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# In Vitro Inhibition of the Infectivity and Replication of Human Immunodeficiency Virus Type 1 by Combination of Antiretroviral 2′,3′-Dideoxynucleosides and Virus-Binding Inhibitors

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**We tested the in vitro inhibitory activities of three 2′,3′-dideoxynucleosides and two inhibitors of viral binding in combinations against the infectivity and cytopathic effect of human immunodeficiency virus type 1. 3′-Azido-2′,3′-dideoxythymidine, 2′,3′-dideoxyinosine, or 2′,3′-dideoxycytidine, combined with recombinant soluble CD4 (sCD4), brought about synergistic antiretroviral activity without toxicity at clinically achievable concentrations. Combinations of 2′,3′-dideoxynucleosides plus dextran sulfate exerted similar synergistic antiviral effects without concomitant increases in toxicities. When sCD4 and dextran sulfate were combined, apparent antagonism was observed. We confirmed that no combination of sCD4 plus 3′-azido-2′,3′-dideoxythymidine, 2′,3′-dideoxyinosine, or 2′,3′-dideoxycytidine significantly increased the inhibitory effect on colony formation of human myeloid-monocytic bone marrow cells in vitro at the concentrations used in this study. These data might have clinical relevance for the treatment of patients infected with human immunodeficiency virus.**

In the past few years, efforts aimed at prevention and control of replication of human immunodeficiency virus (HIV) have gained increasing importance, and potentially useful strategies for therapy against acquired immunodeficiency syndrome (AIDS) have arisen from the accumulated knowledge of the life cycle of HIV (5, 12, 23). Notably, there have been certain successes at a clinical level in using reverse transcriptase inhibitors. For example, 3′-azido-2′,3′-dideoxythymidine (also termed 3′-azido-3′-deoxythymidine or AZT), one member of a broad family of 2′,3′-dideoxynucleosides (ddN) (22, 27), can improve the clinical course and prolong the survival of some patients with advanced AIDS and its related disorders (8, 35, 36). However, one of the major toxicities of AZT is significant bone marrow suppression, which has been a dose-limiting toxicity for many patients (30, 36). A closely related drug, 2′,3′-dideoxycytidine (ddC), which has been shown to exert a potent effect against HIV type 1 (HIV-1) and HIV-2 in cultured T cells (22, 24) and an in vivo virustatic effect in patients with AIDS or the AIDS-related complex (21, 38), has a different dose-dependent toxicity in the form of peripheral neuropathy, which limits its use for some patients (21, 38). Finally, in the treatment of HIV infection, the emergence of drug-resistant virus variants should always be considered possible, and indeed, Larder and co-workers have recently reported that AZT-insensitive HIV-1 variants were isolated from patients who received AZT therapy (18).

There are now several agents with potential usefulness in the treatment of HIV-1 infection (5, 6, 9, 11–14, 23, 31, 33). Logical extension of current therapeutic approaches would be the use of combinations of drugs which have different antiretroviral mechanisms (10, 16). Combination therapy might enhance the efficacy and minimize the toxicity of each drug and also could minimize or retard the emergence of drug-resistant variants. It is also worth noting that judicious application of combination chemotherapies made it possible to successfully treat a variety of microbial and neoplastic diseases which were not treatable with single agents. In the current work, we attempted to evaluate the antiretroviral effects of combinations of three ddNs and two inhibitors of virus binding, all of which have been given to patients infected with HIV.

## MATERIALS AND METHODS

**Viruses and cells.** HIV-1 was pelleted by ultracentrifugation from the culture supernatant of HIV-1/$III_B$-producing H9 cells (29) and prepared to contain $1.2 \times 10^{11}$ virus particles per ml. The 50% tissue culture infective dose per milliliter of the stock cell-free virus preparation was determined by an endpoint titration method using ATH8 cells (see below). The titration was performed in 10 replicate cultures, and 50% tissue culture infective doses were calculated by the method previously described (19). A human T-cell lymphotropic virus type 1-transformed $CD4^+$ T-cell clone (ATH8) and a normal tetanus toxoid-specific helper-inducer T-cell clone (TM11) were used as target cells for infection by HIV-1. The characteristics of clones ATH8 and TM11 have been described elsewhere (24, 27). Cell cultures were not synchronized as to cell cycle.

**Reagents.** 2′,3′-Dideoxyinosine (ddI) and ddC were provided by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. AZT was kindly provided by Wellcome Research Laboratories (Research Triangle Park, N.C.). Recombinant soluble CD4 (sCD4) and dextran sulfate (molecular weight, approximately 8,000) were kindly provided by Genentech Inc. (South San Francisco, Calif.) and Ueno Fine Chemical Co. (Itami, Japan), respectively.

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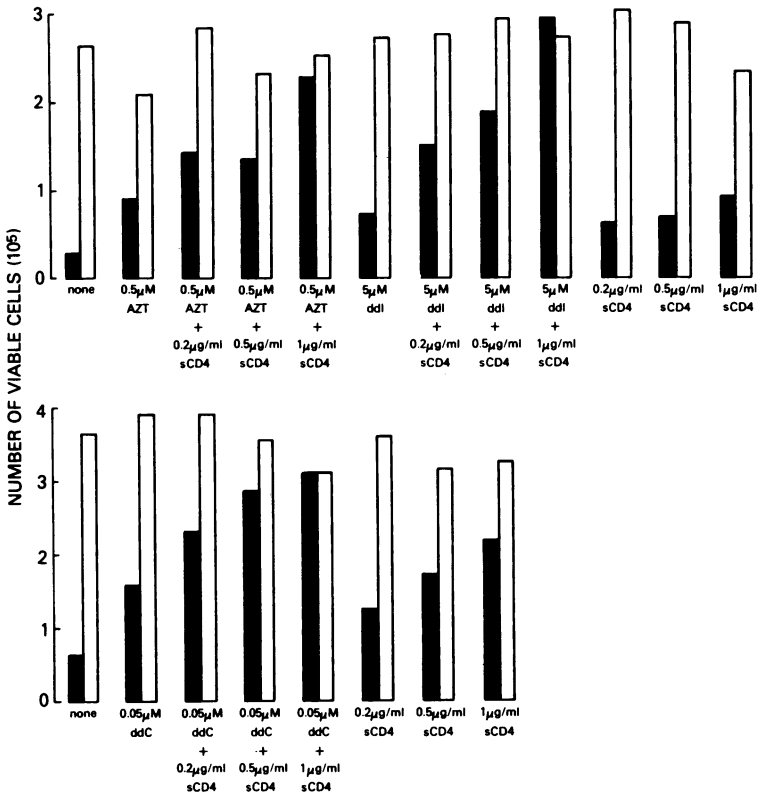


FIG. 1. Inhibition of the infectivity and cytopathic effect of HIV-1 in ATH8 cells by combinations of ddNs plus sCD4. Target ATH8 cells ($2 \times 10^5$) were exposed to HIV-1 (1,000 viral particles per cell) and cultured with or without drugs (solid bars). Control cells were similarly treated but not exposed to the virus (open bars). Total viable cells were counted on day 7. Nine combinations of AZT and sCD4 (AZT at 0.1, 0.5, 2, and 5 μM; sCD4 at 0.2, 0.5, 1, and 5 μg/ml), nine combinations of ddI and sCD4 (ddI at 0.5, 1, 5, and 20 μM; sCD4 at 0.2, 0.5, 1, and 5 μg/ml), and eight combinations of ddC and sCD4 (ddC at 0.01, 0.05, 0.1, and 5 μM; sCD4 at 0.2, 0.5, 1, and 5 μg/ml) were tested. Quantitative analysis of CI revealed that most of the combined drugs showed synergism. Representative data for each combination are shown.

**Assay for inhibition of HIV-1 cytopathic effect.** Inhibition of the HIV-1 cytopathic effect was assessed as previously described (27). Clone TM11 cells were stimulated by 2 limiting flocculation units of tetanus toxoid (Commonwealth of Massachusetts Department of Public Health, Jamaica Plain, Mass.) per ml plus irradiated autologous peripheral blood mononuclear cells 5 days before the assay. The stimulated TM11 cells were cultured in complete medium (RPMI 1640 supplemented with 4 mM L-glutamine, 15% [vol/vol] undialyzed and heat-inactivated fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml) containing 15% (vol/vol) interleukin 2 (lectin depleted; Advanced Biotechnologies, Silver Spring, Md.) and 25 U of recombinant interleukin-2 (Amgen Biological, Thousand Oaks, Calif.) per ml until the HIV cytopathic effect inhibition assay. ATH8 cells were used without antigen stimulation. In the assay, the target ATH8 cells and TM11 cells ($2 \times 10^5$) were exposed to 14,300 (1,000 viral particles per cell) and 28,600 (2,000 viral particles per cell) 50% tissue culture infective doses of HIV-1 per cell, respectively, for 1 h, suspended in 2 ml of fresh complete medium with interleukin-2, and incubated at 37°C in 5% $CO_2$-containing humidified air. Drugs were added to the culture after suspension of target cells following exposure to the virus. Control cells were similarly treated but not exposed to the virus. At various times, viable cells were counted in a hemacytometer

TABLE 1. Percent inhibition of HIV-induced cytotoxicity by combinations of sCD4 plus ddI in TM11 cells[a]

| ddI concn (μM) | % Inhibition (% cytotoxicity)[b] [CIs][c] at an sCD4 concn (μg/ml) of: | | | |
|---|---|---|---|---|
| | 0 | 0.2 | 0.5 | 1 |
| 0 | 0.0 (0.0) | 31.9 (1.7) | 45.0 (0.4) | 56.0 (4.6) |
| 1 | 10.8 (2.8) | 71.1 (0.0) [0.15; 0.16] | 70.1 (3.4) [0.03; 0.03] | 94.3 (0.0) [0.33; 0.35] |
| 5 | 48.0 (0.0) | 97.3 (0.0) [0.04; 0.04] | 80.2 (8.2) [0.28; 0.30] | 94.3 (4.6) [0.09; 0.09] |
| 10 | 61.1 (0.0) | 100.0 (0.0) [0.03; 0.03] | 88.2 (0.0) [0.31; 0.32] | 72.1 (2.2) [1.06; 1.31] |

[a] TM11 cells were exposed to HIV-1 and cultured as described in Materials and Methods. Percent protective effect and percent cytotoxicity were determined by the formulas described in Materials and Methods.
[b] Percents cytotoxicity of less than 10% are not biologically significant in this assay system (22, 25).
[c] CIs were calculated with the classical isobologram equation (entry 1 in brackets) and the conservative isobologram equation (entry 2 in brackets) (2, 3). CI < 1, CI = 1, and CI > 1 indicate synergism, summation (additivism), and antagonism, respectively.

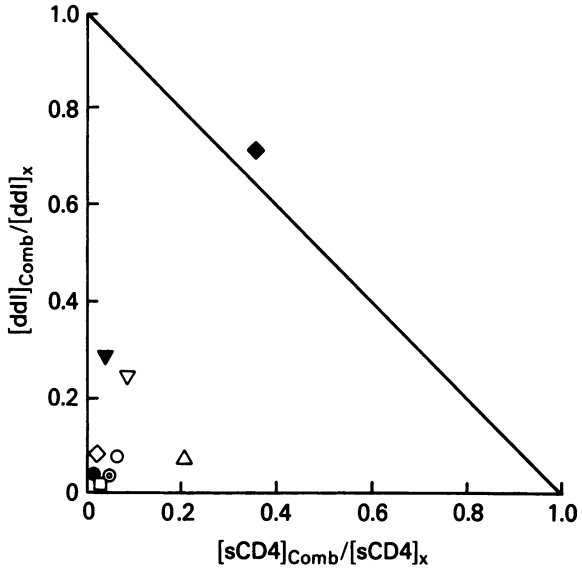


FIG. 2. Isobologram representing synergism by combinations of ddI plus sCD4 in antiviral activity against the infectivity and cytopathic effect of HIV-1 in vitro. A classical isobologram was drawn on the basis of percent antiviral effects of drugs on the survival of ATH8 cells exposed to HIV-1 as described in Table 1, footnote *a*. Nine combinations of both drugs at different concentrations were tested (1 μM ddI plus sCD4 at 0.2 [○], 0.5 [△], or 1 [□] μg/ml; 5 μM ddI plus sCD4 at 0.2 [⊙], 0.5 [▽], or 1 [◇] μg/ml; 10 μM ddI plus sCD4 at 0.2 [●], 0.5 [▼], or 1 [◆] μg/ml). On the *x* and *y* axes, respectively, the concentrations of sCD4 and ddI in combination (Table 1) that gave *x*% inhibition were normalized (divided) by the concentrations of the corresponding drug $[D]_x$ which alone also gave *x*% inhibition. $[D]_x$ can be calculated by the median-effect equation: $[D]_x = D_m[f_x/(1 - f_x)]^{1/m}$, where $f_x$ is percent inhibition/100 and *m* and $D_m$ are the slope and the antilog of the *x* intercept of the median-effect plot (2, 3). In the isobologram, if the point is located below the diagonal straight line (which represents additivism), synergism is indicated, and if the point is located above the line, antagonism is indicated.

by the trypan blue exclusion method. Variability in cell number determinations was ±10% of the values shown (22, 25).

**Calculation of antiretroviral effect and cytotoxicity.** Percentages of antiretroviral effects of drugs on the survival and growth of target T cells exposed to the virus were determined by the following formula: 100 × [(number of viable cells exposed to HIV-1 and cultured with the compound − number of viable cells exposed to HIV-1 and cultured without the compound)/(number of viable cells cultured alone − number of viable cells exposed to HIV-1 and cultured without the compound)]. By this formula, when the number of viable cells exposed to the virus and the compound is equal to or greater than the number of virus-unexposed cells cultured alone, 100% is given. Calculated percentages of ≤0 are expressed as 0%. In quantitative analysis of combination effects, 100 and 0% inhibitions were assumed to be 99.0 and 0.01% inhibitions to facilitate the calculation. Percent cytotoxicity of drugs for growth of ATH8 cells was determined by the following formula: 100 × [1 − (number of total viable cells cultured with the compound/number of total viable cells cultured alone)]. Calculated percentages of ≤0 are expressed as 0%.

**Synergy calculation.** The effects of combined drugs were determined by the method of Chou and Talalay (2, 3). Synergism or antagonism of combined effects was quantitatively represented by the combination index (CI), where CI < 1, CI = 1, and CI > 1 indicate synergism, summation (additivism), and antagonism, respectively. This method, based on the median-effect principle of the mass-action law, consists of the classical and conservative isobologram equations (2, 3). It takes into account not only the potency but also the shapes (*m* values) of the dose-effect curves. Computer software (J. Chou and T.-C. Chou, *Dose-Effect Analysis with Microcomputers* [5.25-in. disk and manual for IBM-PC and Apple II], Elsevier-Biosoft, Cambridge, United Kingdom, 1987) was used for automated analysis and graphics. Isobolograms representing the equieffective graph of the combination of two drugs at their various concentrations were drawn on the basis of the percent antiretroviral effects obtained and the calculation described above.

**Assay for bone marrow toxicity of drugs.** Bone marrow cells obtained from normal volunteers were washed and diluted with sterile phosphate-buffered saline at 4°C, and the mononuclear cell population was further separated by Ficoll-Hypaque gradient centrifugation as previously described (7). Briefly, the cells were suspended in McCoy 5A medium (M.A. Bioproducts, Walkersville, Md.) supplemented with 20% fetal bovine serum, 2 mM glutamine, and 15% (vol/vol) granulocyte-macrophage colony-stimulating factor-containing conditioned medium, derived from an HIV-negative leukemic cell culture (P38 cell line, kindly provided by Z. Salahuddin). The cells were plated by the soft-agar method (28) at final concentrations of 0.3% agar, 20% fetal bovine serum, 15% colony-stimulating factor-containing conditioned medium with $2 \times 10^5$ mononuclear cells per well in a total volume of 1 ml in triplicate with or without drugs that did not exceed 50 μl per well. The agar was allowed to gel for 15 min at 30°C, and the suspension was incubated for 10 to 12 days at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

TABLE 2. Percent inhibition of HIV-induced cytotoxicity by combinations of ddI plus dextran sulfate in ATH8 cells[a]

| ddI concn (μM) | % Inhibition (% cytotoxicity)[b] [CIs][c] at a dextran sulfate concn (μM) of: | | | | |
|---|---|---|---|---|---|
| | 0 | 0.125 | 0.250 | 1.25 | 2.50 |
| 0 | 0.0 (0) | 8.0 (3.5) | 10.8 (4.8) | 47.2 (0) | 92.0 (0) |
| 0.5 | 10.8 (0) | 30.4 (0) [0.57; 0.65] | 44.4 (0) [0.55; 0.62] | 68.2 (0) [1.04; 1.13] | ND |
| 1 | 22.0 (8.6) | 47.2 (0) [0.52; 0.58] | 89.2 (0) [0.15; 0.15] | 96.2 (0) [0.22; 0.23] | ND |
| 5 | 45.8 (0) | 76.6 (0) [0.75; 0.8] | 100.0 (0) [0.07; 0.07] | 100.0 (0) [0.13; 0.14] | ND |
| 20 | 87.8 (0) | ND | ND | ND | ND |
| 50 | 100.0 (0) | ND | ND | ND | ND |

[a] ATH8 cells were exposed to HIV-1 and cultured as described in Materials and Methods. Percent protective effect and percent cytotoxicity were determined by the formulas described in Materials and Methods.
[b] Percents cytotoxicity of less than 10% are not biologically significant in this assay system (22, 25). ND, Not done.
[c] See Table 1, footnote *c*, and Materials and Methods.

TABLE 3. Percent inhibition of HIV-induced cytotoxicity of combinations by ddC plus dextran sulfate in ATH8 cells[a]

| ddC concn (μM) | % Inhibition (% cytotoxicity)[b] [CIs][c] at a dextran sulfate concn (μM) of: | | |
|---|---|---|---|
| | 0 | 0.125 | 0.25 |
| 0 | 0.0 (0.0) | 0.0 (0.0) | 8.8 (0.0) |
| 0.02 | 17.5 (0.0) | 7.0 (0.0) [4.81; 7.12] | 26.3 (0.0) [0.85; 0.97] |
| 0.05 | 12.3 (4.8) | 28.0 (0.0) [0.71; 0.84] | 52.6 (0.0) [0.52; 0.54] |
| 0.1 | 26.3 (0.0) | 71.9 (0.0) [0.20; 0.20] | 100.0 (5.1) [0.12; 0.12] |
| 0.2 | 36.8 (0.0) | 75.4 (8.1) [0.20; 0.20] | 100.0 (0.0) [0.12; 0.12] |

[a] ATH8 cells were exposed to HIV-1 and cultured as described in Materials and Methods. Percent protective effect and percent cytotoxicity were determined by the formulas described in Materials and Methods.
[b] Percents cytotoxicity of drugs less than 10% are not biologically significant in this assay system (22, 25).
[c] See Table 1, footnote *c*, and Materials and Methods.

Colonies of >40 cells were counted by an inverted microscope. Sample wells were stained by the method of Kubota et al. (17) for myeloid morphological examination. Approximately 100 to 200 colonies formed per control well, and each drug, alone and in combination, was tested for inhibitory effect on colony formation. All experiments were performed in triplicate.

## RESULTS

**Synergistic antiretroviral effects exerted by combinations of ddNs and sCD4 on ATH8 cells exposed to HIV-1.** First, we tested the antiviral activities of combinations of the various ddNs plus sCD4 (Fig. 1). We have previously shown that AZT, ddI, and ddC, as single agents, can exert potent inhibitory effects against the infectivity and replication of HIV-1 at doses that do not affect the growth of target cells in the HIV cytopathic effect inhibition assay and can completely suppress the virus at concentrations of ≥1, ≥10, and ≥0.5 μM, respectively (22, 27). It has recently been shown that sCD4 can also suppress the infectivity of HIV-1 in vitro at concentrations of 1 to 5 μg/ml (6, 9, 13, 31, 33). In the current HIV cytopathic effect inhibition assay, in which we used susceptible ATH8 cells and a high multiplicity of infection, eight or nine combinations of sCD4 with several concentrations of each ddN were tested for activity against HIV-1. In this assay, sCD4 alone gave only partial protection in the range of 0.2 to 1 μg/ml (Fig. 1), and each low concentration of a ddN (AZT at 0.5 μM, ddI at 5 μM, and ddC at 0.05 μM) also partially inhibited the cytopathic effect of the virus. However, sCD4 plus any one of the ddNs provided synergistic protection. AZT at 0.5 μM plus sCD4 at 1 μg/ml almost completely protected virus-exposed ATH8 cells without damaging their survival and growth rate, with a CI of 0.53 (Fig. 1, upper panel). The antiviral activity of a combination of ddI and sCD4 was further tested under the same conditions. Although 5 μM ddI alone and sCD4 alone at 1 μg/ml exerted partial protective effects, together, the drugs exhibited a substantial synergistic antiviral effect, with a CI of 0.13. Growth of target ATH8 cells was not significantly affected at any concentration of combined drugs used (Fig. 1, upper panel).

We also tested the effects of combinations of ddC plus sCD4 under the same conditions. ddC at 0.05 μM combined with sCD4 at 0.5 and 1 μg/ml exerted a strong synergistic antiretroviral effect and gave virtually complete protection, with CI values of 0.17 and 0.21, respectively (Fig. 1, lower panel).

**Antiretroviral effects of combinations of ddI plus sCD4 on normal $CD4^+$ TM11 cells exposed to HIV-1.** The antiretroviral effect of ddI combined with sCD4 was further tested in normal helper-inducer T-cell clone TM11 following exposure to HIV-1 (Table 1). With no drugs, HIV-1 exerted a substantial cytopathic effect on the TM11 population by day 15 of culture, resulting in an approximately 60% decrease in the number of viable cells. However, 1 μM ddI combined with sCD4 at 0.2, 0.5, or 1 μg/ml exerted an enhanced antiretroviral effect. The CIs obtained revealed substantial synergistic antiretroviral effects for eight of nine combinations. This synergistic interaction is illustrated as an isobologram in Fig. 2.

**Possible synergistic inhibition of infectivity and replication of HIV-1 by combinations of ddNs and dextran sulfate.** We next tested the antiviral effect of ddI combined with dextran sulfate, an anionic polysaccharide which inhibits the binding of HIV-1 virions to $CD4^+$ T cells in vitro (see below). Individually, ≤5 μM ddI and ≤1.25 μM dextran sulfate exerted only partial protective effects on ATH8 cells exposed to the virus. When combined, however, the drugs exhibited a substantial enhanced antiretroviral effect. For eight of nine combinations, the synergistic protective effect appeared to have taken place as assessed by CI (Table 2). When ddC and dextran sulfate were combined, substantial synergistic antiviral activity was observed for seven of eight combinations (Table 3).

**Effects of combinations of dextran sulfate and sCD4 on replication of HIV-1.** Dextran sulfate can block binding of HIV-1 virions to various target cells, inhibit syncytium formation, and exert a potent inhibitory effect on replication of HIV-1 and HIV-2 in vitro (14, 26, 34). We and others have recently shown that dextran sulfate is capable of inhibiting virion attachment and/or fusion-dependent events which depend on interaction between cellular CD4 molecules and viral gp120 (1, 26). sCD4 has a high binding affinity for gp120, which is comparable to cellular CD4, and can competitively inhibit binding of HIV-1 to $CD4^+$ target cells (31). It is therefore possible that dextran sulfate blocks the interaction of sCD4 and gp120, which may result in reduction of the antiviral activity of sCD4. We then asked whether dextran sulfate could affect the antiviral activity of sCD4 in ATH8 cells exposed to the virus. We found an apparent antagonism with sCD4 plus lower concentrations of dextran sulfate, while with about equipotent combinations (sCD4 at 0.5 to 1 μg/ml plus 0.625 μM dextran sulfate) a weak synergistic effect was also observed (Table 4).

**Bone marrow toxicities of combinations of ddNs plus sCD4.** We tested the toxicities of the drugs in combinations for human myeloid-monocytic bone marrow cells (CFU-granulocyte-macrophage) in vitro. Testing drugs for possible toxicity for bone marrow cells in vitro may often predict bone marrow suppression in patients undergoing therapy with those drugs (20). Indeed, AZT, whose major toxicity is bone

TABLE 4. Percent inhibition of HIV-induced cytotoxicity by combinations of sCD4 plus dextran sulfate in ATH8 cells[a]

| sCD4 concn (μg/ml) | % Inhibition (% cytotoxicity)[b] [CIs][c] at a dextran sulfate concn (μM) of: | | | | |
|---|---|---|---|---|---|
| | 0 | 0.125 | 0.250 | 0.625 | 2.50 |
| 0 | 0.0 (0) | 3.3 (0) | 1.3 (19.3) | 18.6 (16.0) | 78.0 (17.6) |
| 0.2 | 12.8 (0) | 5.2 (5.9) [1.37; 1.78] | 18.6 (0) [0.87; 1.06] | 39.7 (0) [0.83; 0.99] | ND |
| 0.5 | 14.8 (0) | 24.3 (0) [1.16; 1.32] | 35.8 (0) [1.01; 1.19] | 68.4 (14.2) [0.68; 0.80] | ND |
| 1 | 24.3 (12.6) | 24.3 (2.5) [2.16; 2.48] | 45.4 (2.5) [1.46; 1.70] | 79.9 (21.0) [0.80; 0.92] | ND |
| 5 | 100.0 (0) | ND | ND | ND | ND |

[a] ATH8 cells were exposed to HIV-1 and cultured as described in the legend to Fig. 1. Percent protective effect was determined by the formula described in Materials and Methods.
[b] See the legend to Fig. 1. ND, Not done.
[c] See Table 1, footnote *c*, and Materials and Methods.

marrow suppression in patients with AIDS and the AIDS-related complex (30, 36), has been shown to be substantially toxic to bone marrow cells in vitro (4, 15, 32). We then asked whether combinations of ddN and inhibitors of viral binding could be toxic to bone marrow cells in vitro. When normal bone marrow cells were cultured with granulocyte-macrophage colony-stimulating factor and various concentrations of the drugs in combinations, we detected no significant increase in toxicity to bone marrow cells for any combination tested (Table 5).

TABLE 5. Lack of bone marrow toxicity exerted by combinations of ddNs plus sCD4

| Drug(s) and concn(s) | Mean ± SE no. of colonies[a] | % Control[b] |
|---|---|---|
| None (control) | 156 ± 2 | 100 |
| sCD4 | | |
| 0.5 μg/ml | 160 ± 3 | 100 |
| 1 μg/ml | 142 ± 2 | 91 |
| 5 μg/ml | 132 ± 3 | 85 |
| AZT, 0.5 μM | 125 ± 2 | 80 |
| AZT-sCD4 | | |
| 0.5 μM; 0.5 μg/ml | 122 ± 1 | 78 |
| 0.5 μM; 1 μg/ml | 110 ± 3 | 76 |
| ddI | | |
| 5 μM | 182 ± 1 | 100 |
| 10 μM | 145 ± 2 | 93 |
| ddI-sCD4 | | |
| 5 μM; 0.5 μg/ml | 188 ± 5 | 100 |
| 5 μM; 1 μg/ml | 174 ± 2 | 100 |
| 5 μM; 5 μg/ml | 139 ± 1 | 89 |
| 10 μM; 0.5 μg/ml | 140 ± 4 | 90 |
| 10 μM; 1 μg/ml | 139 ± 3 | 89 |
| 10 μM; 5 μg/ml | 134 ± 1 | 86 |
| ddC, 0.05 μM | 149 ± 2 | 96 |
| ddC-sCD4 | | |
| 0.05 μM; 0.5 μg/ml | 150 ± 4 | 96 |
| 0.05 μM; 1 μg/ml | 136 ± 2 | 87 |

[a] Assays were performed in triplicate in three different experiments for each drug, alone or in combinations. Approximately 100 to 200 colonies formed per control well, and the representative data are shown as arithmetic means of triplicate determinations.
[b] When % control is 100% or greater, 100% is given.

## DISCUSSION

Combination chemotherapies have been widely explored as curative approaches to many other diseases, including cancers and infectious diseases, notably for tuberculosis and leukemias-lymphomas. In this study, we demonstrated that combinations of ddNs and two different inhibitors of viral binding, sCD4 and dextran sulfate, can exert at least an additive effect in vitro and, at certain concentrations, can exhibit a synergistic antiviral effect with no significant additive or synergistic toxicity to the target cells or bone marrow cells in vitro.

It is worth noting that cautions should be taken against overinterpretation of the magnitudes of the synergistic and antagonistic effect found in this study. The method of Chou and Talalay (2, 3) used in the present study is based on the median-effect principle of the mass-action law and thus emphasizes the importance of potency. One major feature of this method is to fit the data to the mass-action principle instead of drawing the empirical curve to fit the data. The applicability of data to this principle is routinely checked with the linear correlation coefficient of the median-effect plot without exception (2, 3). In this study, the statistical reliability of the data was thus tested at the beginning of analysis rather than at the end of analysis after many mathematical transformations had occurred. It should also be stressed that experimental and biological variations are also reflected in the CIs we used in this study. However, we emphasize that the CIs for each set of data in this study consistently indicated synergism (or antagonism). In fact, the distribution of CIs in Tables 1 to 4 provided an indication of the general trend of drug interaction of each set of combinations.

sCD4 (6, 9, 13, 31, 33) and dextran sulfate (14, 26, 34) have been shown to be inhibitors of infectivity and replication of HIV-1 in vitro. sCD4 and dextran sulfate may act on an early phase(s) of HIV-1 replication, perhaps on binding of the virus to the target cell or its entry, while ddNs, following phosphorylation in the cytoplasm, appear to serve as substrates for viral reverse transcriptase and function as DNA chain terminators (22, 25), although this may not be the only mechanism. Thus, combinations of these drugs, which target different stages in the HIV-1 replicative life cycle, may synergize each antiviral activity and may also decrease side effects in patients treated with the combination therapy.

Interestingly, when two inhibitors of viral binding, sCD4 and dextran sulfate, were combined, there was apparent antagonism in five of the nine combinations, although at higher concentrations, weak synergy was observed. This suggests that one of the two agents inhibits interaction of the

other agent with the virus. In this regard, it is noteworthy that although 1 μM dextran sulfate inhibits binding of radiolabeled HIV-1 virions to $CD4^+$ cells (27), binding of radiolabeled recombinant gp120 to sCD4 is not inhibited by even 10 μM dextran sulfate (H. Mitsuya et al., unpublished data). These data suggest that dextran sulfate does not affect the CD4 molecule-viral gp120 interaction itself but affects other events of the cell-virion interaction. The antiviral mechanism(s) of dextran sulfate and sCD4 requires further research.

The current data indicate that ddI combined with sCD4 brings about substantial synergism without concomitant increases in toxicities in vitro. This could be noteworthy, since ddI has been shown to suppress HIV-1 replication in patients with AIDS and the AIDS-related complex (37), and this agent appears to be the least toxic of the nucleoside analogs we have tested as antiretroviral drugs. If the optimal effectiveness of sCD4 for clinical application is determined, such a combination could represent a potent antiviral therapy for HIV-1 infection. Combination chemotherapy with ddI and other drugs could also be useful; in particular, it could be most suitable for long-term administration for treatment of individuals infected with HIV.

Taken together, the data in this report have theoretical and clinical implications for the development of antiretroviral therapies for AIDS and its related diseases.

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