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Combination antiretroviral chemotherapy: a potential strategy in AIDS-related malignancy

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There has been considerable interest and controversy over the potential clinical role of combination antiretroviral therapy, primarily in the treatment of patients with established HIV infection. In order to model the hematologic toxicity of high-dose combination antiretroviral therapy, the HL60 myeloid leukemia cell line was exposed to zidovudine, dideoxycytidine and/or didanosine. The results suggest that the myelotoxicity of high-dose combination antiretroviral therapy may be controlled by using very brief periods of drug exposure. Brief intense antiretroviral therapy may offer a useful approach, particularly in the treatment of patients with AIDS-related neoplasms who are also receiving myelotoxic antineoplastic drugs.

Key words: AZT (zidovudine), DDC (dideoxycytidine), DDI (didanosine), hematologic effects, HIV.

Introduction

Zidovudine (AZT) was the first antiretroviral drug to come into use for the treatment of HIV disease. It remains a mainstay of AIDS therapy even though it is a drug with limited efficacy and with significant hematologic toxicity with chronic use. Less intense dose schedules of AZT have helped to limit the long-term toxicity, but of course do not address the common clinical problem of patients whose disease progresses despite ongoing AZT therapy.

Strategies to deal with AZT failure have explored the use of didanosine (DDI) and/or dideoxycytidine (DDC),[1–4] also drugs with known hematologic toxicity.[5] There has been particular interest and controversy regarding the possible use of multiple agents simultaneously,[6] in the hope of improving efficacy against HIV, but it seems logical that combination therapy might also prove more myelotoxic.

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One of the most difficult settings in which to treat malignancy has been that of patients with HIV disease.[7] Such patients may develop high-grade lymphomas or other tumors which, in the absence of HIV, would routinely be treated with vigorous combination antineoplastic chemotherapy. The deoxyribonucleoside analogs which AIDS patients typically receive as antiretroviral therapy may also incidentally have toxicity for both their normal hematopoietic tissue and for their malignant cells. Hematologic malignancies in AIDS patients in turn may further compromise their hematopoietic and immune systems. As a result, patient management options in this setting have been problematic.

The HL60 cell line was derived by others from a human myeloid leukemia, and has been used in this and many other laboratories for many years as an *in vitro* model for the growth and behavior of more normal myeloid cells *in vivo*. (HL60 cells are especially useful for this purpose because, unlike normal myeloid cells, the HL60 cells can be successfully propagated for many cell generations in culture). In the present experiments, HL60 cells were exposed to brief courses of high-dose antiretroviral therapy to study the acute myelotoxicity of such therapy.

Materials and methods

Cells of the HL60 cell line (American Type Culture Collection, Rockville, MD) were grown in a mixture of 79% RPMI 1640 medium with HEPES and L-glutamine (Gibco, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (Sigma, St Louis, MO) and 1% penicillin/streptomycin (Gibco), in a 37°C 5% CO_2 humidified incubator, and carried in culture by serial passage. The antiretroviral drugs used in the experiments were AZT, DDC and DDI (Sigma). Cells were counted by a hemocytometer, with viability evaluted by Trypan blue exclusion, in order to calculate the viable cell counts. Experiments were routinely set up with

simultaneous triplicate samples. Cultures were inspected grossly and microscopically for signs of contamination during the course of the experiments, and any contaminated samples discarded. When one of a triplicate set of experiments had to be discarded, the average of the two remaining samples is reported as a result for 'mean of 2'. Statistical calculations (for standard deviations, ANOVA and HSD testing) were based only on those drug conditions for which triplicate data was obtained.

Within each experiment reported below, drug exposure was done beginning at time zero. The experimental cultures were exposed for the selected period of drug exposure to AZT 0.01 mg/ml (A), DDI 0.01 mg/ml (I) and/or DDC 0.01 mg/ml (C). AI, AC and IC represent the corresponding use of two agents each at 0.01 mg/ml; AIC the use of all three drugs each at 0.01 mg/ml. In the continuous drug exposure experiment (Figure 1 and Table 1), the cells were left in the presence of the retroviral drug(s) for the entire week until they were counted (at $t=7$ days).

One set of experiments looked at the effect of multiple 4 h pulse courses of chemotherapy separated by recovery periods in drug-free media. The first pulse of drug exposure was begun at $t=0$; the cells were resuspended in drug-free media at $t=4$ h. A second pulse of drug exposure was begun at $t=3$ days; 4 h later the cells were again resuspended in drug-free media. At $t=6$ days the third pulse of drug exposure was begun; 4 h later the cells were again resuspended in drug-free media. Viable cell counts were determined at $t=7$ days (Figure 2 and Table 2).

In another variation of the drug exposure protocol, HL60 cells were exposed to chemotherapy for 48 h (Figure 3 and Table 3). At the conclusion of the 48 h pulse, the cells were spun down and resuspended in drug-free medium and cell counts were done at the end of the week ($t=7$ days).

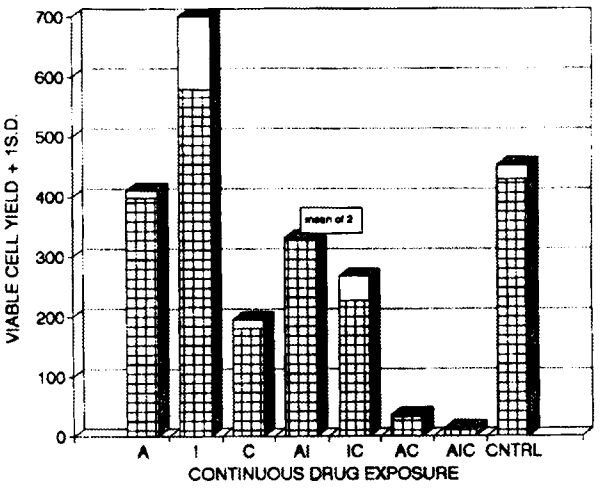

Figure 1. Continuous drug exposure. The cross-hatched bars show the mean viable cell yield after 1 week for the given drug exposure condition, as a percent of the initial cell count. (The light portion atop each bar equals 1 SD.) A represents AZT, I represents DDI and C represents DDC. AI, AC and IC are the corresponding two drug combinations, AIC is the combination of all three drugs.

Data analysis

Data analysis was based on the viable cell counts 1 week after subculturing, because this initial week is a time period where (as shown in previous experimentation from this laboratory) one would expect exponential growth in the absence of antiretroviral drugs. The yield of viable HL60 cells was determined under control (no antiretroviral drugs) or under a variety of drug exposure conditions. Analysis of variance was computed for each set of experimental data, using the viable cell counts at day 7. The α value for the F test was set at 0.01 to test the null hypothesis that there was no significant difference produced by any of the choices of chemotherapy exposure. If this preliminary testing refuted the null hypothesis, then Tukey's honestly

Table 1. Continuous drug exposure: table of mean differences

	A	I	C	IC	AC	AIC	Control
A	—	180	217*	171	365*	385*	31
I	180	—	398*	351*	465*	565*	149
C	217*	398*	—	46	148	168	249*
IC	171	351*	46	—	194	214*	202*
AC	365*	546*	148	194	—	20	397*
AIC	385*	565*	168	214*	20	—	417*
Control	31	149	249*	202*	397*	417*	—

Variance among the group means was demonstrated at the 0.01 level by analysis of variance. (VR of 35.34, exceeding the F.99 critical value of 4.46.) Pairwise differences in cell yield exceeding 200, marked by an asterisk, are significant at the $p<0.01$ level by the HSD test.

significant difference (HSD) test was used to determine, at an α level of 0.01, which pairs of experimental conditions were associated with significantly different viable cell counts on day 7.[8]

The graph in each figure shows the viable cell yield at $t = 7$ days as a percent of the count at $t = 0$. A cell yield of 100 means that the viable cell count at the end of the experiment was equal to that at the start of the experiment; a value below 100 represents a decrease in viable cell count; one above 100 an increase in count. The HSD tables accompanying each figure show the difference in mean viable cell yield between a given pair of exposure conditions, with an asterisk placed by those which were significant at the 0.01 level by the Tukey HSD test.

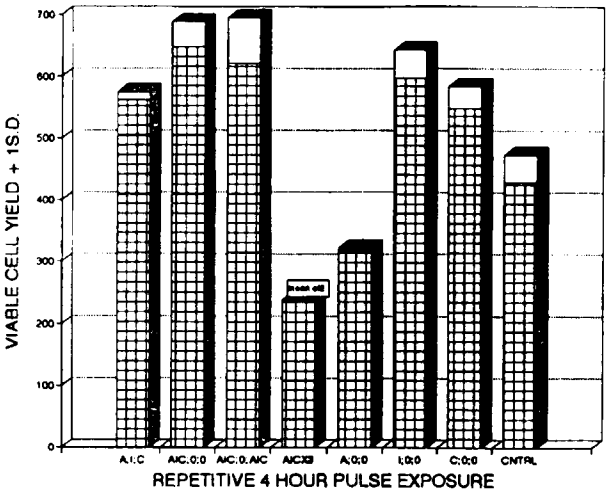

Figure 2. Repetitive 4-h pulse exposures. Viable cell yield. Drug abbreviations as in Figure 1.

Condition	First pulse	Second pulse	Third pulse
A;I;C	A	I	C
A;I;C;0;0	AIC	0	0
AIC;0;AIC	AIC	0	AIC
AIC × 3	AIC	AIC	AIC
A;0;0	A	0	0
I;0;0	I	0	0
C;0;0	C	0	0
Control	0	0	0

Results

In each of the experiments reported here (Figures 1–3) analysis of variance demonstrated that the null hypothesis was refuted at the 0.01 level. This result confirmed that retroviral therapy was able to perturb the growth pattern of the HL60 cell line when administered on any of a variety of schedules (7 day continuous exposure, 48 h pulse exposure or repetitive 4 h pulse exposure).

HL60 cells demonstrated a complex pattern of response to 7 days of continuous exposure to AZT 0.01 mg/ml, DDC 0.01 mg/ml and/or DDI 0.01 mg/ml. Effective inhibition of growth was seen when all three drugs were used together, as well as with the two-drug combination of AZT and DDC. The HL60 cells were able to grow, however, in the face of continuous exposure to any of the three drugs as a single agent (Figure 1 and Table 1).

Other experiments explored the toxicity of these concentrations of the antiretroviral drugs during less sustained exposure. One set of experiments examined the effect of three sequential 4 h pulses of drug, separated by recovery periods in drug-free media. When the period of drug exposure was limited to sequential 4 h pulses, even the triple drug combination had only a modest inhibitory effect on cell growth compared with the drug-free control (Figure 2 and Table 2).

Another set of experiments used a single 48 h exposure to drug(s) followed by resuspension in drug-free media. Cell counts at 1 week after the initial drug exposure demonstrated modest (but statistically significant) inhibition of cell growth by the three-drug combination, but not by any of the drugs as single agents (Figure 3 and Table 3).

In almost all of the experimental conditions reported here, the yield of viable cells at the end

Table 2. Repetitive 4 h pulse exposures: table of mean differences

	A;I;C	AIC;0;0	AIC;0;AIC	A;0;0	I;0;0	C;0;0	Control
A;I;C	—	86	60	243*	36	13	132
AIC;0I;0	86	—	26	329*	51	99	210*
AIC;0;AIC	60	26	—	303*	24	73	192*
A;0;0	243*	329*	303*	—	278*	230*	111
1;0;0	36	51	24	278*	—	48	167
C;0;0	13	99	73	230*	48	—	119
CNTRL	132	218*	192*	111	167	119	—

Variance among the group means was demonstrated at the 0.01 level by analysis of variance. (VR of 15.00, exceeding the F.99 critical value of 4.46). Pairwise differences in cell yield exceeding 170, marked by an asterisk, are significant at the $p < 0.01$ level by the HSD test.

Table 3. Forty-eight hour pulse exposure: table of mean differences

	A	I	C	AI	IC	AC	AIC	Control
A	—	14	12	191	101	58	435*	100
I	14	—	25	205	114	44	449	86
C	12	25	—	179	89	69	423*	112
AI	191	205	179	—	90	249*	244	291*
IC	101	114	89	90	—	159	334*	201
AC	58	44	69	249*	159	—	493*	42
AIC	435*	449*	423*	244	334*	493*	—	535*
Control	100	86	112	291*	201	42	535*	—

Variance among the group means was demonstrated at the 0.01 level by analysis of variance. (VR of 19.50, exceeding the F.99 critical value of 4.03). Pairwise differences in cell yield exceeding 244, marked by an asterisk, are significant at the $p > 0.01$ level by the HSD test.

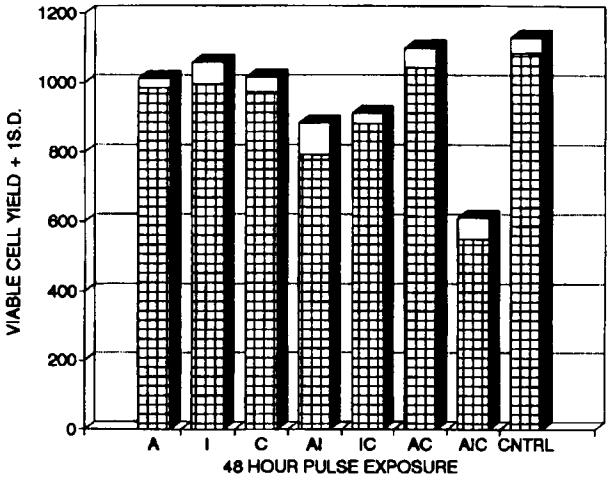

Figure 3. Forty-eight hour pulse exposure. Viable cell yield. Drug abbreviations as Figure 1.

of the experiment was over 100, indicating that any cell kill produced by the drug(s) was more than offset by new cell growth. The only conditions which produced a net increase in cells (viable cell yield below 100) were ones which involved continuous exposure to combination drug therapy (Figure 1 and Table 1).

Discussion

There are only limited data in the literature on brief exposure of humans to very high-dose antiretroviral drug therapy, in the form of patient overdoses with AZT as a single antiretroviral agent.[9] This anecdotal experience has indicated generally good human tolerance of high-dose pulse AZT exposure; some patients have had questionable neurologic effects after zidovudine overdose, but these have been of brief duration. The present experiments likewise suggest that one might use high doses of any one of the three single antiretroviral agents with acceptable myelotoxicity, at least for short exposures.

The doses of the three drugs were kept equal by weight; because of the similar molecular weights (AZT 267.2, DDC 211.2, DDI 236.2) of these three dideoxynucleosides, the doses of the three agents are roughly equivalent on a molar basis. At nearly 40 μm AZT, for example, the AZI dose is somewhat higher than typical peak therapeutic levels, but within the range that would be associated with episodes of patient overdoses with AZT.

The theoretical advantage of combining several agents is that one might thereby circumvent the problem of resistance to a particular one of the antiretroviral drugs.[10] It is less clear whether combination antiretroviral pulse exposure would be as well tolerated as high doses of any single drug. The data presented here suggest that combinations of high doses of antiretroviral drugs may have important myelotoxicity with prolonged exposure. This result is consistent with previous findings by others in an animal model.[11] Similarly, previous *in vitro* experiments by Dornsife and colleagues have demonstrated that continuous exposure to AZT and/or DDI is markedly toxic for human bone marrow progenitors even at lower doses than those studied in the present work.[12]

The assay system used here looked only at hematologic toxicity. Combination antiretroviral therapy may also increase the risk of toxicity for other organ systems.[13] In particular, one would anticipate that any prolonged use of DDC at the dose attempted here would be prevented by significant non-hematologic toxicity such as neuropathy.

The *in vitro* data presented here suggest that keeping the patient's exposure period very brief is a promising strategy to explore to moderate the toxicity of combination high-dose retroviral therapy. The *in vitro* data presented here should not alter routine clinical practice, but they do suggest a

potential strategy to explore in the research setting. In particular, AIDS patients have tolerated aggressive cancer chemotherapy approaches poorly and it is important to consider how to coordinate their overall therapy in a way which they can tolerate. In part, this has involved less vigorous cytotoxic therapy, but the overall strategy should include consideration of how to tailor their antiviral therapy as well.

The data here presented suggest that a useful strategy to explore for the antiretroviral therapy of patients with AIDS-associated malignancies might be to use brief but intense combination antiretroviral chemotherapy pulses. The most convenient strategy might be to administer the antiretroviral therapy simultaneously with the pulses of chemotherapy for the underlying malignancy, if simultaneous administration proved tolerable. Questions which would need to be addressed in the clinical setting are whether pairing combination antiretroviral therapy pulses with combination antineoplastic therapy produces a clinically significant exacerbation in the intensity or duration of the hematopoietic toxicity, and whether such toxicity can still be successfully ameliorated with cytokine therapy.

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