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Inhibition of human immunodeficiency virus type 1 infection in vitro by combination of delavirdine, zidovudine and didanosine[1]

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Abstract

Delavirdine (DLV), a non-nucleoside reverse transcriptase inhibitor (RTI) of human immunodeficiency virus type 1 (HIV-1), was evaluated in two and three-drug combinations against acute and co-culture infections of HIV-1_{JRCSF} in human peripheral blood mononuclear cells. DLV combined with didanosine (DDI) at 1:10 and 1:30 ratios were statistically synergistic (combination indices (CI) < 1) at $> 75\%$ inhibition levels. However, at 1:100 ratio, the interaction appeared to be additive. Three-drug combinations of zidovudine (ZDV), DLV, and DDI (at a ratio of 1:2:333) were synergistic at 50–99% inhibition levels. The three-drug group also showed significantly ($P < 0.01$) lower p24 levels in acute cultures than two-drug combination groups (DLV + ZDV, DLV + DDI, ZDV + DDI). In co-culture studies, the extent of viral inhibition was dependent on drug dose and the duration of treatment. Combination of DLV, ZDV, and DDI at IC_{95} concentration of the individual drugs showed complete inhibition of viral growth in co-culture after 19 days, but not after 7 or 12 days of treatment. The combinations studied did not show additive or synergistic drug toxicity. These data provide an in vitro basis for beneficial use of DLV in combinations with DDI and ZDV in HIV-1 infected patients. © 1997 Elsevier Science B.V.

Keywords: Drug Combination; Delavirdine; Reverse transcriptase inhibitors; HIV-1

1. Introduction

The emergence of resistant virus during antiviral drug therapy presents a major challenge for the development of antiviral agents for the control of human immunodeficiency virus type 1 (HIV-1) infection. HIV-1 exists in vivo as a large popula-

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tion of genetic variants due to a high rate of viral replication and mutation accompanied by a broad tropism for a variety of host cells (von-Briesen et al., 1987; Goodenow et al., 1989; Meyerhans et al., 1989; Groenink et al., 1991; Ball et al., 1994). From this heterogenous virus population, pre-existing drug resistant variants are rapidly selected during drug treatment, particularly when a single antiviral agent has been used (Mohri et al., 1993; Nájera et al., 1994; Kozal et al., 1995; Nájera et al., 1995). In theory, it should be possible to suppress the emergence of resistant virus by employing a highly potent inhibitor that can completely inhibit viral replication (Richman, 1996). However, this level of control of viral replication in vivo is more likely to be accomplished by the combined use of several drugs that might suppress or delay the development of drug resistance (de Jong et al., 1996). Combination therapy for HIV-1 infection has also shown other benefits such as an additive or synergistic drug effect, reduced drug toxicity, and enhanced range of viral targets (recently reviewed by Caliendo and Hirsch, 1994; De Clercq, 1994a; Hammer et al., 1994; Johnson, 1994; de Jong et al., 1996).

A proven target for HIV-1 therapy is the reverse transcriptase (RT), a viral encoded multifunctional enzyme. There are currently two major classes of HIV-1 reverse transcriptase inhibitors (RTIs): the nucleoside RTIs and a structurally diverse group of non-nucleoside RTIs (NNRTIs). Until recently, the only approved HIV-1 RTIs were nucleoside analogs such as zidovudine (ZDV), zalcitabine (DDC), didanosine (DDI), stavudine (D4T), and lamivudine (3TC). Several NNRTIs including delavirdine (DLV), nevirapine and loviride (α-APA) are in late stages of clinical trials (De Clercq, 1994b). Currently, nevirapine is approved by the FDA for combination therapy with nucleoside RT inhibitors. Therapy using single agents of nucleoside or non-nucleoside RTIs have been shown to result in viral resistance, but cross-resistance between these two classes of RTIs has not been observed (Balzarini et al., 1993a; Balzarini et al., 1995; Mellors et al., 1995; Arts and Wainberg, 1996). Thus, it is not surprising that enhanced antiviral effects in vitro are often observed with the combined use of various nucleoside and non-nucleoside RTIs (De Clercq, 1994b). Preliminary results from recent clinical trials have also shown the beneficial effect of combining DLV or nevirapine with various nucleoside RTIs (D'Aquila et al., 1995; Murphy et al., 1995; Freimuth et al., 1996a; Freimuth et al., 1996b). Furthermore, other investigators have reported that the combined use of nucleoside and non-nucleoside RTIs may delay or reduce the emergence of drug-resistant viral strains in vitro (Larder, 1992; Balzarini et al., 1995). For example, Larder (1992) reported that drug resistance to a NNRTI induced in culture containing a NNRTI and ZDV is associated with restoration of drug susceptibility to ZDV. These results suggest that further studies are needed to more fully explore the clinical utility of combined nucleoside and non-nucleoside RTIs.

We previously reported that DLV is synergistic when used in two-drug combinations in vitro with ZDV, DDC, protease inhibitor U-75875, or interferon-α (Chong et al., 1994; Pagano and Chong, 1995). Although DDI has been widely used in combination therapy, it has only recently been recommended by the FDA Antiviral Drugs Advisory Committee for use in first line monotherapy and may gain much wider use in patients with AIDS. We describe here the in vitro antiviral activity and cytotoxicity of DLV in combination with DDI or with DDI and ZDV in human peripheral blood mononuclear cells (PBMC) infected with a molecularly cloned clinical isolate of HIV-1. The purpose of our in vitro study is to provide rationale for prioritizing drug combinations for clinical trial. Currently, DLV is in Phase II/III clinical trials in combinations with ZDV and DDI (Freimuth et al., 1994; Davey et al., 1995). Since high concentrations of various non-nucleoside RTIs have been shown to completely suppress viral replication (Balzarini et al., 1993b), we also report the effect of combined DLV, DDI, and ZDV on recovery of viral replication after drugs were removed from treated cultures.

2. Materials and methods

2.1. Compounds

Stocks of ZDV, DDI (Sigma), and DLV (Pharmacia & Upjohn) were prepared as 10 mM solutions in dimethylsulfoxide and stored at −80°C. Stock solutions were further diluted in culture medium on the day of assay.

2.2. Virus and cells

HIV-1_{JRCSF} (Dr Irvin Chen) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This virus was originally isolated from the cerebrospinal fluid of a patient with AIDS dementia and stock virus was obtained from transfection of a molecular clone into 729 B cells and rescued by cocultivation with human PBMC. In cell culture, HIV-1_{JRCSF} replicates readily in primary human lymphocytes or mononuclear phagocytes but has not been adapted to replicate in cell lines (Koyanagi et al., 1987). For this study, stock viruses of HIV_{JRCSF} from infected human PBMC cultures were harvested as culture supernatants and titered in PBMC using a p24 enzyme-linked immunosorbent assay (ELISA) as previously described (Chong et al., 1994).

2.3. Acute infection antiviral assay

PBMC were obtained from fresh plasmapheresis preparations taken from HIV-1 sero-negative donors and prepared by density gradient centrifugation with Ficoll/Hypaque (Organon Teknika, Durham, NC). The cells were collected, washed, and incubated for 3 days in culture medium containing 4 μg/ml phytohemagglutinin (PHA). PHA-treated cells were then cultured in medium containing recombinant human interleukin-2 (80 U/ml; Gibco, Gaithersburg, MD). Antiviral assays were set up by seeding 7.5×10^4 cells into each well of a 96-well microtiter plate. PBMC were infected with HIV-1_{JRCSF} by incubation for 2 h with diluted stock virus at multiplicity of infection (MOI) of 0.005. After 5 days of incubation, culture supernatant showed a p24 concentration of 200 ng/ml (equivalent to 70% of peak viral titer achievable on day 7 post infection). For assay of antiviral activity, infected cells in duplicate or triplicate wells were maintained in the continued presence of test compounds for 5 days at 37°C in a humidified CO_2 incubator. Culture supernatants were harvested and the extent of p24 core antigen production was measured by an ELISA assay specific for HIV-1 p24 antigen (Coulter Diagnostics, Hialeah, FL). The percent inhibition of HIV replication by the compound was determined by comparing HIV p24 antigen levels in the supernatant of infected cells treated with inhibitor versus supernatant from the control cultures without compound. The concentrations of inhibitor that gave 50 and 90% inhibition (IC_{50}, IC_{90}) of HIV-1 activity were obtained using linear regression analysis.

2.4. Cell viability and survival of HIV challenged PBMC

Cell viability was measured by the formation of formazan, a tetrazolium dye, in a 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay as previously described (Chong et al., 1994). A non-infected PBMC control group was also assayed in parallel. Cell viability on various days after the start of experiments was determined and expressed as a percent of the non-infected cell control.

2.5. Viral co-culture assay

HIV-1_{JRCSF} infected PBMC (MOI of 0.005) were maintained in culture for 7 days to achieve a peak titer of ≥ 250 ng of p24 antigen per ml culture medium. Cells obtained from this infected culture were washed twice to remove cell-free virus and mixed with uninfected PBMC at 1:100 ratio. A total of 4.6×10^5 mixed cells were seeded into each well of a 24-well tissue culture plate containing 1 ml of culture medium containing 80 U/ml IL-2 with or without (drug-free virus control) the test compound(s). The uninfected cell control wells were similarly set up, but in drug-free culture medium. Every 3–4 days, 500 μl of cell-free supernatant were harvested for determi-

nation of HIV-1 p24 production. Cells in the individual wells were then resuspended and 50 μl (representing 10% of cells) were harvested and assayed for viability by MTT measurements. The medium removed from the test wells was replaced with medium containing drug(s) and the control wells received drug-free culture medium. After 7, 12 or 19 days depending on experiment design, drug treatments were removed from the cells by two washes each containing 10 ml of drug-free culture medium. Cell pellets were resuspended in 1 ml of drug free culture medium containing 80 U/ml IL-2 and transferred to new wells. Both infected and uninfected cell control wells were subjected to the same wash procedure. Following drug removal, cells and supernatant were harvested every 3–4 days for p24 antigen and MTT assays as described above. For culture treated for 19 days, beginning at 2 days after drug removal, portions of culture supernatants with undetectable p24 antigen levels were also added to fresh PBMC to determine whether infectious HIV-1 was still present.

2.6. Combination studies

The inhibition of acute HIV-1 replication by combinations of DLV, DDI, and ZDV was evaluated in experiments involving several molar ratios of the drugs. Four or five concentrations of each drug (DLV at 0.6, 2, 6, 20 and 60 nM; DDI at 100, 300, 1000 and 3000 nM; ZDV at 0.3, 1, 3 and 10 nM) or drug combinations were assayed either in duplicate wells (two-drug combination) or in triplicate wells (three-drug combination). For each molar ratio, 3–4 datapoints were analyzed for combination indices (CI) by the multiple drug effect equation of Chou and Talalay (1984) using the more conservative mutually non-exclusive drug interaction condition. The CI values at various fractional inhibitions (50, 75, 95, and 99%) were used to determine whether the combinations were synergistic ($CI < 1$), additive ($CI = 1$) or antagonistic ($CI > 1$). Estimates of accuracy of CI were calculated with Monte Carlo simulations (Belen'kii and Schinazi, 1994) and were computed using a new window-based software for dose effect analysis called Calcusyn (Biosoft, Cambridge, UK). The 95% confidence interval at each level of F_a is calculated by the formula: $CI \pm (1.96 \times S.D.)$, where the values $CI + (1.96 \times S.D.)$ and $CI - (1.96 \times S.D.)$ correspond to the upper and lower boundaries of the confidence interval, respectively. Separately a two-sample t-test at the 95% confidence level was used to compare drug effects between selected treatment groups.

In co-culture experiments, in which uninfected PBMC were mixed with infected PBMC at a 1:100 ratio, we evaluated individual compounds at IC_{50} and IC_{95}. Two to three experiments were performed for each combination regimen and assays were run in duplicate wells. Each culture well was drug-treated and assayed for p24 and cell survival separately. Comparison of the effect of different drug regimens on viral growth and cell survival was calculated using the two samples Student's t-test.

2.7. Polymerase chain reaction (PCR) analysis of culture

Culture supernatants that showed undetectable p24 antigen (< 0.01 ng/ml) were added to fresh PBMC cultures. After seven and 10 days incubation, culture supernatant were analyzed for p24 antigen and cells were analyzed for proviral DNA. Total cellular DNA isolated from $1–2 \times 10^6$ PBMC was amplified with HIV-1 specific primers, GAG3 and GAGB6 (GenSet, Paris, France) from the HIV-1 gag region as previously reported (Wathen et al., 1996). The specific amplified sequences were detected by liquid hybridization with horseradish peroxidase labeled probe GPR5 (Synthetic Genetics, San Diego, CA) for gag-specific PCR products. In all PCR analysis, an HIV-1 positive control and negative control and a buffer control containing all reagents except DNA were included.

3. Results

3.1. Effects of single agent DLV, ZDV, DDI against acute and co-culture infection in PBMC.

In preliminary experiments, we determined the

Table 1
Inhibition of HIV-1 p24 antigen production in acutely infected PBMC treated with DLV and DDI alone or in two drug combinations

DLV (nM)	DDI (nM)	Experiment 1		Experiment 2	
		p24[a] (ng/ml)	% Inhibition[b]	p24[a] (ng/ml)	% Inhibition[b]
100		14.6 ± 0.6	93.6	15.7 ± 0.8	93.0
30		85.8 ± 5.9	62.4	140.0 ± 41.2	38.1
10		165.0 ± 25.3	27.6	183.0 ± 3.2	19.0
3		194.0 ± 4.8	14.9	236.0 ± 36.8	0.0
	3000	61.6 ± 2.8	73.0	53.9 ± 13.8	76.2
	1000	206.0 ± 0.6	9.6	146.0 ± 27.7	35.4
	300	202.0 ± 22.2	11.4	205.0 ± 10.8	9.3
	100	222.0 12.0	2.6	234.0 ± 39.8	0.0
100	3000	0.9 ± 0.4	99.6	0.4 ± 0.1	99.8
30	3000	8.5 ± 2.0	96.3	20.0 ± 9.1	91.1
10	3000	19.8 ± 9.1	91.3	24.0 ± 3.1	89.4
3	3000	38.9 ± 5.6	82.9	63.6 ± 2.2	71.9
100	1000	1.4 ± 0.7	99.4	2.3 ± 1.4	99.0
30	1000	40.7 ± 10.1	82.2	42.2 ± 6.6	81.3
10	1000	50.6 ± 1.9	77.8	101.0 ± 7.1	55.3
3	1000	139.0 25.8	39.0	113.0 ± 27.1	50.0
100	300	9.4 ± 0.7	95.9	4.6 ± 0.5	98.0
30	300	47.9 ± 16.3	79.0	92.9 ± 34.0	58.9
10	300	96.6 ± 27.2	57.6	147.0 ± 4.8	35.0
3	300	192.0 ± 4.7	15.8	185.0 ± 45.9	18.1
100	100	11.9 ± 1.5	94.8	12.1 ± 2.8	94.6
30	100	81.8 ± 38.4	64.1	83.0 ± 40.5	63.3
10	100	141.0 ± 11.3	38.2	144.0 ± 3.9	36.3
3	100	223.0 ± 31.6	2.2	209.0 ± 38.0	7.5

[a] Data represent mean values ± S.E. of the mean of 2 replicate culture wells. Each well was infected, treated, and assayed for p24 antigen separately. The mean HIV-1 p24 antigen production in untreated control cultures (6 replicates) was 228.0 ± 23.9 and 226.0 ± 18.7 ng/ml of supernatant with coefficient of variation of 10.5 and 8.3% for experiment 1 and 2, respectively. The mean coefficient of variations for all treated wells (2 replicates) was 17.8 and 19.7% for experiment 1 and 2, respectively.
[b] Data represents percentage inhibited compared with untreated control.

dose effect of each drug used alone. In human PBMC cultures acutely infected with HIV_{JRCSF}, the mean IC_{50} and IC_{90} (with mean coefficients of variation of 20%) of test compounds were: 8 and 40 nM for DLV; 1000 and 3000 nM for DDI; 5 and 20 nM for ZDV. From this data, comparable concentration ranges of individual agents were selected to yield a wide-range of drug interactions in subsequent combined therapy experiments. In co-culture infection in which infected and uninfected PBMC were mixed at 1:100 ratio, a peak viral titer of 400–600 ng p24 per ml culture supernatant was achieved on day 6 of infection. For this assay, the mean IC_{50} and IC_{95} (with mean coefficient of variations of $< 10\%$) of test compounds were: 60 and 200 nM for DLV; 6000 and 10 000 nM for DDI; 20 and 100 nM for ZDV. Drug doses representing about 50 and 95% inhibitory concentrations of individual agents were then selected for the subsequent combined therapy experiments under similar assay conditions.

3.2. Effects of two- and three-drug combinations of DLV, DDI, and ZDV on acutely infected PBMC

We studied the effects of combinations of DLV and DDI on HIV-1 replication while monitoring potential cytotoxicity of these agents to PBMC.

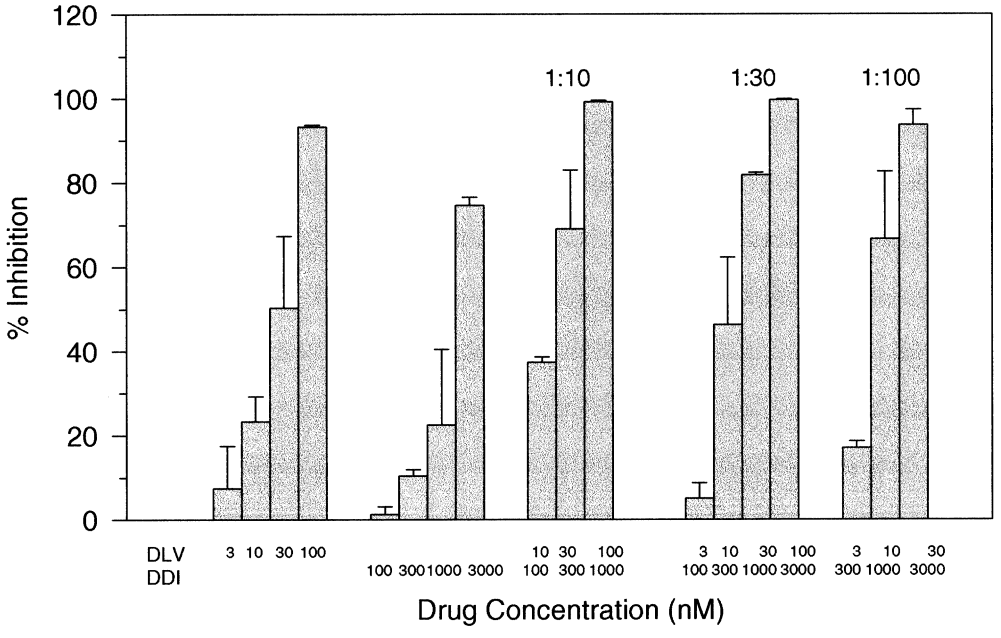

Fig. 1. Inhibition of HIV-1 p24 antigen production in acutely infected PBMC treated with DLV and DDI either alone or in two-drug combinations (ratios of DLV to DDI of 1:10, 1:30, and 1:100). HIV-1 p24 antigen concentrations in culture supernatant were measured on day 5 after infection from drug treated and nontreated control wells. Data are mean percent inhibition ± S.E. of two separate experiments each including two replicate wells treated and assayed for p24 antigen separately.

Combination of DLV and DDI in the dose range tested were consistently more effective than either agent alone (Table 1 and Fig. 1). For more quantitative analysis of drug interactions, the dose-effects for each single agent and the combinations were used to make a series of median effect plots. From these plots, we computed combination indices at combination ratios of DLV to DDI of 1:10, 1:30 and 1:100 because DLV was about 50-fold more active than DDI on a molar basis. At 1:10 and 1:30 ratios, both experiment 1 and 2 showed statistically synergistic interaction at > 75% inhibition levels. However, at 1:100 ratio, experiment 1 showed synergistic interaction whereas experiment 2 showed additive to slight antagonistic effect. Overall, we interpret the combination at 1:100 ratio as additive (Table 2).

In another experiment, we evaluated DLV, DDI, and ZDV alone and as two and three-drug combinations in acutely infected PBMC. The two-drug combinations of DLV + ZDV, DDI + ZDV and DLV + DDI were more suppressive than the corresponding drugs used alone. Combination regimens containing ZDV were slightly more inhibitory than regimens without ZDV due to the greater effective doses of ZDV selected for study (Table 3). The three-drug groups were generally more effective than the two-drug groups, which had greater antiviral activity than the corresponding single-drug groups (Table 3). Both two and three-drug combination groups showed more than 95% inhibition of viral growth at the highest drug levels tested. However, at lower drug levels, three drug combinations were significantly more inhibitory ($P < 0.01$) than two drug combinations (Fig. 2). When these drug effects were subjected to mathematical analysis, it was clear that both combinations of two or three drugs were synergistic at 95 and 99% inhibition levels (Table 2). At lower inhibition levels, the three-drug groups also showed significant synergistic effect but the two-drug groups were showing additive to slight antagonistic effect. These combinations did not show any additive or synergistic cytotoxicity as determined by MTT assay (data not shown) at the highest drug concentrations evaluated (ZDV = -

Table 2
Combination index (CI) values for two and three-drug combination of DLV, DDI and ZDV in acutely infected PBMC

Treatment (drug ratio)	Experiment No.	Parameter[a]		C.I[b] ± 1.96 S.D. at fractional inhibition of:			
		m	*r*	0.50	0.75	0.95	0.99
DLV	1	1.24 ± 0.18	0.980				
	2	1.94 ± 0.24	0.986				
DDI	1	1.19 ± 0.41	0.899				
	2	1.66 ± 0.10	0.996				
DLV/DDI (1:10)	1	2.44 ± 0.43	0.985	0.95 ± 0.28	0.68 ± 0.21	0.34 ± 0.14	0.20 ± 0.11
	2	2.26 ± 0.77	0.945	0.77 ± 0.34	0.77 ± 0.40	0.56	0.48
DLV/DDI (1:30)	1	2.52 ± 0.31	0.985	0.97 ± 0.29	0.70 ± 0.23	0.35 ± 0.17	0.19 ± 0.13
	2	2.44 ± 0.41	0.973	0.70 ± 0.24	0.63 ± 0.21	0.46 ± 21	0.43 ± 0.40
DLV/DDI (1:100)	1	2.15 ± 0.17	0.997	0.78 ± 0.28	0.50 ± 0.22	0.28 ± 0.16	0.21 ± 0.16
	2	1.66 ± 0.14	0.996	1.00 ± 0.15	1.03 ± 0.17	1.17 ± 0.31	1.35 ± 0.55
ZDV	3	1.51 ± 0.04	0.999				
DLV	3	1.78 ± 0.27	0.966				
DDI	3	1.33 ± 0.26	0.948				
DLV + DDI (1:166.7)	3	2.00 ± 0.05	0.999	1.72 ± 0.71	1.31 ± 0.41	0.85 ± 28	0.64 ± 0.28
ZDV + DLV (1:2)	3	1.94 ± 0.15	0.992	1.13 ± 0.23	0.98 ± 0.17	0.75 ± 0.14	0.64 ± 0.17
ZDV + DDI (1:333)	3	1.83 ± 0.11	0.995	1.51 ± 0.43	1.20 ± 0.29	0.85 ± 21	0.67 ± 0.21
ZDV + DLV + DDI (1:2:333)	3	1.82 ± 0.25	0.974	0.75 ± 0.29	0.61 ± 0.20	0.47 ± 14	0.40 ± 0.14

[a] *m* is the slope ± S.E. and *r* is the correlation coefficient, as determined from the median effect plot.
[b] CI < 1, = 1 or > 1 indicates synergy, additivity and antagonism. CI values were determined for a mutually non-exclusive interaction. Confidence intervals were calculated with the Monte Carlo technique using $n = 500$, where *n* is the number of statistical computations.

100 nM, DLV = 200 nM, and DDI = 10 000 nM). This suggests that the enhanced effects are due to specific antiviral activities.

3.3. Effects of combination of DLV, DDI, and ZDV on co-culture infection in PBMC

In HIV-1 infected individuals, new cycles of infection are initiated in previously uninfected cells by the spread of circulating cell-free virus or from direct cell-to-cell transmission. To more closely mimic these aspect of infection in vivo, we evaluated the effect of drug treatment in assays in which uninfected PBMC were co-cultured with infected PBMC. We tested individual drugs at non-toxic doses of ZDV at 20 nM, DLV at 60 nM and DDI at 6000 nM, that produced about 50 to 70% inhibition on day 6 of culture. Single agents at these concentrations were partially inhibitory leading to complete viral recovery when drug treatments were terminated. The combined drugs were highly inhibitory showing only 0.2% of control viral replication on day 6 of drug treatment but viral replication returned by day 5 after drug removal (Fig. 3B). Monotherapy with ZDV at three-fold IC_{50} concentration was much less active, showing 85% inhibition on day 6 of infection and a complete recovery of viral replication 1 day after drug removal (Fig. 3B). In contrast, the three-drug combination group not only delayed viral recovery, but produced significantly greater ($P < 0.05$) cell survival than with any of the groups treated with a single agent (Fig. 3A). The mean cell viability, calculated as percent of the mock-infected cell control, was 84% for the combination treated group and 67% for the untreated virus alone group.

We next evaluated combinations of individual drugs at higher doses (DLV at 200 nM, DDI at 10 000 nM and ZDV at 100 nM). At these drug levels, individual drugs produced about 95% inhibition of viral-spread on day 5 of culture. Fig. 4B shows that three-drug combination of DLV, DDI, and ZDV, at IC_{95} concentrations of the individual

Table 3
Inhibition of HIV-1 p24 antigen production in acutely infected PBMC treated with DLV, ZDV, and DDI alone or in combination

ZDV (nM)	DLV (nM)	DDI (nM)	p24[a] (ng/ml)	% Inhibition[b]
100			0.3 ± 0.1	99.8
30			1.1 ± 0.3	99.1
10			6.4 ± 1.8	95.0
3			30.3 ± 8.3	76.5
1			86.2 ± 18.0	33.2
	200		0.3 ± 0.2	99.8
	60		9.8 ± 1.2	92.4
	20		56.8 ± 4.7	56.0
	6		103.0 ± 11.7	20.2
	2		115.0 ± 25.4	10.9
		10,000	0.9 ± 0.1	99.3
		3,000	15.8 ± 2.7	87.7
		1,000	66.2 ± 10.7	48.7
		300	84.6 ± 16.0	34.4
		100	103.0 ± 44.1	20.2
30	60		0.2 ± 0.0	99.8
10	20		0.9 ± 0.3	99.3
3	6		25.6 ± 5.7	80.2
1	2		63.2 ± 1.7	51.0
0.3	0.6		19.0 ± 35.0	7.8
	60	10,000	0.2 ± 0.0	99.8
	20	3,000	2.5 ± 0.3	98.0
	6	1,000	30.2 ± 10.6	76.6
	2	300	86.5 ± 32.0	33.0
	0.6	100	123.0 ± 1.5	4.7
30		10,000	0.2 ± 0.0	99.8
10		3,000	0.8 ± 0.1	99.4
3		1,000	10.3 ± 3.6	92.0
1		300	50.3 ± 1.9	61.0
0.3		100	113.0 ± 1.4	12.4
30	60	10,000	0.2 ± 0.0	99.9
10	20	3,000	0.2 ± 0.0	99.9
3	6	1,000	5.5 ± 2.6	95.8
1	2	300	18.6 ± 0.7	85.6
0.3	0.6	100	94.1 ± 5.6	27.0

[a] Data represents mean values ± S.E. of the means of 3 replicate culture wells. Each well was infected, treated, and assayed for p24 antigen separately. The mean HIV-1 p24 antigen production in untreated control cultures (6 replicates) was 129.0 ± 16.3 ng/ml of supernatant with coefficient of variation of 12.6%. The mean coefficient of variation for all treated wells (2 replicates) was 19.4%.
[b] Data represents percentage inhibited as compared with untreated control.

drugs, produced nearly complete (99.99%) inhibition of viral growth on day 5 of drug treatment. This further delayed viral recovery but viral growth resumed by 13 days after the removal of drugs. This combination of drugs did not affect the survival of cells. For all groups, the mean cell viability remained at about 90–100% of non-infected cell control throughout the duration of experiments (Fig. 4A). Thus, the more lasting antiviral effect was not due to any non-specific toxicity effect on cell proliferation.

We performed two additional experiments in which the treatment time was increased to 12 and 19 days while maintaining IC_{95} concentration of drugs. Increased treatment duration from 7 to 12 days further suppressed viral replication but viral replication still resumed after drug removal (data not shown). However, after 19 days of drug treat-

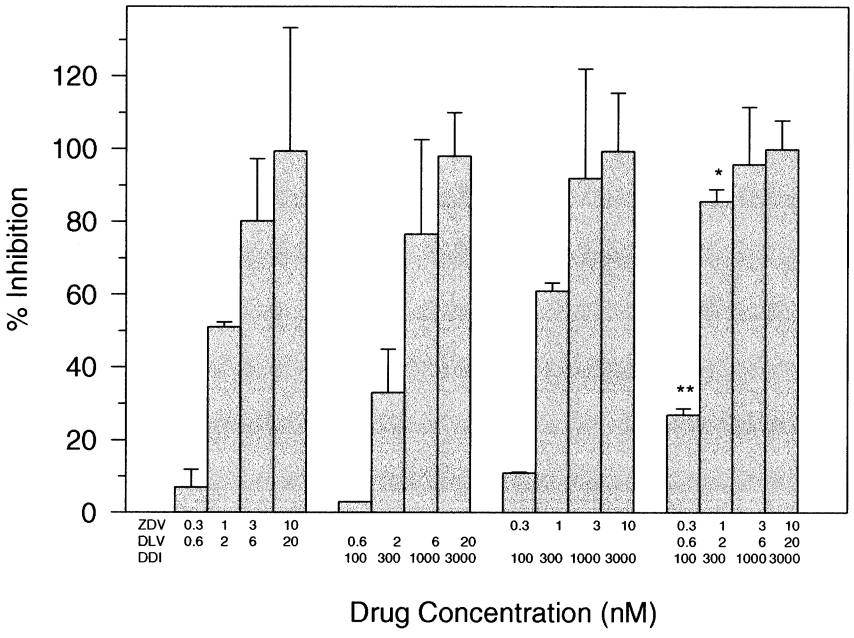

Fig. 2. Inhibition of HIV-1 p24 antigen production in acutely infected PBMC treated with DLV, ZDV, and DDI in two- or three-drug combinations. HIV-1 p24 antigen concentrations in culture supernatant were measured on day 5 post-infection from drug-treated and non-treated control wells. Data are mean percent inhibition ± S.E. of the mean of three replicate wells separately infected, treated and assayed for p24 antigen. Three-drug combination groups were significantly more active than the two-drug treatment groups (two samples t-test: $^{*}P < 0.01$ vs. DLV + DDI; $P < 0.05$ versus DLV + ZDV, and DDI + ZDV; $^{**}P < 0.001$ vs. DLV + DDI; $P < 0.01$ vs. DLV + ZDV, and DDI + ZDV).

ment, viral growth was completely inhibited with no detectable viral p24 antigen on day 18 of treatment and for another 64 days in drug-free media (Fig. 5). The replication of virus was also not detected by p24 measurement when undiluted culture supernatant samples harvested on or after day 21 (day 24, 28, 32, 35, 40, 43, 48, 63, 74 and 83) were used to infect fresh PBMC under drug-free condition. The absence of HIV-1 at these time points in the cultures was further confirmed using PCR assays that showed no proviral DNA in cellular DNA obtained from $1-2 \times 10^6$ cells (Fig. 5).

4. Discussion

We evaluated DLV in combination with DDI and ZDV against acute and co-culture infections of HIV-1_{JRCSF} in PBMC. Two and three-drug combinations of DLV, DDI and ZDV at the drug levels studied did not result in any toxicity to PBMC. Combinations of DLV and DDI showed statistically significant synergistic inhibition of viral replication in acutely infected cells at the lower drug ratios of 1:10 and 1:30 especially at $> 75\%$ inhibition levels. Three-drug combinations of DLV, DDI and ZDV showed significant synergy at 50–99% inhibition levels. However their respective two-drug combinations showed synergy only at $\geqslant 95\%$ inhibition levels. Since current therapeutic approach favors more complete suppression of viral load, we believe that CI values at 95 and 99% inhibitory levels are more relevant to clinical situations. The three-drug combination was significantly more suppressive of viral replication than the corresponding two-drug combinations. Other investigators have also reported that the addition of a NNRTI to two nucleoside RTIs results in improved antiviral effect in vitro (Chow et al., 1993; Mazzulli et al., 1994; Rusconi and Merrill, 1994; St. Clair et al., 1995). However, in

these studies the combined effects of three drugs were not analyzed by mathematical methods.

The co-culture assays represented higher viral load than the acutely infected cultures as evidenced by a two to three-fold increase in peak viral titer. Moreover, the co-culture assay required a five-fold higher drug dose to achieve the same level of viral inhibition as seen in acute infection assays. Combinations of DLV, DDI and ZDV at various drug levels were significantly more effective than any one drug in reducing viral spread in co-culture assays. The extent of viral inhibition was dependent on drug dose and the

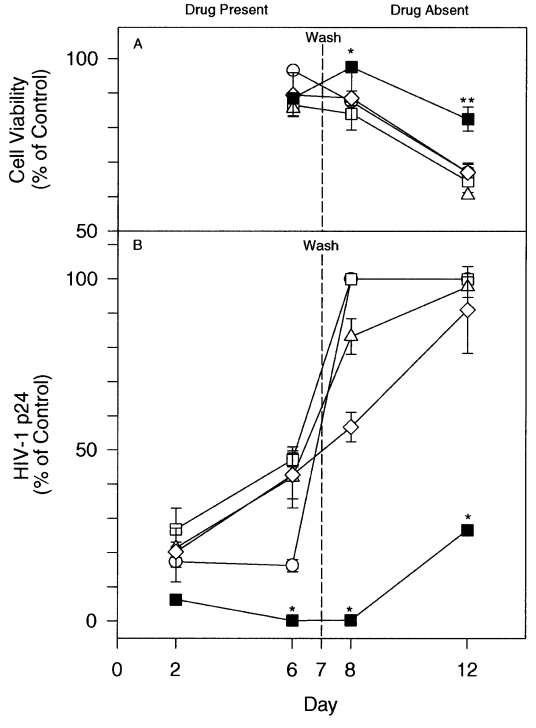

Fig. 3. Effects of IC_{50} concentrations of ZDV (□), DDI (◇), and DLV (△) alone and in three-drug combination (■) on co-culture infection in PBMC. ZDV was also tested at $3\times$ IC_{50} concentration (O). Cell viability (A) and HIV-1 p24 antigen levels in culture supernatant (B) were measured on various days post-infection. The vertical broken line shows day 7 post-treatment when drugs were removed from cultures by washing twice with drug-free medium. Data are mean ± S.E. of two separate experiments; symbols with no error bars indicate small standard error. The three-drug group showed significantly greater antiviral activity (two samples t-test: $^*P <$ 0.01) and cell survival ($^*P < 0.01$; $^{**}P < 0.05$) than any single-drug groups.

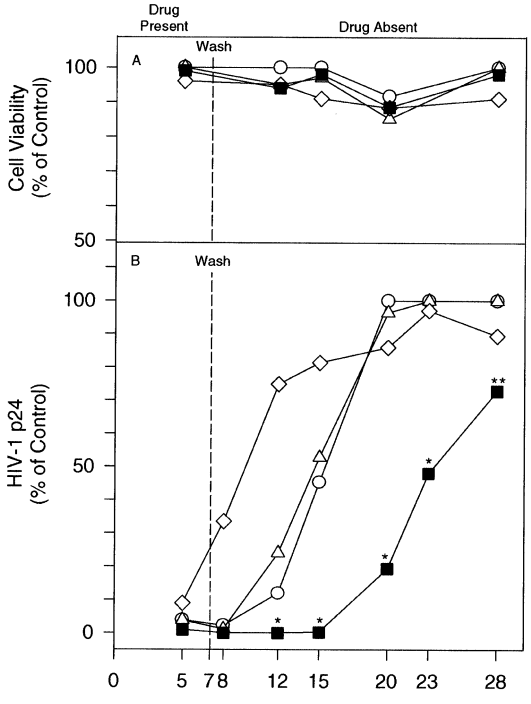

Fig. 4. Effects of higher concentrations of drugs on HIV-1 growth in co-cultured PBMC. Drug were used at IC_{95} concentrations: ZDV (○), DDI (◇), and DLV (△) alone and in three-drug combination (■). Cell viability (A) and HIV-1 p24 antigen levels in culture supernatant (B) were measured on various days post-infection. Drugs were removed on day 7 as described for Fig. 3. Data are mean of two replicate wells, treated and assayed for p24 antigen separately. The three-drug group was significantly more inhibitory than any single-drug groups (two samples t-test: $^*P < 0.01$; $^{**}P > 0.05$). Differences in cell viability (A) were not significant.

duration of treatment. Greater inhibitions were observed when individual drugs were used at IC_{95} than at $IC_{50\text{-}70}$ concentrations. After 19 days of triple drug treatment at IC_{95} concentrations, the culture appeared to be 'cured' as evidenced by a lack of detectable viral p24 antigen in co-culture maintained for 64 days in drug-free media. It seemed that viral infection was completely eliminated from cultures since we were unable to detect cellular proviral DNA by PCR. Moreover, we were not able to culture secondary virus from these drug-free cultures after the termination of drug treatment.

Recently, various investigators have reported that non-nucleoside RTIs can completely suppress

viral replication in cultures. Specifically, a BHAP derivative U-88204 has been shown to completely inhibit viral spread in HIV-1_{LAV}-infected MT-2 cells (Vasudevachari et al., 1992). In addition, Balzarini et al. (1993b) have also shown that high concentrations of various NNRTIs (DLV, nevirapine, HEPT, TIBO, and TSAO derivatives) can completely suppress HIV-1_{IIIB} growth and spread in CEM cell cultures. Other studies have shown that combined regimens of nucleoside and non-nucleoside RTIs can completely inhibit HIV-1 replication in cultures (Dueweke et al., 1993; Chow et al., 1993; Mazzulli et al., 1994; St. Clair et al., 1995). These investigators appeared to have successfully eliminated virus from infected cultures. However, it is not clear whether this effect is due to drug treatment alone or due to the combined effects of drug treatment and the dilution of cultures. In these studies, drug treatment was accompanied by a large dilution of culture due to the frequent sub-culture that maintain cells in logarithmic growth (1:3–1:6 split, two or three times a week). Therefore, it is presumed that drug treatments effectively suppressed the infection of new cells while the cumulative effect of dilution removed the input virus from dead infected cells in cultures. To reduce the role of culture dilution, we modified the experimental design so that cultures were not maintained in logarithmic growth. This procedure involved the replacement of half the media with no removal of cells from cultures. We showed that the complete elimination of virus from cultures was dependent on the concentration of drug and the duration of treatment. Our assay procedure still cannot rule out the effect of culture dilution on viral elimination, but it represents an initial attempt to show viral elimination without frequent sub-culturing of cells.

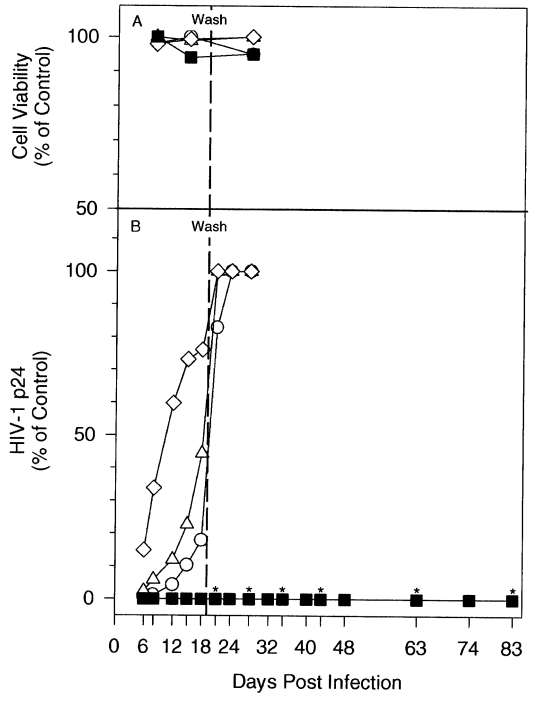

Fig. 5. Effects of extending treatment duration on HIV-1 growth in co-cultured PBMC. Cell viability (A) and HIV-1 p24 antigen levels in culture supernatant (B) were measured on various days post-infection. Drugs were used at IC_{95} concentrations: ZDV (○), DDI (◇), and DLV (△) alone and in three-drug combination (■). The vertical broken line shows day 19 post-treatment when drugs were removed from cultures by washing twice with drug-free medium. Data are mean of two replicate wells treated and assayed for p24 antigen separately. Culture supernatants that showed undetectable p24 antigen (< 0.01 ng/ml) were added to fresh PBMC cultures; supernatant p24 antigen and cellular proviral DNA were not detected in inoculated cultures (denoted by *). Differences in cell viability (A) were not significant.

The combination of three-drugs at IC_{50} drug levels, not only improved antiviral effect, but also significantly increased cell survival compared to the single agent treated group. The higher dose combination groups further suppressed viral growth but showed little or no improvement in cell survival compared to the individual agents treated groups. At higher drug levels (i.e., IC_{95}) individual agents are more protective of cells as shown by 85–95% cell survival for the entire 28-day duration of experiments. In our assays, cell counts varied by 10–12% in both control and treated cultures due to assay errors and the normal fluctuation in cell density under culture conditions. Therefore, our assays are probably not sensitive enough to detect any differences in cell survival under such culture conditions.

Our results provide an in vitro basis for the clinical trial of DLV in combination with ZDV and DDI. Recent results from various on-going clinical trials also support the use of treatment regimens involving one non-nucleoside and two

nucleoside RTIs. Specifically, combination of ZDV, DDI with DLV; ZDV, DDI with nevirapine or ZDV, 3TC and loviride were shown to result in a significant decline in HIV-1 markers for a period of six to twelve months (Staszewski et al., 1994; Wei et al., 1994; Davey et al., 1995).

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