ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1990, p. 1061–1067
0066-4804/90/061061-07$02.00/0
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Vol. 34, No. 6

# Activities of 3′-Azido-3′-Deoxythymidine Nucleotide Dimers in Primary Lymphocytes Infected with Human Immunodeficiency Virus Type 1

RAYMOND F. SCHINAZI,[1,2]* JEAN-PIERRE SOMMADOSSI,[3] VANESSA SAALMANN,[1,2] DEBORAH L. CANNON,[1,2] MENG-YU XIE,[3] GEORGINA C. HART,[4] GREGORY A. SMITH,[4] AND ELLIOT F. HAHN[4]

*Veterans Affairs Medical Center, Decatur, Georgia 30033[1]*; Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30303[2]; Department of Pharmacology, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama 35294[3]; and IVAX Corporation, Miami, Florida 33178[4]*

Received 18 October 1989/Accepted 28 February 1990

**The relative antiviral potencies of five nucleotide heterodimers of 3′-azido-3′-deoxythymidine (AZT), 3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-adenylic acid (AZT-P-ddA), 3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-inosinic acid (AZT-P-ddI), and the corresponding 2-cyanoethyl congeners AZT-P(CyE)-ddA and AZT-P(CyE)-ddI, were determined in primary human peripheral blood mononuclear cells infected with human immunodeficiency virus type 1. The homodimer 3′-azido-3′-deoxythymidilyl-(5′,5′)-3′-azido-3′-deoxythymidilic acid (AZT-P-AZT) was also included for comparison. The potencies of the compounds were AZT-P-ddA ≥ AZT-P-ddI > AZT-P(CyE)-ddA ≥ AZT-P(CyE)-ddI ≥ AZT > AZT-P-AZT. Whereas AZT-P-ddA and AZT-P-ddI had in vitro therapeutic indices greater than that of AZT, the homodimer of AZT had a low therapeutic index. AZT-P-ddI exhibited the lowest toxicity in peripheral blood mononuclear, Vero, or CEM cells. Combination studies between AZT and 2′,3′-dideoxyinosine (ddI) at nontoxic concentrations indicated a synergistic interaction at a drug ratio of 1:100. At higher ratios (1:500 and 1:1,000), the interactions were synergistic only at concentrations that produced up to 75% virus inhibition. At higher levels of antiviral effects, this combination was antagonistic, as determined by the multiple drug effect analysis method. AZT-P-ddI was about 10-fold less toxic than AZT to human granulocyte-macrophage progenitor cells. However, no significant difference was apparent when the compounds were evaluated against cells of the erythroid lineage. The greater antiviral activity and lower toxicity of this compound could not be attributed to the extracellular decomposition of the dimer in media at physiological temperature and pH. However, in acidic solutions, AZT-P-ddI decomposed in a pH-dependent manner. Advanced preclinical studies with this heterodimer of two clinically effective antiretroviral agents should be considered.**

Among the antiviral agents that are currently being developed for the treatment of human immunodeficiency virus type 1 (HIV-1) infections and acquired immunodeficiency syndrome (AIDS), nucleoside analogs represent the largest group (13, 19). Once phosphorylated to the 5′-triphosphate form, nucleotide analogs are potent and specific inhibitors of the viral encoded reverse transcriptase (RT). Targeting of the viral enzymes that are necessary for viral replication represents one of the most successful rational approaches of modern antiviral chemotherapy and has proven to be effective in producing compounds for the treatment or suppression of several viral infections, including HIV-1. For example, 3′-azido-3′ deoxythymidine (AZT) is a potent antiviral drug that extends life and shows clinical benefits in individuals with AIDS (8, 17). Although the rapid approval of this drug is considered a milestone in the development of antiretroviral agents, much remains to be done to increase its efficacy and reduce its toxic effects (18). Toward this goal, several approaches, such as combination chemotherapy or the use of natural nucleosides for preventing its toxicity, have been suggested, and recent experimental data indicate that some of these modalities should be considered for use in humans (1, 7, 11, 12, 19, 20, 24).

Recently, Busso and co-workers (2) and Hahn et al. (10) showed that several nucleotide dimers of potentially useful antiretroviral agents inhibited HIV-1-induced cytopathic effects, RT production, and the expression of HIV-1 p24 antigens in the absence of toxic effects. It is not clear whether these novel dimers function as dimers or monomers. It is also not clear how the dimers dissociate intracellularly. Theoretically, if the molecule dissociates by cleavage of the phosphate bond that links the two known antiviral monomers, one nucleoside and one 5′-monophosphate form of the second nucleoside are released. Presumably, the levels of cellular kinases, phosphatases, and phosphorylases, as well as pharmacodynamic parameters, dictate the metabolic and anabolic fates of the components and their antiviral effectiveness as single or combined agents. The objective of this study was to ascertain the relative potencies of five different nucleotide dimers against HIV-1 replication in primary human lymphocytes and to determine the cytotoxicities of these dimers in various cell systems. A dimer consisting of the pyrimidine nucleoside AZT coupled to the purine nucleoside 2′,3′-dideoxyinosine (ddI) (26) was studied in detail to determine its stability, its in vitro bone marrow toxicity, and the interaction of its antiviral components alone and in combination in primary human lymphocytes infected with HIV-1. ddI is a nucleoside that is undergoing extensive studies in humans infected with HIV-1.

* Corresponding author.

## MATERIALS AND METHODS

**Compounds.** 3′-Azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-adenylic acid (AZT-P-ddA); 3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-adenylic acid, 2-cyanoethyl ester [AZT-P(CyE)-ddA]; 3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-inosinic acid (AZT-P-ddI); 3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxy-5′-inosinic acid, 2-cyanoethyl ester [AZT-P(CyE)-ddI]; and 3′-azido-3′-deoxythymidilyl-(5′,5′)-3′-azido-3′-deoxythymidilic acid (AZT-P-AZT) were provided by IVAX Corp., Miami, Fla., through the Developmental Therapeutic Branch, AIDS Program, National Institutes of Health, Rockville, Md. AZT, which was used as a positive control in the studies, was synthesized in our laboratory by the method of Lin and Prusoff (16) and was greater than 99% pure, as determined by high-pressure liquid chromatography. ddI and 2′,3′-dideoxyadenosine (ddA) were obtained from U.S. Biochemical Corp., Cleveland, Ohio.

**Antiviral assays.** The procedures for the antiviral assays in human peripheral blood mononuclear cells (PBMCs) have been published previously (20). Briefly, uninfected phytohemagglutinin-stimulated human PBMCs were infected with HIV-1 (strain LAV-1) (about 63,000 disintegrations of RT activity per minute per $10^7$ cells per 10 ml of medium). The drugs were then added to duplicate or triplicate cultures. Uninfected and untreated PBMCs were grown in parallel at equivalent cell concentrations as controls. The cultures were maintained in a humidified 5% $CO_2$–95% air incubator at 37°C for 6 days after infection, at which point all cultures were sampled for supernatant RT activity. Previous studies indicated that maximum RT levels are obtained at that time (20, 25). The supernatant was clarified, and the viral particles were then pelleted at 40,000 rpm for 30 min by using a rotor (70.1 Ti; Beckman Instruments, Inc., Fullerton, Calif.) and suspended in virus-disrupting buffer. The RT assay was performed by a modification of the method of Spira et al. (25) in 96-well microdilution plates by using $(rA)_n \cdot (dT)_{12-18}$ as the template primer. The RT results were expressed in disintegrations per minute per milliliter of originally clarified supernatant.

**Cytotoxicity assays in lymphocytes.** The compounds were evaluated for their potential toxic effects on uninfected phytohemagglutinin-stimulated human PBMCs and also in CEM and Vero (African green monkey kidney) cells. PBMCs were obtained from whole blood of healthy HIV-1- and hepatitis B virus-seronegative volunteers and collected by single-step Ficoll-Hypaque discontinuous gradient centrifugation. CEM (CEM-CCRF) cells are a T-lymphoblastoid cell line that was obtained from the American Type Culture Collection, Rockville, Md. The CEM cells were maintained in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The PBMCs and CEM cells were cultured with and without drug for 6 days, at which time portions were counted for cell proliferation and viability by the trypan blue exclusion method (24). Only the effects on cell growth are reported, since these correlated well with cell viability. The toxicity of the compounds in Vero cells was assessed after 3 days of treatment with a hemacytometer as described previously (22).

**Assay of CFU of granulocyte-macrophages or burst-forming units of erythroids for drug cytotoxicity studies.** Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers and treated with heparin, and the mononuclear population was separated by Ficoll-Hypaque gradient centrifugation as described previously (24). Cells were washed twice in Hanks balanced salt solution and counted with a hemacytometer, and their viabilities were >98%, as assessed by trypan blue exclusion. The culture assays were performed by using a bilayer soft agar or methylcellulose method as described recently (23, 24). McCoy 5A nutrient medium supplemented with 15% dialyzed fetal bovine serum (heat inactivated at 56°C for 30 min; GIBCO Laboratories, Grand Island, N.Y.) was used in all experiments. This medium was devoid of thymidine and uridine. Human recombinant GM-CSF (50 U/ml; Genzyme, Boston, Mass.) or erythropoietin (1 U/ml; Connaught, Swiftwater, PA) was used as colony-stimulating factors. After 14 days of incubation at 37°C in a humidified atmosphere of 5% $CO_2$ in air, colonies (≥50 cells) were counted by using an inverted microscope.

**Drug stability studies.** Five samples (pH 3 to 7) were prepared by dissolving 40 mg of AZT-P-ddI in 1 ml of the appropriate 0.2 M phosphate buffer ($KHPO_4$, KOH, or $H_3PO_4$). Portions (200 μl) of each solution at a concentration of 0.07 μM were stored at either 4 or 22°C. The remainder (600 μl) was stored at 37°C. At the designated time intervals, 10-μl portions were diluted to 10 ml with the corresponding buffer and analyzed by high-pressure liquid chromatography. The concentration of the dimer was calculated as a percentage of the total area under the curve for the dimer and presumed decomposition products.

**High-pressure liquid chromatography.** A reversed-phase column (5 mm by 12.5 cm; Lichrospher RP-18; VWR Scientific, Miami, Fla.) was used to determine the stability of AZT-P-ddI. The mobile phase was 10% acetonitrile in 0.01 M ammonium acetate. An isocratic flow rate of 1 ml/min was used, and the peaks were monitored with a UV detector set at 254 nm. The retention times for AZT, ddI, and AZT-P-ddI were 7.8, 2.3, and 3.5 min, respectively.

**Data analyses.** The median effective concentrations ($EC_{50}$s) and inhibitory concentrations ($IC_{50}$s) were derived from the computer-generated median effect plot of the dose-effect data, as described previously (3). From the slope of the dose-effect plot and the $EC_{50}$, the computer program also generated the 90% effective concentration ($EC_{90}$). The ratios of the drugs selected for the combination studies were based on the relative potencies of the individual compounds. The combination index (CI) for the combined effects of the drugs was also determined by using the same computer program. For the analyses we used constant ratios of the drugs (7, 20, 21). The CI values were determined from the median effect plot by using a conservative, mutually nonexclusive equation. CI values of <1, 1, or >1 indicate synergism, additivism, or antagonism, respectively (3, 21).

## RESULTS

The heterodimers were all potent inhibitors of HIV-1 replication in PBMCs with $EC_{50}$s below 0.4 nM compared with AZT, which had an $EC_{50}$ of 5.6 nM (Table 1). The potencies ($EC_{50}$s) of the compounds were AZT-P-ddA ≥ AZT-P-ddI > AZT-P(CyE)-ddA ≥ AZT-P(CyE)-ddI > AZT > AZT-P-AZT >> ddA > ddI. AZT-P-ddA and AZT-P-ddI were more potent on a molar basis than their individual components, i.e., AZT, ddA, or ddI (see Table 7). The heterodimers containing the phosphate bridge were at least fourfold more potent than the corresponding compounds containing the 2-cyanoethyl ester bridge. Dimers containing the ddA moiety appeared to be more potent at the $EC_{50}$s than those containing ddI (see Tables 1 and 7). The homodimer AZT-P-AZT was markedly less potent than AZT.

TABLE 1. Effect of drugs against HIV-1 (strain LAV-1) in human PBMCs

| Treatment | Concn (nM) | RT activity (mean kdpm/ml ± SD)[a] | % Inhibition |
|---|---|---|---|
| None | | 944 ± 299 | 0 |
| AZT | 0.1 | 1,035 | −9.7 |
| | 1 | 392 | 58.6 |
| | 10 | 93.1 | 90.4 |
| | 100 | 16.9 | 98.4 |
| AZT-P-ddA | 0.01 | 613 | 35.1 |
| | 0.1 | 368 | 61.2 |
| | 1 | 84.3 | 91.3 |
| | 10 | 32.3 | 96.8 |
| | 100 | 12.9 | 98.9 |
| AZT-P(CyE)-ddA | 0.1 | 505 | 46.6 |
| | 1 | 345 | 63.6 |
| | 10 | 91.9 | 90.5 |
| | 100 | 67.5 | 93.1 |
| | 1,000 | 11.5 | 99.0 |
| AZT-P-ddI | 0.1 | 469 | 50.4 |
| | 1 | 108.5 | 88.7 |
| | 10 | 29.6 | 97.1 |
| | 100 | 11.6 | 99.0 |
| | 1,000 | 38.6 | 96.1 |
| AZT-P(CyE)-ddI | 0.1 | 573 | 39.4 |
| | 1 | 487 | 48.5 |
| | 10 | 61.8 | 93.7 |
| | 100 | 21.9 | 97.9 |
| | 1,000 | 11.7 | 99.0 |
| Blank | | 2.24 ± 0.61 | |
| Uninfected control | | 5.89 ± 0.23 | |

[a] Each value represents the arithmetic mean of duplicate or triplicate determinations of virus present in the supernatant 6 days after infection, as determined by a RT assay.

TABLE 2. Effect of AZT and nucleotide dimers on the growth of uninfected mitogen-stimulated human PBMCs, CEM cells, and Vero cells

| Treatment | Concn (μM) | % Growth inhibition in: | | |
|---|---|---|---|---|
| | | PBMCs[a] | CEM cells[b] | Vero cells[c] |
| AZT | 1 | −2.2 | −1.4 | 10.2 |
| | 10 | 5.5 | 49.3 | 20.7 |
| | 50 | 16.8 | 51.6 | 47.4 |
| | 100 | 28.4 | 60.9 | 66.3 |
| AZT-P-ddA | 1 | 6.1 | −7.0 | −6.7 |
| | 10 | 11.8 | 8.9 | −12.3 |
| | 100 | 10.1 | 48.8 | 20.0 |
| | 200 | ND[d] | 56.6 | 22.8 |
| AZT-P(CyE)-ddA | 1 | −3.0 | 0.5 | 4.6 |
| | 10 | 2.6 | 0 | 30.5 |
| | 100 | 10.5 | 48.4 | 46.0 |
| | 200 | 24.5 | 65.3 | ND |
| AZT-P-ddI | 1 | −7.1 | −11.3 | −4.6 |
| | 10 | −13.0 | 4.2 | 19.3 |
| | 100 | 17.8 | 33.3 | 39.0 |
| AZT-P(CyE)-ddI | 1 | −9.1 | −4.2 | −1.8 |
| | 10 | 23.7 | 38.5 | 8.8 |
| | 100 | 63.5 | 52.1 | 18.6 |
| | 200 | ND | 83.0 | 32.6 |
| AZT-P-AZT | 1 | 16.6 | ND | −7 |
| | 10 | 65.8 | ND | 16 |
| | 100 | 70.9 | ND | 96 |
| | 200 | 60.0 | ND | 97 |

[a] PBMCs were counted after drug exposure for 6 days by the trypan blue exclusion method. Untreated cultures had $(4.93 \pm 0.11) \times 10^5$ cells per ml.
[b] CEM cells were counted after drug exposure for 6 days. Untreated cultures had $(2.13 \pm 0.11) \times 10^5$ cells per ml.
[c] Vero cells were counted after drug exposure for 4 days. Untreated cultures had $(3.56 \pm 0.19) \times 10^5$ cells per ml.
[d] ND, Not determined.

AZT, AZT-P-ddA, AZT-P(CyE)-ddA, AZT-P(CyE)-ddI, and AZT-P-AZT exhibited marked toxicity in one or more of the cell systems used when tested at 100 μM (Table 2). The toxicity of the compounds appeared to be more pronounced in CEM cells compared with those in PBMCs or Vero cells. AZT-P-ddI was the least toxic dimer in PBMCs, Vero cells, or CEM cells; a mild toxicity was apparent in CEM and Vero cells. Moreover, AZT-P-ddA and AZT-P-ddI had greater in vitro therapeutic indices (defined as the ratio of $IC_{50}$:$EC_{90}$) than that of AZT. The homodimer of AZT was more toxic to PBMCs and Vero cells than was AZT (Table 2).

As reported previously (23), AZT is found to be toxic to human granulocyte-macrophage precursor cells. However, AZT-P-ddI was significantly less toxic than AZT to human granulocyte-macrophage progenitor cells (Table 3). Whereas AZT displayed a 50% suppression of colony formation at concentrations of 1.14 μM, the dimer AZT-P-ddI required a 10-fold greater concentration to produce the same effect. The difference was more pronounced when the 90% suppression values were compared (11.5 versus 208 μM). In contrast to the difference obtained with the results with granulocyte-macrophage precursor cells, there was no significant difference between the two compounds when erythroid progenitor cells were used (Table 3).

The effects of AZT and ddI, alone and in combinations, on the virus yield, as determined by the RT activity of disrupted virus obtained from cell culture supernatants, are shown in Table 4. A 50% reduction in RT activity, or $EC_{50}$, was observed at 0.0039, 0.46, and 0.00014 μM for AZT, ddI, and AZT-P-ddI, respectively, when the drugs were tested alone. Table 5 summarizes the $EC_{50}$s, as well as slopes and correlation coefficients, for the median effect plots. Since the slope for AZT was different than that for ddI or the combination of AZT and ddI, a mutually nonexclusive drug effect is indicated (i.e., independent modes of action). The interaction of AZT and ddI was determined by calculating the CI values of the drugs at ratios of 1:100, 1:500, and 1:1,000. These ratios were selected since they approximated the ratio of the $EC_{50}$s for AZT and ddI. The CI values of each combination giving 50, 75, and 90% reduction of RT activity were all well below 1 for the drugs at a 1:100 ratio, suggesting synergy (Table 5). At higher ratios (1:500 and 1:1,000), synergy was apparently only at concentrations that produced up to 75% virus inhibition. At a ratio of 1:1,000 the combination of AZT and ddI produced no toxicity greater than that of the agents alone to uninfected proliferating PBMCs (data not shown).

A study was conducted to determine the stability of AZT-P-ddI over broad pH (3 to 7) and temperature (4, 22, and 37°C) ranges. The results indicate that decomposition of the dimer accelerated with increasing temperature and decreasing pH. After 4 days, at pH 3 and 4°C, only 65% of the

TABLE 3. Relative effects of AZT and AZT-P-ddI on human myeloid (CFU of granulocyte-macrophages) and erythroid (burst-forming units of erythroids) progenitor cells by clonogenic assays

| Treatment | Concn (μM) | % of control ± SD[a] | | $IC_{50}/IC_{90}$ (μM) | |
|---|---|---|---|---|---|
| | | CFU-GM | BFU-E | CFU-GM | BFU-E |
| AZT | 0.1 | 85.57 ± 10.16 | 66.74 ± 12.84 | 1.14/11.5 | 0.69/97.4 |
| | 1 | 57.31 ± 10.37 | 45.90 ± 4.27 | | |
| | 10 | 30.71 ± 8.80 | 33.16 ± 6.09 | | |
| | 100 | 0.58 ± 0.69 | 7.36 ± 5.26 | | |
| AZT-P-ddI | 0.1 | 83.26 ± 7.42 | 76.93 ± 5.99 | 11.1/208 | 1.41/85.7 |
| | 1 | 72.10 ± 8.87 | 52.60 ± 5.71 | | |
| | 10 | 51.72 ± 9.01 | 43.89 ± 6.46 | | |
| | 100 | 16.05 ± 9.52 | 5.79 ± 3.43 | | |

[a] Results represent mean values of three separate experiments with cells from different donors performed in triplicate. CFU-GM, CFU of granulocyte-macrophages; BFU-E, burst-forming units of erythroids.

TABLE 4. Combinations of drugs against HIV-1 (strain LAV-1) in human PBMCs

| Treatment (drug ratio) | Concn (nM) | RT activity (mean kdpm/ml ± SD)[a] | % Inhibition |
|---|---|---|---|
| None | | 1,184 ± 145 | 0 |
| AZT | 0.1 | 1,187 | −0.3 |
| | 1 | 983 | 17.0 |
| | 10 | 244.5 | 79.5 |
| | 100 | 30.1 | 97.7 |
| ddI | 10 | 1,257 | −6.2 |
| | 100 | 944 | 20.3 |
| | 1,000 | 516 | 56.6 |
| | 10,000 | 35 | 97.2 |
| AZT-P-ddI | 0.1 | 674 | 43.1 |
| | 1 | 338 | 71.6 |
| | 10 | 30.2 | 97.6 |
| | 100 | 24.3 | 98.1 |
| AZT/ddI | 0.1/10 | 902 | 23.8 |
| 1:100 | 0.5/50 | 860 | 27.4 |
| | 1/100 | 656 | 44.7 |
| | 5/500 | 160 | 86.6 |
| | 10/1,000 | 54.8 | 95.6 |
| | 50/5,000 | 16.7 | 98.8 |
| 1:500 | 0.1/50 | 737 | 37.8 |
| | 0.5/250 | 572 | 51.8 |
| | 1/500 | 483 | 59.3 |
| | 5/2,500 | 114.5 | 90.5 |
| 1:1,000 | 0.05/50 | 785 | 33.7 |
| | 0.2/200 | 708 | 40.2 |
| | 0.4/400 | 489 | 58.8 |
| | 0.8/800 | 344 | 71.1 |
| | 1.6/1,600 | 177 | 85.2 |
| Blank | | 2.43 | |
| Uninfected control | | 8.94 | |

[a] Each value represents the arithmetic mean of duplicate or triplicate determinations of virus present in the supernatant 6 days after infection, as determined by a RT assay.

initial concentration of the dimer remained. At the same pH but at 37°C, the intact dimer concentration was 75% after 1 h, and it had completely decomposed within 24 h. However, much less degradation occurred at either pH 6 or 7, with less than 5% decomposition noted at pH 7 even after 3 weeks at 37°C. Table 6 summarizes the results obtained at different pHs and temperatures.

## DISCUSSION

Human PBMCs are routinely used in our laboratory for evaluating potential anti-HIV-1 compounds, as they have the advantage of being one step closer to the patients infected with HIV-1 compared with continuous lymphocytic cell lines cultured in the laboratory (16). High virus yields are achieved rapidly in these primary cells, which are also commonly used to isolate HIV-1 from clinical specimens because of their sensitivity to the virus. In our study, peak RT levels of close to $10^6$ dpm/ml occurred 6 days after infection at relatively low virus input (Table 1). In agreement with the report of Busso et al. (2) and Hahn et al. (10), who tested three of the five dimers described here in MT-2 cells, all the dimers also showed significant activity against HIV-1 in PBMCs (Table 7).

TABLE 5. Median effective concentration and CI values for AZT and ddI alone and in combination or as the dimer AZT-P-ddI

| Treatment (drug ratio) | Parameter[a] | | | CI at $F_a$ of[b]: | | |
|---|---|---|---|---|---|---|
| | $m$ ± SE | $EC_{50}$ (μM) | $r$ | 0.50 | 0.75 | 0.90 |
| AZT | 1.21 ± 0.04 | 0.0039 | 0.99 | | | |
| ddI | 1.07 ± 0.12 | 0.46 | 0.99 | | | |
| AZT-P-ddI | 0.67 ± 0.14 | 0.00014 | 0.96 | 0.018[c] | 0.038[c] | 0.078[c] |
| AZT/ddI | | | | | | |
| 1:100 | 1.31 ± 0.50 | 0.11 | 0.99 | 0.58 | 0.51 | 0.45 |
| 1:500 | 0.49 ± 0.20 | 0.06 | 0.87 | 0.16 | 0.61 | 2.58 |
| 1:1,000 | 0.70 ± 0.13 | 0.20 | 0.95 | 0.51 | 0.92 | 1.73 |

[a] $m$ is the slope, $EC_{50}$ is the median effective concentration, and $r$ is the correlation coefficient, as determined from the median effect plot.

[b] CI of <1 indicates synergy (see text). $F_a$ is a component of the median effect equation referring to the fraction of the system affected (e.g., 0.50 means the CI at a 50% reduction of RT activity). $F_a$ values were determined for a mutually nonexclusive interaction.

[c] Assuming that AZT-P-ddI dissociates to AZT and ddI in a ratio of 1:1.

TABLE 6. Stability of AZT-P-ddI as a function of pH and temperature

| pH | % AZT-P-ddI intact after 7 days at: | | |
|---|---|---|---|
| | 4°C | 22°C | 37°C |
| 7 | 100 | 100[a] | 98[a] |
| 6 | 100 | 97[a] | 90 |
| 5 | 100 | 92 | 39 |
| 4 | 96 | 18.5 | 6[b] |
| 3 | 65[b] | 0 | 0 |

[a] AZT-P-ddI was found to be stable (>95%) through 23 days under these conditions.
[b] Data for study day 4 are provided.

Consistent with our previous observations, the compounds were shown to be markedly more potent in PBMCs than in MT-2 cells (4). For example, whereas the $EC_{50}$ for AZT-P-ddI was 1.0 μM in MT-2 cells, the $EC_{50}$ in PBMCs was 10,000-fold lower. The PBMC system used in these studies had the added advantage of allowing the determination of the relative potencies of the dimers compared with that of AZT, a feat which was not possible with the MT-2 system, because the $EC_{50}$s of all the drugs were found to be in a narrow range of 0.7 to 1.5 μM (2, 10). It is of interest that the homodimer of AZT was markedly less potent than AZT, suggesting that AZT-P-AZT is not equivalent to two molecules of AZT or a molecule of AZT and AZT-5'-monophosphate. The intracellular decomposition of this dimer and its metabolism deserve further studies.

In the toxicity studies, three different host cells were used. Primary human PBMCs were selected, because these lymphocytes represent slow-growing cells, and were also used in the antiviral assay as host cells. The lymphocytic CEM cells were selected, as they are a continuous nontransformed cell line that replicates quickly and is commonly used for anti-HIV-1 assays. Vero cells were used, as they not only grow rapidly but are also anchored. In addition, AZT has been previously reported to be toxic in Vero cells (R. F. Schinazi, C. K. Chu, P. Feorino, and J.-P. Sommadossi, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1092, 1986); the toxicities in these cells may indicate potential problems with this type of nucleoside. Furman et al. (9) reported that AZT is toxic at 170 μM in Vero cells, whereas we found that AZT was clearly toxic at 3.4-fold lower concentrations (Table 2). De Clercq et al. (6) have shown that AZT was toxic to primary rabbit kidney cells ($IC_{50}$, 26 μM) and L1210 cells ($IC_{50}$, 36 μM) by measuring the incorporation of $[^3H]$thymidine into cellular DNA. It is conceivable that the inhibitory effect may have resulted from the competition of labeled thymidine with AZT. With the exception of AZT-P-ddI, all the other dimers exhibited some toxicity in PBMCs, CEM cells, or Vero cells (Tables 2 and 7). The low toxicity of some of the heterodimers in Vero cells compared with that of AZT suggests that these compounds either penetrate the cell membrane by a different mechanism or they are metabolized differently from AZT in uninfected cells (Table 7). A comparison of the toxicity of the dimers at 100 μM with that of AZT at 50 μM suggests that this could be the case for AZT-P-ddA and AZT-P(CyE)-ddI.

Toxicity to human bone marrow cells, especially cells of the erythroid lineage, is a limitation associated with the chronic administration of AZT (18). Sommadossi and Carlisle (23) were the first to demonstrate that in vitro toxicity of antiviral agents to human granulocyte-macrophage and erythroid progenitor cells may be a useful prognosticator of what may happen in humans. The toxicity profiles of AZT and AZT-P-ddI with respect to these cells are presented in Table 3. The dimer was about 10-fold less toxic than AZT. Since ddI is essentially not toxic to these cells, with an $IC_{50}$ greater than 100 μM (J.-P. Sommadossi, Z. Zhu, R. Carlisle, M.-Y. Xie, D. A. Weinder, and M. H. El Kouni, Pharmacol. Ther., in press), the toxicity of the dimer is probably primarily due to the AZT component in this compound. It is of interest that no marked difference was noted between the two compounds when they were evaluated against erythroid progenitor cells. This difference between bone marrow precursor cells is not readily apparent and requires further investigation of the mechanism of toxicity of this dimer.

Because these molecules may act as prodrugs of potent anti-HIV-1 drugs, it was essential to compare the dimers with their individual and combined antiviral components. Experiments to determine the effects of combinations of different constant ratios of AZT and ddI in infected PBMCs were performed. Results of the data for synergy with determination of the CI are presented in Table 5. Since AZT is at least 100-fold more potent than ddI, it was not possible to evaluate this combination at a ratio of 1:1. However, if it is assumed that AZT-P-ddI is composed of equal ratios of AZT and ddI and an analysis of the combined effects of the drugs is performed by using the median effect equation, a determination of the CI indicates synergy at all levels of antiviral effects. Similarly, for AZT and ddI at a 1:100 ratio, synergy was observed. However, at higher ratios (1:500 and 1:1,000) the interactions were synergistic only at concentrations that produced up to 75% virus inhibition. It appears that high concentrations of ddI in combination with AZT do not result in synergy and can, in fact, produce antagonism. The reasons for these observations are unclear. The antagonism

TABLE 7. Summary of antiviral and cytotoxicity studies

| Treatment | Anti-HIV-1 activity in PBMCs at: | | Cytotoxicity ($IC_{50}$ [μM]) in: | | |
|---|---|---|---|---|---|
| | $EC_{50}$ (nM) | $EC_{90}$ (nM) | PBMCs | CEM cells | Vero cells |
| AZT | 3.9–5.6 | 15.8–24.0 | >100 | 14.3 | 50.6 |
| AZT-P-ddA | 0.03 | 1.1 | >100 | 128.1 | >200 |
| AZT-P(CyE)-ddA | 0.15 | 24.8 | >200 | 130.5 | ≥100 |
| AZT-P-ddI | 0.07–0.14 | 2.0–3.7 | >100 | >100 | >100 |
| AZT-P(CyE)-ddI | 0.34 | 9.0 | 47.0 | 56.6 | >200 |
| AZT-P-AZT | 10.0 | 48.0 | 16.5 | ND[a] | 13.5 |
| ddA[b] | 500 | 2,140 | >100 | >100 | >100 |
| ddI | 460 | 3,700 | >100 | >100 | >100 |

[a] ND, Not determined.
[b] Data were not shown in Table 1 or 4.

observed cannot be attributed directly to toxic effects at the highest concentrations of AZT and ddI. Baba et al. (1) reported that the combination of AZT and ddA interacted synergistically against HIV-1 in MT-4 cells. ddA is rapidly deaminated to ddI and, thus, can be considered a prodrug of ddI (5). Our results with a similar combination demonstrate that at concentrations that are easily attained in vivo, combinations of the two clinically useful compounds AZT and ddI can interact synergistically or antagonistically in inhibiting HIV-1 replication in human PBMCs. This interaction was dependent on the ratio of the drugs; lower ratios of effective concentrations of the drugs (1:100) produced a synergistic interaction (Table 5). The mechanism(s) by which ddI enhances the antiviral activity of AZT at certain drug ratios and concentrations remains to be elucidated.

The antiviral activity and low toxicity of AZT-P-ddI could not be attributed to the extracellular decomposition of AZT-P-ddI in media at physiological temperature and pH (Table 6). However, in acidic solutions, this dimer decomposed in a pH-dependent manner. These results again reinforce the possibility that the dimer must either interact with the cell membrane or affect viral replication after it is transported into the cell and subsequently is dissociated in certain intracellular compartments. This hypothesis is being validated or refuted by using radiolabeled dimer.

In summary, results of the present comparison of specific dimers and AZT in both the antiviral and cytotoxicity studies indicate that these highly potent compounds are worthy of further preclinical studies to determine their effectiveness in animal retroviral models and to determine their pharmacological profiles. AZT-P-ddI deserves additional study, as it appears to be one of the most potent and least toxic dimers evaluated to date. Of particular importance for this dimer is the finding that AZT-resistant variants appear to be susceptible to ddI (14, 26), reducing the possibility for these resistant variants to develop in the presence of the dimer. Results presented in this report suggest that studies of synergism can be used to rationally design novel single agents that are formed by chemically linking two synergistic drugs to form a larger molecule with significant benefits when compared with that of the single agents.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI 26055 (to R.F.S.), AI 25899 (to R.F.S.), HL 42125 (to J.-P.S.), and AI 25784 (to J.-P.S.) from the National Institutes of Health and a grant from the Department of Veterans Affairs (to R.F.S.). J.-P.S. is the recipient of a Junior Faculty Research Award from the American Cancer Society.

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