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RAPID COMMUNICATION

Favorable Interaction of β-L(-) Nucleoside Analogues with Clinically Approved Anti-HIV Nucleoside Analogues for the Treatment of Human Immunodeficiency Virus

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ABSTRACT. The combination of L(-)-2',3'-dideoxy-3'-thiacytidine (L(-)SddC, 3TC), L(-)-2',3'-dideoxy-5-fluorocytidine (L(-)FddC), or L(-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (L(-)FTC) with 3'-azido-3'-deoxythymidine (AZT) synergistically inhibited replication of human immunodeficiency virus (HIV) *in vitro*. Similar synergistic activity was also obtained when these compounds were used in combination with 2',3'-didehydro-2',3'-dideoxythymidine (D4T). In terms of 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC), only additive anti-HIV activity was observed. None of the β-L(-) nucleoside analogues had additive toxicity in cell culture, and they could protect against the delayed mitochondrial toxicity associated with AZT, D4T, ddC, and ddI in drug-treated cells. Thus, combinations of β-L(-) nucleoside analogues with any of the approved anti-HIV drugs could have a potentially beneficial outcome.
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KEY WORDS. anti-HIV nucleoside; synergy; mitochondrial DNA

Nucleoside analogues represent the major chemotherapeutic approach for the treatment of AIDS[†]. Unfortunately, clinical isolates of HIV-1 with reduced drug susceptibility can be recovered from patients after single-agent antiretroviral therapy with AZT, ddC, or ddI [1, 2]. Although the relationship of drug-resistant HIV-1 clinical isolates to failure of therapy is unclear, it is likely responsible for disease progression in AIDS patients treated with anti-HIV nucleoside analogues. Much research has been directed toward combinations of various anti-HIV agents to achieve complete virus suppression and limit, or at least delay, the emergence of drug resistance [3-6]. Several clinical trials are in progress to evaluate various drug combinations in HIV-infected patients.

In the search for agents with improved therapeutic indices and pharmacological profiles, β-L(-) nucleoside analogues have emerged as an exciting new class of compounds with potent anti-HIV activity and low toxicity. The first such compound, L(-)SddC or 3TC [7], is currently undergoing clinical trials against HIV-1 infection in combination with AZT. L(-)FTC and L(-)FddC are additional β-L(-) nucleoside analogues with potent and selective activity against HIV-1 [8, 9]. We report here the biological antiviral activity of these β-L(-) enantiomers against HIV-1 when used in combination with either AZT, D4T, ddC, or ddI. The effects of these drug combinations on cell growth including mitochondrial toxicity are also discussed.

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[†]Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus type 1; L(-)SddC, L(-)-2',3'-dideoxy-3'-thiacytidine; AZT, 3'-azido-3'-deoxythymidine; ddC, 2',3'-dideoxycytidine; ddI, 2',3'-dideoxyinosine; D4T, 2',3'-didehydro-2',3'-dideoxythymidine; L(-)FTC, L(-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine; L(-)FddC, L(-)-2',3'-dideoxy-5-fluorocytidine; MOI, multiplicity of infection; TCID, tissue culture infective dose; IC_{50}, 50% inhibitory concentration; FBS, fetal bovine serum; SSC, saline sodium citrate; and HIV-RT, HIV reverse transcriptase.

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MATERIALS AND METHODS

Drugs were tested using MT-2 cells infected with HIV-1 strain IIIB as described [10] with some modifications. Briefly, triplicate wells of 96-well plates containing 1 x 10^4 MT-2 cells were infected with HIV-1 strain IIIB at an MOI of 0.1 TCID/cell. Serial dilutions of drug were added immediately after infection. Cell viability was quantitated by the tetrazolium-dye reduction model [11] 5 days after infection. The percentage of protection was calculated with the formula [(*a*-*b*/*c*-*b*) x 100], where *a* = the A_{595} of drug-treated, virus-infected wells, *b* = A_{595} of no-drug, infected wells, and *c* = the A_{595} of no-drug, uninfected wells. The IC_{50} for each drug was calculated from linear-log_{10} plots of the percentage of protection versus drug concentration. Isobolograms are defined as the percent change in the IC_{50} of the first agent alone (e.g. D4T) when in combination with the second agent (e.g. L(-)FddC) plotted against the percent change in the IC_{50} of the second agent alone (e.g. L(-)FddC) when in combination with the first agent (e.g. D4T).

To determine the effect of nucleoside analogues on cell growth and mtDNA content, CEM cells were seeded at 2 x 10^5 cells/mL in RPMI 1640 medium supplemented with 10% dialyzed FBS. CEM cells were treated with various concentrations of drugs as single-agents or in combination for 8 days with medium changes on day 4 and day 6. On day 4 cell growth was assessed using a Coulter counter. Cells (1 x 10^5) were harvested on day 8, washed once with PBS, and resuspended in 25 mM Tris-HCl, 1 mM EDTA, pH 8.0. The cells were lysed by 5 cycles of freeze/thaw. DNase-free RNase A was added to the cell lysate at a final concentration of 0.1 mg/mL and incubated for 1 hr at 37°, followed by the addition of proteinase K at 0.1 mg/mL for 3 hr at 50°. The lysate was brought to a final concentration of 10x SSC, boiled for 10 min, and applied to a nylon membrane presoaked in 2x SSC using a slot blot apparatus. To detect mtDNA, the blots were hybridized with an 880 base mtDNA fragment spanning nucleotide positions 13,370 to 14,258 radiolabeled with a Stratagene random primer kit using [^{32}P]-dCTP. All blots were normalized for loading using an *Alu* probe (American Type Culture Collection, Rockville, MD). Blots were exposed to X-ray film and quantitated using a Molecular Dynamics Personal Densitometer SI.

RESULTS AND DISCUSSION

Antiviral assays were performed in cell culture with L(-)SddC, L(-)FddC, and L(-)FTC in combination with either D4T, AZT, ddI, or ddC. Effects of the drug combinations were evaluated on the basis of the anti-HIV activity of the compounds when tested alone. In each experiment, the data for a single agent and various drug combinations were used to plot an isobologram. Additive antiviral protection is represented graphically in Fig. 1 by linearity between the IC_{50} value of each compound as a single agent. When combinations of the β-L(-) deoxycytidine analogues with ddC or ddI were examined, we observed no synergistic anti-HIV activity (Fig. 1).

Experimentally determined antiviral protection for a drug combination resulting in a curve below the line between the IC_{50} value of each compound as a single agent in the isobologram plots indicates antiviral synergy or inhibition greater than the additive effect of each single drug. In all cases, combinations of D4T plus either L(-)SddC, L(-)FddC or L(-)FTC demonstrated synergistic activity at blocking HIV-1 induced cell killing (Fig. 2). Similarly, synergistic antiviral activity was observed with AZT in combination with either L(-)SddC, L(-)FddC, or L(-)FTC. Inhibition of cell proliferation or viability was not apparent with any of the above combinations. These results indicate that combinations of these β-L(-) deoxycytidine analogues with the anti-HIV thymidine analogues produce synergistic anti-HIV interactions *in vitro*.

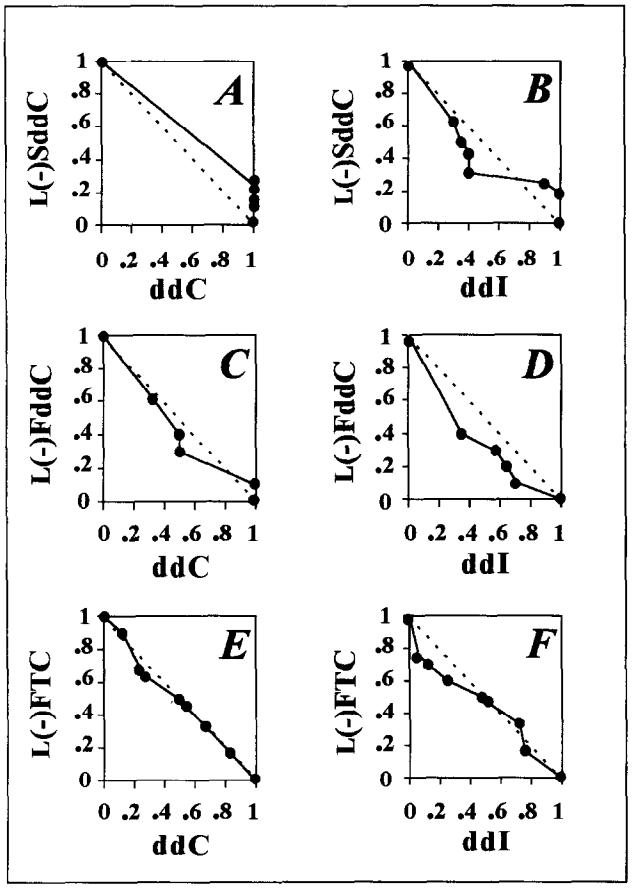

FIG. 1. Antiviral isobolograms of drug combination data obtained in MT-2 cells with L(-)SddC + ddC (*A*), L(-)SddC + ddI (*B*), L(-)FddC + ddC (*C*), L(-)FddC + ddI (*D*), L(-)FTC + ddC (*E*), and L(-)FTC + ddI (*F*). Symbols on the axis represent the results when the agents are used alone at an IC_{50} of 20 μM ddI, 0.6 μM ddC, 3 μM L(-)SddC, 0.45 μM L(-)FddC, or 0.6 μM L(-)FTC; symbols not on the axes represent the results when the agents were used in combination at a percentage of their antiviral IC_{50}.

To examine the biochemical mechanism(s) underlying this antiviral synergy, we measured the *in vitro* metabolic profile of L(-)FddC + D4T and L(-)FddC + AZT in cell culture. The combination of an equimolar concentration of L(-)FddC with either radiolabeled D4T or AZT did not appear to alter intracellular levels of the D4T or AZT phosphorylated metabolites in uninfected MT-2 cells (data not shown). Similarly, the metabolism of radiolabeled L(-)FddC was not affected by combination with an equimolar concentration of D4T or AZT. These results suggest that increases in intracellular 5'-triphosphate levels do not play a role in the synergistic activity seen against HIV in cell culture by combinations of L(-)FddC and the anti-HIV thymidine analogues. Several mechanisms could account for the synergy observed with these drug combinations. Recent studies show that the synergistic interaction of AZT + ddC against HIV in cell culture is not related to synergistic inhibition of HIV-RT, alteration of natural dNTP pool sizes, or altered metabolism of either nucleoside analogue [12,13]. The role of these factors in the synergistic antiviral interaction of the β-L(-) deoxycytidine analogues and anti-HIV thymidine analogues is under investigation.

To study potential adverse effects of these drug combinations, acute cytotoxicity was assessed by cell growth assays using CEM cells after 4 days of drug exposure (Table 1). We observed that L(-)FddC in combination with any of the anti-HIV nucleoside analogues did not demonstrate acute cytotoxicity. Similarly, combinations of L(-)SddC + D4T and L(-)SddC + ddC were not cytotoxic. Table 1 represents a typical experiment demonstrating the well known mitochondrial toxicity of the anti-HIV drugs, whereas the β-L(-) deoxycytidine analogues had a minimal effect on mtDNA content. More important, we observed no additive toxicity towards mtDNA content with these drug combinations. Indeed, these results suggest that at least a partial reversal of drug-induced decreases in mtDNA content may occur. Further studies will be required to establish if these β-L(-) deoxycytidine analogues may play a role in protecting against nucleoside analogue induced mitochondrial toxicity.

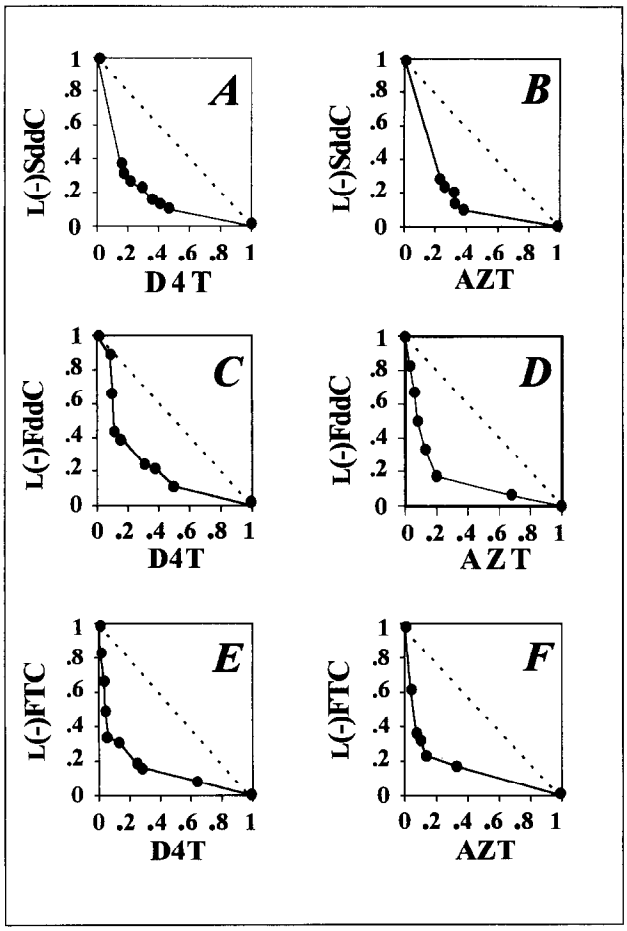

FIG. 2. Antiviral isobolograms of drug combination data obtained in MT-2 cells with L(-)SddC + D4T (*A*), L(-)SddC + AZT (*B*), L(-)FddC + D4T (*C*), L(-)FddC + AZT (*D*), L(-)FTC + D4T (*E*), and L(-)FTC + AZT (*F*). Symbols on the axis represent the results when the agents were used alone at an IC_{50} of 6 μM D4T, 0.09 μM AZT, 3 μM L(-)SddC, 0.45 μM L(-)FddC, or 0.6 μM L(-)FTC; symbols not on the axes represent the results when the agents were used in combination at a percentage of their antiviral IC_{50}.

The ideal agents for use in combination regimens should at least be synergistic *in vitro*, have nonoverlapping toxicity profiles, and lack viral cross-resistance. The combination of L(-)SddC + AZT meets these criteria and is the most recent exciting drug combination in clinical trials to date. The study by Larder *et al.* suggests that the sustained *in vivo* anti-HIV activity of AZT + L(-)SddC combination therapy may result from not only a lack of cross-resistance but also from the presence of an L(-)SddC associated mutation of codon 184 to valine in HIV-RT that confers increased sensitivity to AZT in HIV containing AZT resistance mutations [14]. The studies reported herein suggest that the anti-HIV synergistic activity of L(-)SddC + AZT may also contribute to the antiviral activity of this combination protocol. Furthermore, L(-)SddC not only has no significant additive toxicity with AZT, but also, at least in part, reverses or protects cells from AZT-induced mitochondrial toxicity, which may play an important role in therapy [15]. The other anti-HIV β-L(-) deoxycytidine analogs, L(-)FddC and L(-)FTC, behaved like L(-)SddC with respect to their interaction with AZT. Thus, the combination of L(-)FddC or L(-)FTC with AZT could also be entertained for therapy. In addition, development of HIV resistance to L(-)FddC and L(-)FTC has been observed *in vitro* [16, 17]. Reverse transcriptases from these resistant viruses were consistent with a change at codon 184 to isoleucine with L(-)FddC and a change to valine with L(-)FTC. Thus, a phenotypic suppressive effect on AZT resistance may also be observed with AZT in combination therapy with L(-)FddC or L(-)FTC similar to that reported for AZT + L(-)SddC. With regard to the effects of these β-L(-) deoxycytidine analogs with D4T, they are similar to the effects found when these same analogues are combined with AZT in cell culture. Thus, the combination of β-L(-) deoxycytidine analogs with D4T should also be considered. However, it should be noted that a 184 mutation associated with resistance to L(-)SddC, L(-)FddC, and L(-)FTC has not been reported to suppress a

TABLE 1. Effects of β-L(-) deoxycytidine analogues and anti-HIV nucleoside analogue combinations on cell growth and mtDNA content

	Cell growth (% of control)			mt DNA content (% of control)		
	Alone	Plus 1 μM L(-)FddC	Plus L(-)SddC	Alone	Plus 1 μM L(-)FddC	Plus L(-)SddC
0.01 μM ddC	97	101	100 (0.2 μM)*	52	81	70 (0.2 μM)
50 μM ddI	97	105	ND†	44	93	ND
3 μM D4T	93	94	93 (3 μM)	55	74	87 (3 μM)
5 μM AZT	95	91	ND	66	97	ND
1 μM L(-)FddC	94			101		
3 μM L(-)SddC	96			97		

Treatment of CEM cells with drugs was performed as described in Materials and Methods. Cell growth was determined after 4 days of drug exposure. The CEM cells were harvested on day 8 to assess mtDNA content.

*Concentration of L(-)SddC in combination.

†Not determined.

D4T-resistant phenotype as it does an AZT-resistant phenotype. In the case of ddI or ddC, there was only additive and no synergistic antiviral activity with the β-L(-) deoxycytidine analogs. However, given the protective activity of β-L(-) deoxycytidine analogs against ddI and ddC action on mtDNA, it could be conceivable that the combination will decrease the delayed toxicity of ddI or ddC. Thus, clinical studies could also be considered for combinations of β-L(-) deoxycytidine analogs and ddI or ddC. In summary, the favorable interactions of β-L(-) deoxycytidine analogs in combination with clinically approved anti-HIV drugs should be actively pursued for the treatment of HIV infection.

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