did not last throughout the 14-day experiments and varied with the method and dose of virus inoculum. The CAS concentrations required to inhibit HIV replication in PBMCs were also greater than those required in H9 cells. In experiments 3 and 4, where the virus inoculum was added simultaneously with CAS, inhibition of HIV-1 and HIV-2 replication occurred at concentrations of 169 to 339 μM only on days 7 and 10. In experiment 5, in which a high virus inoculum was preincubated with the PBMCs before drug addition, CAS concentrations of 339 μM were minimally

TABLE 3. Combination indices for CAS and AZT experiments with HIV-1 and HIV-2

Expt	Day	HIV type	Cell type	Assay[a]	CI[b] at following % inhibition:	
					90	95
1	7	1	H9	p24	0.68	0.54
1	10	1	H9	p24	0.65	0.55
2	12	1	H9	p24	0.65	0.59
2	12	1	H9	VYA	0.86	0.91
3b	10	2	PBMC	RT	0.54	0.42
4a	10	1	PBMC	p24	0.39	0.44
4b	7	2	PBMC	RT	0.66	0.54
5a	7	1	PBMC	p24	0.77	0.73
5a	10	1	PBMC	p24	0.87	0.81

[a] p24, p24 core immunoassay; VYA, virus yield assay; RT, RT assay.
[b] Combination indices (CI) are calculated from experimental data by using computer simulation of the isobologram equation as previously described (3–5, 12). Values of <1, =1, and >1 indicate synergism, additive effects, and antagonism, respectively.

TABLE 4. Inhibition of HIV-1 p24 antigen production by CAS and AZT in PBMCs[a]

AZT (μM)	HIV-1 p24 antigen level at the following concn of CAS (μM):					
	0	21	42	85	169	339
0	53.5	57.0	52.5	48.5	44.5	40.5
0.01	33.5	33.0				
0.02	10.7		6.6			
0.04	4.1			3.5		
0.08	3.3				1.3	
0.16	1.0					0.6

[a] Results from day 7 of experiment 5a are shown. HIV-1 p24 core antigen enzyme-linked immunosorbent assay determinations are given in nanograms of protein per milliliter. Similar results were obtained when the data were adjusted for viable cell numbers.

effective throughout the course of the experiments. No CAS cellular toxicity was seen in H9 cells or PBMCs in the concentration ranges tested (data not shown).

Combined CAS and AZT antiviral effects. In acutely infected H9 cells, combinations of CAS (21 to 339 μM) and AZT (0.04 to 0.64 μM) inhibited HIV-1 synergistically, as measured by p24 antigen production (Table 2), RT activity, and infectious virus yield (data not shown). The results of the synergy analysis are shown in Table 3. In experiment 1 on day 10 (Table 2), synergy was demonstrated at several different drug concentrations. In PBMCs, combinations of CAS (42 to 339 μM) and AZT (0.02 to 0.16 μM) synergistically inhibited both HIV-1 and HIV-2 on day 7, as noted by p24 antigen production (Table 4) and RT activity (Table 5), when analyzed mathematically (Table 3). Although the antiviral effects of CAS as a single agent were less evident after 7 to 10 days in PBMCs, the antiviral effects of AZT were still potentiated in combination with CAS (data not shown). The combination did not exhibit toxicity in H9 cells or PBMCs (data not shown).

DISCUSSION

Optimal HIV therapy may require the use of combinations of agents that exhibit synergistic antiviral effects. Ideally, such combinations should target different sites in the HIV replicative cycle, affect viral replication in a broad range of cell types, and yet not display additional toxicity in combination. Combination therapies may prevent the emergence of drug-resistant HIV mutants and may also allow use of individual drugs below their toxic concentrations.

Our results show a synergistic interaction between AZT and a glycosylation inhibitor, CAS, in both acutely HIV-infected H9 cells and PBMCs. As expected in biologic

TABLE 5. Inhibition of HIV-2 RT activity by CAS and AZT in PBMCs[a]

AZT (μM)	RT activity (10^4 cpm/ml) at the following concn of CAS (μM):					
	0	21	42	85	169	339
0	47.0	40.0	43.0	18.0	9.9	3.7
0.01	13.0	7.8				
0.02	6.7		3.5			
0.04	4.1			2.5		
0.08	1.7				0.4	
0.16	1.1					0.1

[a] Results from day 7 of experiment 4b are shown. Similar results were obtained when the data were adjusted for viable cell numbers.

assays, the concentrations of CAS and AZT required to fully inhibit HIV-1 and HIV-2 replication, as single agents and in combination, varied in each experiment depending on the viral isolate used, the input virus inoculum, the cell type tested, the timing of the drug addition, and the sensitivity of the HIV replication assay utilized. Thus, no attempt was made to compare degrees of synergism across multiple experiments. At the concentration ranges tested, no effect on cell proliferation or viability was noted by the drugs alone or in combination. Our experiments suggest that dose reductions of AZT and CAS may be possible when the two drugs are used in combination.

The synthesis and function of HIV envelope glycoproteins are critical for its cytopathicity (17, 18, 30). Because the HIV exterior glycoprotein is heavily glycosylated, it is an attractive antiviral target for inhibitors of N-linked glycosylation (1). CAS inhibits both HIV-1-induced gp160 envelope precursor processing, with a subsequent reduction in gp120 cell surface expression required for effective cell-to-cell fusion, and virion infectivity (34). Similarly, HIV envelope mutants which prevent the intracellular endoproteolytic cleavage of gp160 to gp120 and gp41 during viral maturation have been shown to lose their infectivity and their ability to form multinucleated giant cells (19). It is also likely that gp160 envelope precursor processing is a critical step required for the subsequent membrane fusion events after HIV gp120-CD4 receptor binding which allow HIV biologic activity and subsequent replication (19, 34).

In these experiments, CAS in the concentration range of 42 to 339 μM demonstrated more dramatic and longer-lasting antiviral effects in acutely infected H9 cells than in PBMCs under test conditions with a high multiplicity of infection. Because acutely HIV-1-infected H9 cells more readily form syncytia when compared with PBMCs, our results suggest that a critical mechanism of action of CAS involves its interruption of envelope-mediated cell fusion events in the reduction of virus spread.

Because the use of CAS as monotherapy at higher doses may be limited by toxic effects on normal cellular metabolism (2) and altered glycogen distribution (28), we evaluated combinations containing low CAS concentrations. Concentrations of CAS as low as 21 to 42 μM resulted in synergistic interactions with AZT in vitro, without apparent cellular toxicity. The results of animal toxicity studies will help further clarify the future role of CAS as a single agent or in combination. In addition, the development of newer synthetically derived glycosylation inhibitors with enhanced specificities may increase the possibilities for nontoxic chemotherapeutic interventions in combination with other antiretroviral agents.

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