Antiviral Research, 22 (1993) 295–308

AVR 00663

Antiviral Research

Lack of synergy in the inhibition of HIV-1 reverse transcriptase by combinations of the 5′-triphosphates of various anti-HIV nucleoside analogs

E. Lucile White*, William B. Parker, Larry J. Ross and William M. Shannon

Kettering-Meyer Laboratory, Southern Research Institute, 2000 Ninth Ave. S., Birmingham, AL 35205, USA

(Received 6 May 1993; accepted 4 August 1993)

Summary

3′-Deoxy-3′-azidothymidine (AZT) has been shown to synergistically inhibit the replication of human immunodeficiency virus type 1 (HIV-1) in cell culture when combined with several other 2′,3′-dideoxynucleoside analogs. In an effort to understand the biochemical mechanism of this synergy, we have examined the effect of combinations of the 5′-triphosphate of AZT (AZT-TP) with either ddCTP, ddATP, or the 5′-triphosphate of the carbocyclic analog of 2′,3′-didehydro-2′,3′-dideoxyguanosine (carbovir) on both the RNA-directed and DNA-directed DNA polymerase activity of HIV-1 reverse transcriptase. Kinetic studies, which evaluated the ability of these combinations to competitively inhibit the enzyme, showed that AZT-TP could not bind to the enzyme with either the RNA or DNA template at the same time as either of the other three inhibitors. None of these analogs could affect the incorporation of another analog into the DNA chain by the HIV-1 reverse transcriptase. These results indicated that synergistic inhibition of the HIV-1 reverse transcriptase is not responsible for the synergistic antiviral activity seen in cell culture with combinations of these nucleoside analogs.

HIV-1; Reverse transcriptase; Drug combinations; 3′-Deoxy-3′-azidothymidine; 2′,3′-Dideoxyinosine; 2′,3′-Dideoxycytidine; Carbovir

*Corresponding author. Fax: +1 (205) 5812877.

Introduction

To date, only three drugs have been approved by the FDA for use in the treatment of HIV-1 infection, 3′-deoxy-3′-azidothymidine (Zidovudine, AZT; Yarchoan et al., 1986), 2′,3′-dideoxycytidine (Zalcitabine, ddC; Yarchoan et al., 1988), and 2′,3′-dideoxyinosine (Didanosine, ddI; Ahluwalia et al., 1987). All three compounds are nucleoside analogs generally considered to inhibit viral replication by the same mechanism (Mitsuya et al., 1990; Connolly and Hammer, 1992a). After metabolism to the appropriate 5′-triphosphate (TP), they compete with the natural nucleotides for the HIV-1 reverse transcriptase and are incorporated into the HIV DNA resulting in chain termination. Inhibition of viral DNA synthesis prevents replication and subsequent integration of the proviral genome into the host cell DNA.

The clinical efficacy of each of these drugs is limited due to toxicities associated with their use (Schinazi et al., 1992). In addition, drug-resistant virus strains emerge in patients after fairly short periods of treatment with these compounds (Japour et al., 1991; Richman, 1992; Shirasaka et al., 1993). Although the relationship of these resistant isolates to failure of therapy is unclear, the patient is frequently treated with another nucleoside once they appear (Tudor-Williams and Emery, 1992; Boucher et al., 1992; Gotzsche et al., 1992). Consequently, intensive efforts are underway to improve therapy for HIV-1 infected individuals, both by developing new drugs and by investigating different treatment regimes with the existing drugs (Connolly and Hammer, 1992b). Combination chemotherapy has been successfully used in the treatment of other diseases to address the problems associated with the development of resistance and drug toxicities.

Surprisingly, AZT when combined with either of the two other FDA approved drugs, ddC and ddI, has synergistic antiviral activity in in vitro cell culture tests (Schinazi, 1992; Eron et al., 1992; Dornsife et al., 1991; Cox et al., 1992). Observations of synergistic anti-HIV activity have been expanded to include other nucleoside analogs in combinations with AZT, including the carbocyclic analog of 2′,3′-didehydro-2′,3′-dideoxyguanosine (carbovir, CBV; Shannon, 1990; Smith et al., 1993) and 3′-fluoro-3′-deoxythymidine (FLT; Harmenberg et al., 1990; Cox et al., 1992). Recently, three drug combinations of AZT, ddI, and one of the nonnucleoside reverse transcriptase inhibitors, L697661, were found to be very effective in limiting HIV-1 replication in vitro (Chow et al., 1993). Attempts to determine the mechanism of antiviral synergy of combinations of nucleoside analogs have been unsuccessful to date. Combination of another nucleoside with AZT does not appear to affect the metabolism of either nucleoside or to significantly affect the levels of the natural triphosphate pools, even when these studies are carried out in HIV-infected cells of the type in which the antiviral synergy was measured (Parker et al., 1993; Cox, 1992).

Although it was anticipated that two nucleotide analogs would not interact with the reverse transcriptase to produce synergistic inhibition of this enzyme,

it has not been possible until recently to actually test this assumption for the unique activity of this enzyme, its RNA-directed DNA polymerase activity. Assay systems that employ a homopolymer RNA template can only test compounds with a complementary base as inhibitors of the enzyme. By using ribosomal RNA as the template we have been able to test combinations of the 5′-triphosphates of different bases for their ability to synergistically inhibit HIV-1 reverse transcriptase. In addition, we have examined these combinations for their ability to inhibit the DNA-directed DNA polymerase activity of HIV-1 reverse transcriptase.

Materials and Methods

Chemicals

dATP, dGTP, dCTP, dTTP, ddCTP, ddATP, Sephadex G-25, and T4 polynucleotide kinase were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). (±) CBV-TP was prepared from (±) CBV as described (White et al., 1989). AZT-TP was a generous gift of Burroughs Welcome Co. (Research Triangle Park, NC). [*Methyl*,1,2′-^{3}H]dTTP (100 Ci/mmol) and [8,5′-^{3}H]dGTP (31.9 Ci/mmol) in Tricine buffer were obtained from New England Nuclear Research Products (Wilmington, DE). [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). The 16S/23S *E. coli* ribosomal RNA and one DNA oligomer complementary to 15 bases on the 16S rRNA were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). The sequence of this 15mer (A), used for the primer extension assays, was 5′-ACGGGCGGTGTGTRC-3′, where R can be either A or G. A second DNA primer (B) was purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX). The sequence of this 15-base primer, used for the enzyme inhibition studies and some of the primer extension assays, was 5′-TAACCTTGCGGCCGT-3′. GF/A chromatography paper was from Whatman International Ltd. (Maidstone, England).

Reverse transcriptase from HIV-1

Purified recombinant HIV-1_{BH10} reverse transcriptase was obtained from the University of Alabama at Birmingham, Center for AIDS Research, Gene Expression Core Facility. Briefly the recombinant enzyme was prepared by performing site-directed mutagenesis to create a *Nco*I 5′ cloning site and adding Met-Ala to the N-terminus. A 3′ *Bam*HI cloning site and a strong stop codon were added for the C-terminus of the reverse transcriptase. The reverse transcriptase coding sequence was cloned into the T7 expression plasmid pET11d to create pGEC16. For purification of the reverse transcriptase, *E. coli* BL21(DE3) containing pGEC16 was induced with isopropyl-β-D-thiogalactopyranoside and the cells harvested 3 h post-induction. The culture pellet was disrupted by sonication and the soluble reverse transcriptase purified by ion-exchange chromatography followed by gel-filtration. Upon SDS-PAGE

analysis, the purified reverse transcriptase contained approximately 60% full length reverse transcriptase peptide (p66) and 40% processed reverse transcriptase peptide (p51).

Steady-state enzyme inhibition assays

Template · primers were prepared as described (Parker et al., 1991). To determine the effect of combinations of inhibitors on reverse transcriptase, 10 μl of a solution containing the test compound was added to 35 μl of a mixture containing the assay buffer, template · primer, and substrates. The reaction was started by the addition of 5 μl of a solution containing the enzyme. The assay on the ribosomal RNA template contained 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 3.33 μg/ml of ribosomal RNA · primer (equivalent to 7.5 nM 3′-hydroxyl primers annealed to RNA), 0.88 μM dATP, 2.8 μM dCTP, 2.8 μM dGTP, 2.5 μM dTTP, and 3 nM HIV-1 reverse transcriptase. The assay on a gapped duplex DNA template contained 50 mM Tris-HCl (pH 7.4), 125 mM KCl, 2 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 1.8 μg/ml of gapped duplex DNA, 1.4 μM dATP, 3.4 μM dCTP, 2.6 μM dGTP, 3.6 μM dTTP, and 1 nM HIV-1 reverse transcriptase. The concentrations of the template and the four natural deoxynucleotide substrates were kept constant at 3.5 times their K_m value. The ranges of the inhibitor concentrations were chosen for each combination of compounds so that the total inhibition of the enzyme was kept between 10–90%.

Extension of DNA primer annealed to 16S rRNA

The extension of a ^{32}P-labeled DNA primer annealed to *E. coli.* 16S rRNA was done as described by Parker et al. (1991). The ratio of primer to template was approximately 1 to 1. The extension of the primers by HIV-1 reverse transcriptase was done in 10 μl-mixtures containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 50 nM HIV-1 reverse transcriptase, natural deoxynucleotides and nucleotide analogs as defined in each experiment, and 0.05 mg/ml of ^{32}P-labeled primer annealed to 16S rRNA. The extension produts of the primers, separated by high voltage electrophoresis on a 20% polyacrylamide gel containing 7 M urea, were visualized by autoradiography and quantitated with the aid of a densitometer.

Results

Recombinant HIV-1 reverse transcriptase (data not shown) has similar optimal assay conditions on the ribosomal RNA template and gapped duplex DNA template as enzyme partially purified from virions (Parker et al., 1991). The major difference in the kinetic data between the enzymes was their K_m for the templates. The K_ms for both templates with the virion derived enzyme was less than that for the recombinant. The enzyme purified from the virion may

TABLE 1

Kinetic constants for recombinant HIV-1 reverse transcriptase

Substrate	K_m[a]	
	RNA Template	DNA Template
template	0.95 μg/ml	0.51 μg/ml
dATP	0.3 μM	0.4 μM
dCTP	0.8 μM	1.0 μM
dGTP	0.8 μM	0.7 μM
dTTP	0.7 μM	1.2 μM

[a]The kinetic constants were determined from linear plots of 1/V versus 1/[substrate]. Each value is the average of at least two experiments.

contain other 'accessory factors' from the virus that could promote the tighter template binding seen with this enzyme (Andreola et al., 1992). Kinetic constants for the two templates using the recombinant HIV-1 reverse transcriptase are presented in Table 1. The K_m for the deoxynucleotide substrate dTTP was similar to that found for the virion derived enzyme. For both templates, dATP had a lower K_m than that for any of the other three triphosphates.

The active antiviral metabolites of AZT, ddC, ddI, and CBV are AZT-TP, ddCTP, 2',3'-dideoxyadenosine 5'-triphosphate (ddATP), and CBV-TP, respectively (Connolley and Hammer, 1992a; De Clercq, 1992; Faulds and Brogden, 1992). All four triphosphates have been shown to be potent inhibitors of HIV-1 reverse transcriptase when tested individually for their ability to compete with the appropriate natural 5'-triphosphate as substrates for the enzyme. Using the 16S rRNA as the template, we tested combinations of AZT-TP with ddCTP, ddATP or CBV-TP for their ability to interact and consequently produce synergistic inhibition of HIV-1 reverse transcriptase. If the two inhibitors cannot bind to the enzyme at the same time, i.e., their binding is mutually exclusive, then the slope of the line of a plot of 1/V vs. the concentration of one inhibitor will be independent of the concentration of the other inhibitor (Segel, 1975). If the two inhibitors can simultaneously bind to the enzyme, i.e., their binding is nonexclusive, then the family of lines created from Dixon plots at different fixed concentrations of the second inhibitor will intersect. No assumption about the type of inhibition, e.g., competitive, mixed or noncompetitive, is necessary for these analyses of the data. The data for the combinations with the RNA template are presented in Figs. 1–3, panels A and B. The parallel lines are consistent with a mutually exclusive model of binding to the reverse transcriptase for each compound in the combination of inhibitors. Therefore, none of these combinations synergistically inhibit the RNA-directed DNA polymerase activity of HIV-1 reverse transcriptase.

These compounds could also interact synergistically to inhibit the DNA-directed DNA polymerase activity of the reverse transcriptase. This site of activity was tested using the gapped duplex DNA. The data for the system,

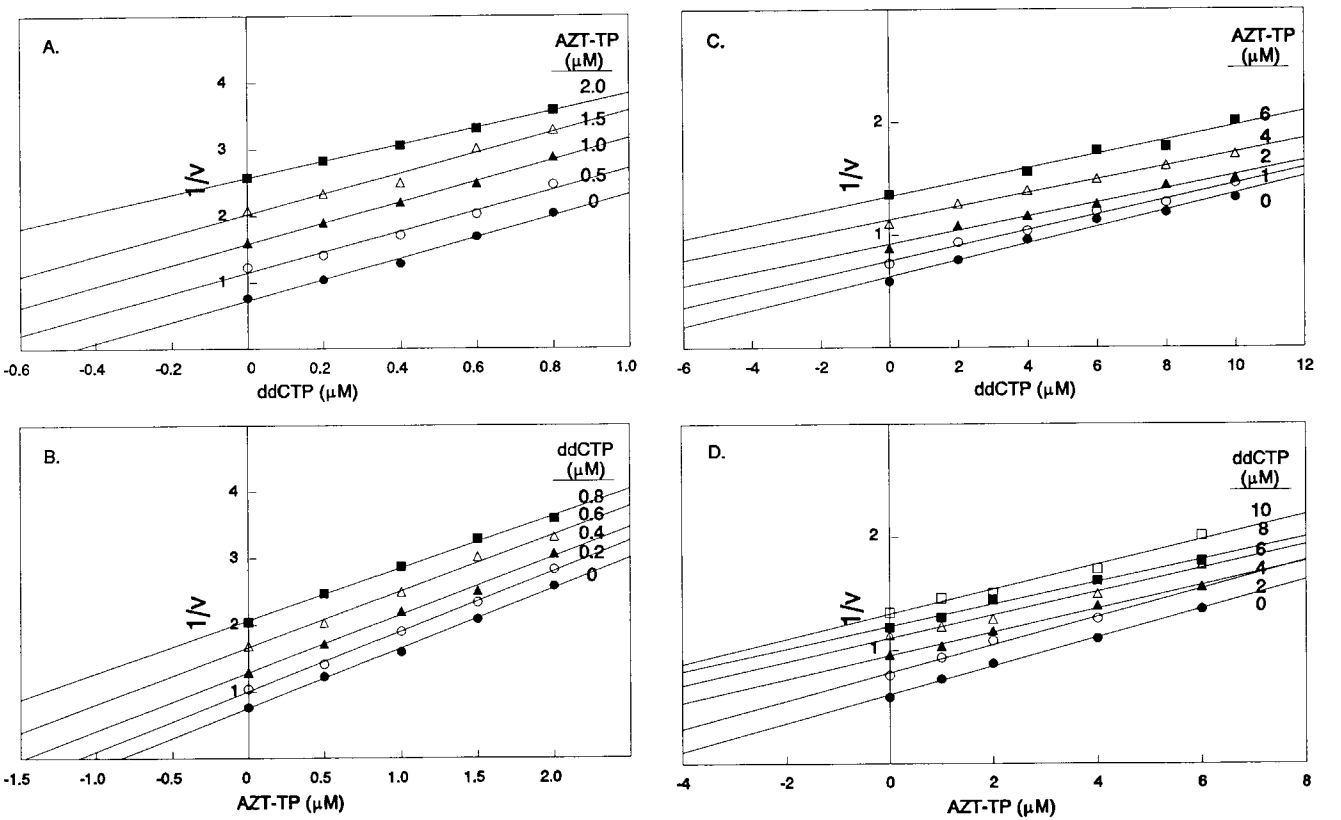

Fig. 1. Inhibition of HIV-1 reverse transcriptase by combinations of AZT-TP and ddCTP. HIV-1 reverse transcriptase was assayed by the methods described in the text using ribosomal RNA (panels A and B) or gapped duplex DNA (panels C and D) as the template. Reaction velocity (V) is expressed as picomoles of [^{3}H]dGTP (31.9 Ci/mmol) incorporated per h. Unweighted least squares method was used to fit the lines to the data.

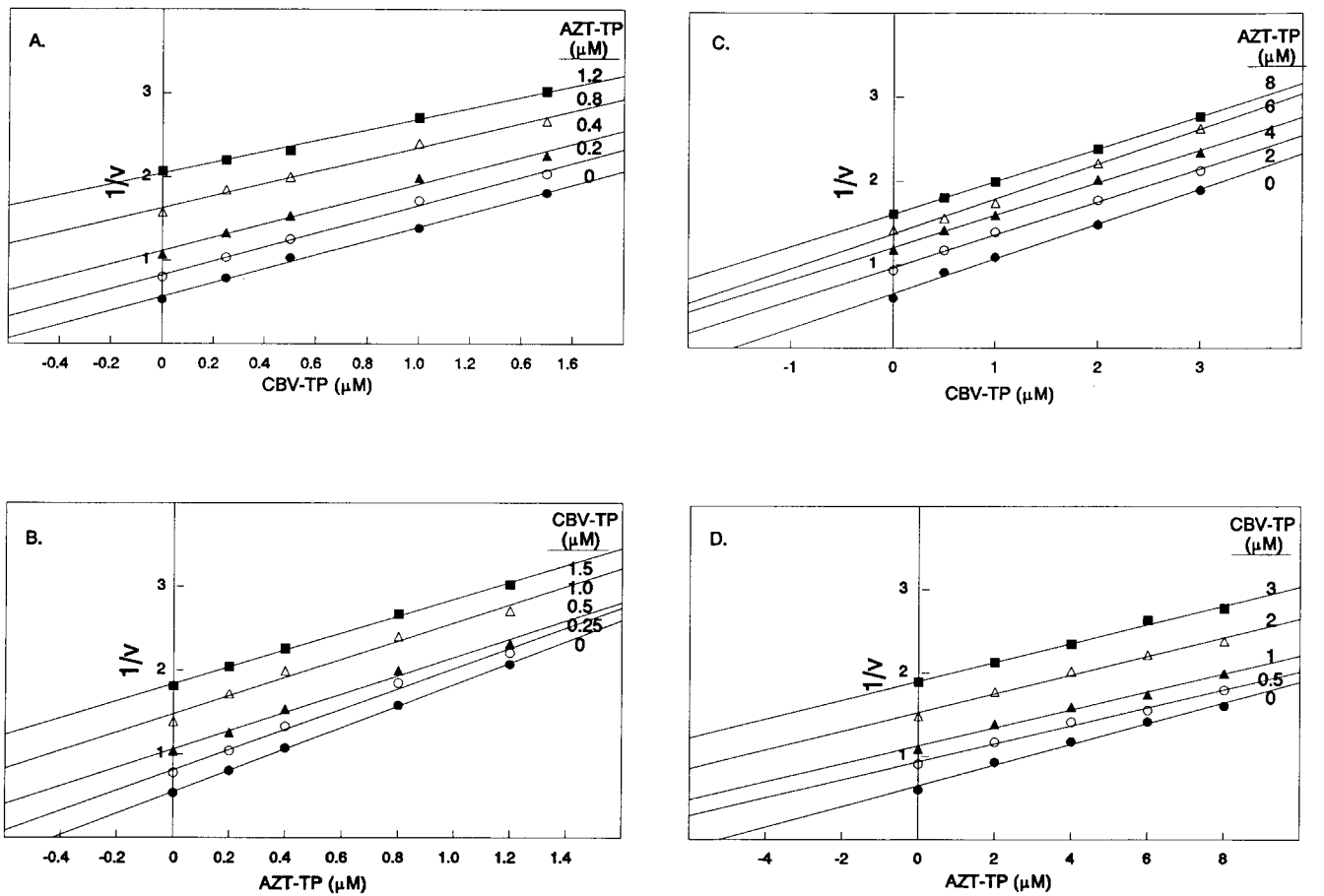

Fig. 2. Inhibition of HIV-1 reverse transcriptase by combinations of AZT-TP and CBV-TP. HIV-1 reverse transcriptase was assayed by the methods described in the text using ribosomal RNA (panels A and B) or gapped duplex DNA (panels C and D) as the template. Reaction velocity (V) is expressed as picomoles of [^{3}H]dATP (58.2 Ci/mmol) incorporated per h. Unweighted least squares method was used to fit the lines to the data.

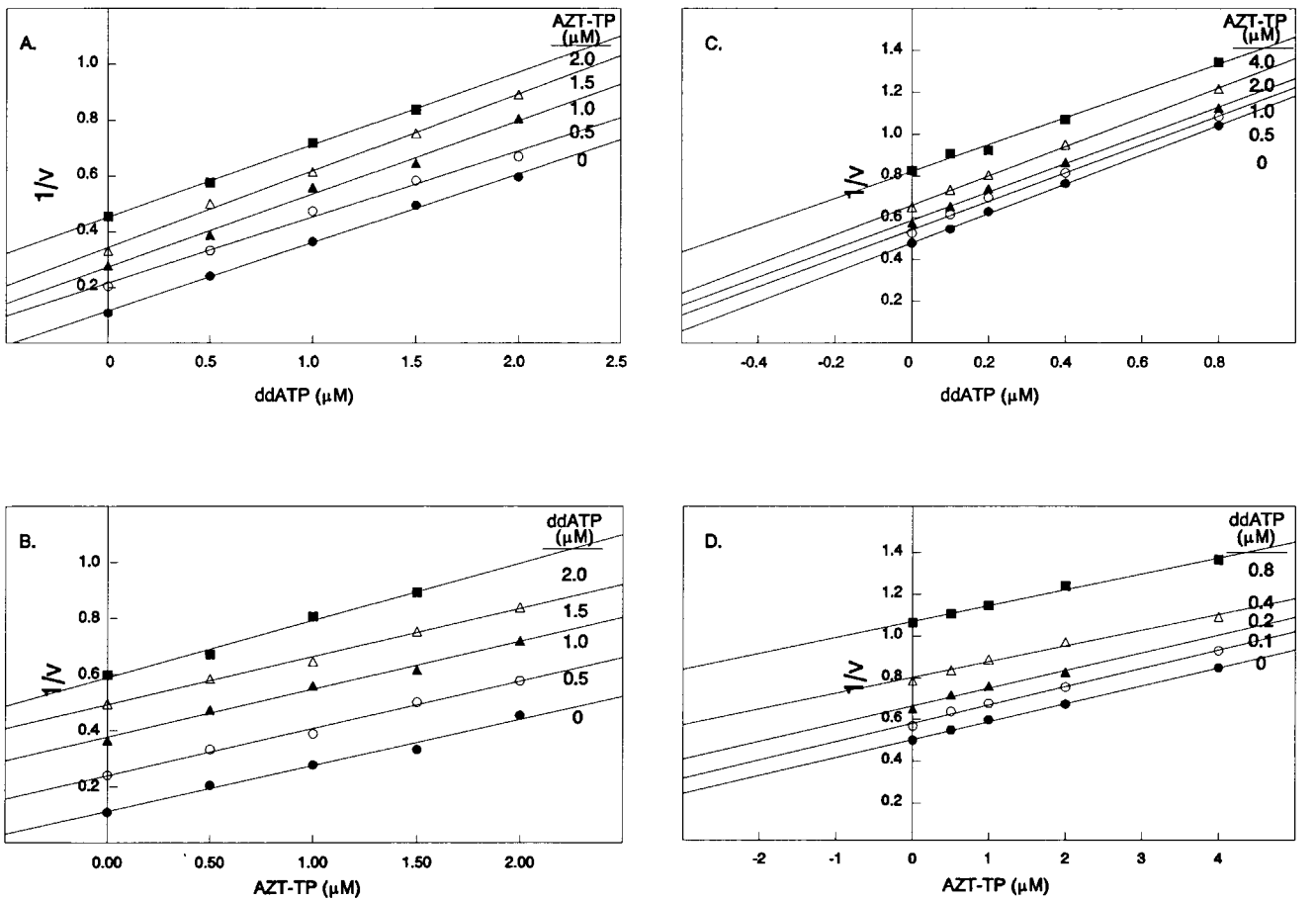

Fig. 3. Inhibition of HIV-1 reverse transcriptase by combinations of AZT-TP and ddATP. HIV-1 reverse transcriptase was assayed by the methods described in the text using ribosomal RNA (panels A and B) or gapped duplex DNA (panels C and D) as the template. Reaction velocity (V) is expressed as picomoles of [^{3}H]dGTP (31.9 Ci/mmol) incorporated per h. Unweighted least squares method was used to fit the lines to the data.

which approximates positive strand synthesis, is given in Fig. 1–3, panels C and D. Again, the parallel lines, which are definitive for mutually exclusive binding for each inhibitor in the combinations, show that none of these combinations synergistically inhibit the enzyme with this template.

Since each of these analogs has also been shown to be a substrate for the HIV-1 reverse transcriptase, addition of the analog to the growing DNA chain results in termination of chain synthesis (Parker et al., 1991; Connolley and Hammer, 1992a; De Clercq, 1992; Goff, 1990). Another way of evaluating whether their synergistic antiviral activity is a result of inhibition of the reverse

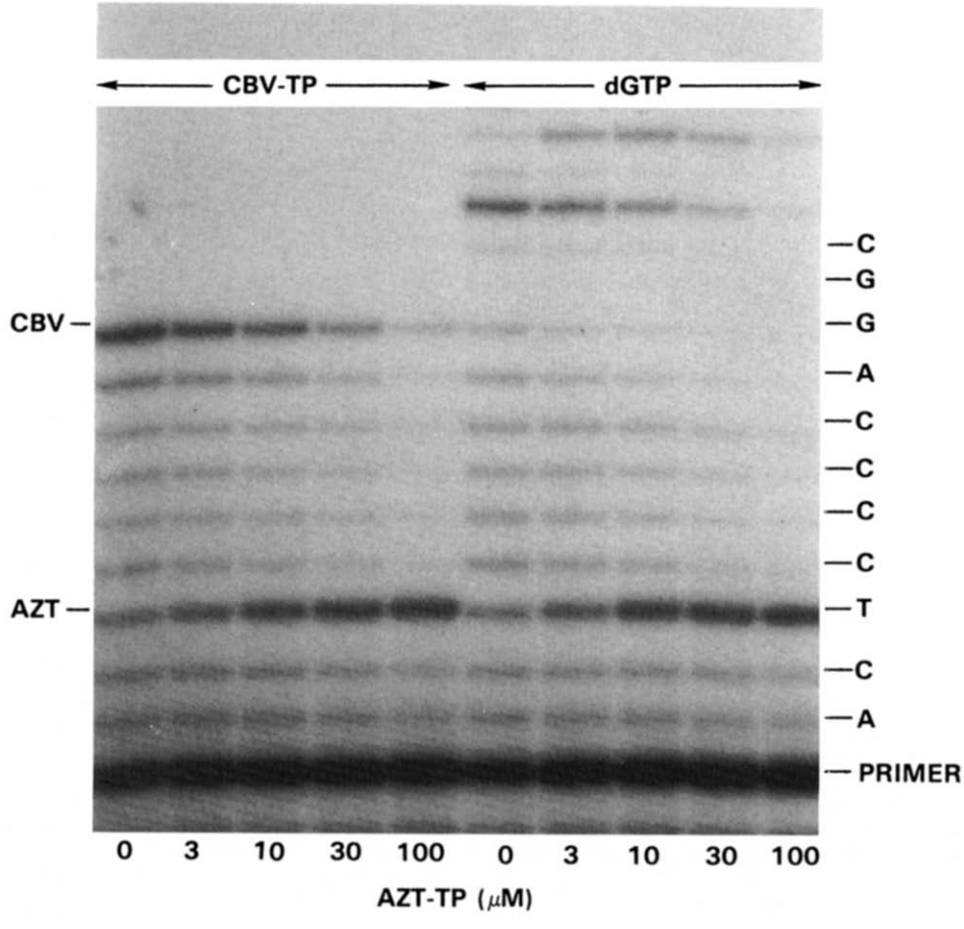

Fig. 4. Effect of CBV-TP on the incorporation of AZT-TP into DNA by HIV-1 reverse transcriptase. The enzyme was incubated with either 50 μM CBV-TP or 50 μM dGTP in the presence of AZT-TP (0–100 μM), 50 μM dATP, 50 μM dCTP, 10 μM dTTP, and the ^{32}P-labeled DNA primer (B) annealed to ribosomal RNA. The products are visualized by autoradiography after electrophoresis. The five lanes on the left contained 50 μM CBV-TP and the five lanes on the right contained 50 μM dGTP. The sequence of the nucleotides added to the primer is shown to the right of the figure.

transcriptase is to determine the effect of one analog on the incorporation of a second analog. The effect of CBV-TP on the incorporation of AZT-TP into the growing DNA chain was studied with a ^{32}P-labeled DNA primer annealed to ribosomal RNA (Fig. 4). Without CBV-TP, AZT-MP reaches maximum incorporation into the DNA chain at approximately 20 μM, and the concentration of AZT-TP at which half maximum incorporation was achieved was 4.1 ± 0.62 μM (Table 2). When 50 μM CBV-TP was present, the concentration of AZT-TP at which half maximum incorporation was achieved was the same (6.4 ± 1.4 μM). Fifty μM CBV-TP was greater than 100 times the K_i for inhibition of the enzyme or the K_m for the incorporation of CBV-MP into the growing chain with either a running or standing start (Parker et al., 1991). When other combinations of analogs were examined this way, no effect of one analog on the incorporation of another could be detected (Table 2).

Discussion

Kinetic analysis of the interaction of combinations of dideoxynucleotide analogs failed to show synergistic inhibition of HIV-1 reverse transcriptase. In assays designed to measure the effect on the steady-state inhibition of either the RNA-directed or DNA-directed DNA polymerase activity of HIV-1 reverse

TABLE 2

Effect of AZT-TP and CBV-TP on the incorporation of AZT-TP, CBV-TP, or ddCTP into DNA by HIV-1 reverse transcriptase

First Nucleotide	50% V_{max} Concentration (μM)[a]		
	AZT-TP	CBV-TP	ddCTP
dTTP	–	6.1 ± 2.5	9.7 ± 4.0
AZT-TP	–	5.9 ± 0.2	6.9 ± 4.4
dGTP	4.1 ± 0.62	–	18 ± 11
CBV-TP	6.4 ± 1.4	–	16.3 ± 4

[a]To determine the effect of AZT-TP on the incorporation of CBV-TP, HIV-1 reverse transcriptase was incubated with 50 μM of either dTTP or AZT-TP with 10 μM dGTP, 50 μM dCTP, 50 μM dATP, ^{32}P-labeled primer A annealed to rRNA, and varying concentrations of CBV-TP. To determine the effect of AZT-TP on the incorporation of ddCTP, HIV-1 reverse transcriptase was incubated with 50 μM of either dTTP or AZT-TP with 50 μM dGTP, 10 μM dCTP, 50 μM dATP, ^{32}P -labeled primer A annealed to rRNA, and varying concentrations of ddCTP. To determine the effect of CBV-TP on the incorporation of AZT-TP, HIV-1 reverse transcriptase was incubated with 50 μM of either dGTP or CBV-TP with 10 μM dTTP, 50 μM dCTP, 50 μM dATP, ^{32}P-labeled primer B annealed to rRNA, and varying concentrations of AZT-TP. To determine the effect of CBV-TP on the incorporation of ddCTP, HIV-1 reverse transcriptase was incubated with 50 μM of either dGTP or CBV-TP with 50 μM dTTP, 10 μM dCTP, 50 μM dATP, ^{32}P-labeled primer B annealed to rRNA, and varying concentrations of ddCTP. The DNA products were separated from the primer by gel electrophoresis and were visualized by autoradiography as shown in Fig. 4. The band representing the incorporation of the nucleotide analog was quantitated with the aid of a densitometer and the concentration of nucleotide required for half maximal incorporation was determined. The data are the average ± S.D. for three experiments.

transcriptase, AZT-TP and either ddATP, ddCTP, or CBV-TP showed mutually exclusive binding, indicating that only one inhibitor could bind to the enzyme at a time. In other assays designed to look at the chain termination effect of these dideoxynucleotides on DNA chain elongation, none of the analogs affected the ability of another analog to serve as a substrate for the reverse transcriptase. Although we did not test all the combinations of deoxynucleotide analogs that have been found to have synergistic antiviral activity in cell culture against HIV-1, we believe that it is reasonable to extrapolate from our results and conclude that synergistic inhibition of HIV-1 reverse transcriptase is not the basis for the synergistic antiviral activity seen in cell culture with combinations of deoxynucleotide reverse transcriptase inhibitors.

Although it is possible that the in vitro HIV-1 reverse transcriptase assay may not accurately reflect the complex cellular environment in which reverse transcription of the HIV-1 viral RNA takes place, the results presented in this paper are consistent with what is known about the structure of the reverse transcriptase (Goff, 1990; Reardon and Miller, 1990) and the catalytic functioning of other DNA polymerases (Fry and Loeb, 1986). Catalytically active HIV-1 reverse transcriptase, and the form of the enzyme believed to be present in the virion, is a heterodimer with one subunit containing an RNase H and a polymerase domain (p66) and the other subunit containing only the polymerase domain (p51) (Goff, 1990). However, extensive experimental evidence indicates that, during catalysis, deoxynucleotides bind only to the single binding site on the p66 subunit of the reverse transcriptase. A kinetic scheme for HIV-1 reverse transcriptase polymerase function has been proposed which is similar to other DNA polymerases in which only one deoxynucleoside 5′-triphosphate can occupy the substrate site at a time (Reardon and Miller, 1990). The likelihood that some unknown cellular or viral factor would change the basic catalytic functioning of the enzyme such that two deoxynucleotide analogs could bind to the enzyme at the same time seems very small.

This study leaves unanswered the question of what is the mechanism of the synergistic anti-HIV activity of combinations of two nucleotide analogs in cell culture tests. Reardon (1992) has recently calculated that for these obligate chain terminators the intracellular level of each analog deoxynucleoside 5′-triphosphate and the ratios of the natural deoxynucleotide triphosphates to the analog triphosphates are such that each compound alone is sufficiently potent to inhibit viral genome replication by greater than 80%. Given the potency of these chain terminators as single agents, it is difficult to visualize how combination drug treatment could produce a synergistic antiviral effect at the reverse transcriptase step. If one accepts the antiviral synergy seen in cell culture, then this unexplained synergy is one more piece of evidence that suggests that AZT may have a second site of action other than the inhibition of HIV-1 reverse transcriptase (Buckheit et al., 1992; Tudor-Williams and Emery, 1992; Lacey et al., 1992; Larder, 1992).

Acknowledgements

We thank Dr. Robert Vince (University of Minnesota) for providing the carbovir 5′-triphosphate through funding by his National Institutes of Health (NIH) grant RO1 CA-23263. Expression and purification of the recombinant reverse transcriptase was supported in part by the NIH Centers for AIDS Research program grant P30 AI27767 and the enzyme is available through the NIH AIDS Research and Reference Repository. This work was partially supported by NIH grant RO1 AI29157 and UO1 AI26054.

References

Ahluwalia, G., Conney, D.A., Mitsuya, H., Fridland, A., Flora, K.P., Hao, Z., Dalal, M., Broder, S. and Johns, D.G. (1987) Initial studies on the cellular pharmacology of 2′-3′-dideoxyinosine, an inhibitor of HIV infectivity. Biochem. Pharmacol. 36, 3797–3800.

Andreola, M.L., Nevinsky, G.A., Barr, P.J., Sarih-Cotton, L., Bordier, B., Fournier, M., Litvak, S. and Tarrago-Litvak, L. (1992) Interaction of $tRNA^{Lys}$ with the p66/p66 form of HIV-1 reverse transcriptase stimulates DNA polymerase and ribonuclease H activities. J. Biol. Chem. 267, 19356–19362.

Boucher, C.A.B., Lange, J.M.A., Miedema, F.F., Weverling, G.J., Koot, M., Mulder, J.W., Goudsmit, J., Kellam, P., Larder, B.A. and Tersmette, M. (1992) HIV-1 biological phenotype and the development of zidovudine resistance in relation to disease progression in asymptomatic individuals during treatment. AIDS 6, 1259–1264.

Buckheit Jr., R.W., Germany-Decker, J., Qualls-Goodwin, K., Bowdon, B.J. and Shannon, W.M. (1992) 3′-Azido-3′-deoxythymidine-induced reduction in the ability of uninfected CD4-expressing cells to participate in syncytium formation. Proc. Nat. Acad. Sci. USA 89, 8361–8365.

Chow, Y-K, Hirsch, M.S., Merrill, D.P., Bechtel, L.J., Eron, J.J., Kaplan, J.C. and D'Aquila, R.T. (1993) Use of evolutionary limitations of HIV-1 multidrug resistance to optimize therapy. Nature 361, 650–654.

Connolly, K.J. and Hammer, S.M. (1992a) Antiretroviral therapy: reverse transcriptase inhibition. Antimicrob. Agents Chemother. 36, 245–254.

Connolly, K.J. and Hammer, S.M. (1992b) Antiretroviral therapy: strategies beyond single-agent reverse transcriptase inhibition. Antimicrob. Agents Chemother. 36, 509–520.

Cox, S. (1992) Metabolism of 3′-azido-3′-deoxythymidine and 3′-fluoro-3′-deoxythymidine in combination in human immunodeficiency virus infected lymphoblastoid cells. Antiviral Chem. Chemother. 3, 165–170.

Cox, S.W., Albert, J., Wahlberg, J., Uhlen, M. and Wahren, B. (1992) Loss of synergistic response to combinations containing AZT in AZT-resistant HIV-1. AIDS Res. Hum. Retroviruses 8, 1229–1234.

De Clercq, E. (1992) HIV inhibitors targeted at the reverse transcriptase. AIDS Res. Hum. Retroviruses 8, 119–134.

Dornsife, R.E., St. Clair, M.H., Huang, A.T., Panella, T.J., Koszalka, G.W., Burns, C.L. and Averett, D.R. (1991) Anti-human immunodeficiency virus synergism by zidovudine (3′-azidothymidine) and didanosine (dideoxyinosine) contrasts with their additive inhibition of normal human marrow progenitor cells. Antimicrob. Agents Chemother. 35, 322–328.

Eron Jr., J.J., Johnson, V.A., Merrill, D.P., Chou, T.C. and Hirsch, M.S. (1992) Synergistic inhibition of replication of human immunodeficiency virus type 1, including that of a zidovudine-resistant isolate, by zidovudine and 2′,3′-dideoxycytidine in vitro. Antimicrob. Agents Chemother. 36, 1559–1562.

Faulds, D. and Brogden, R.N. (1992) Didanosine. A review of its antiviral activity, pharmacokinetic

properties and therapeutic potential in human immunodeficiency virus infection. Drugs 44, 94–116.

Fry, M. and Loeb, L.A. (1986) Animal cell DNA polymerases. CRC Press, Boca Raton, FL.

Goff, S.P. (1990) Retroviral reverse transcriptase: synthesis, structure, and function. J. Acquir. Immune Defic. Syndr. 3, 817–831.

Gotzsche, P.C., Nielsen, C., Gerstoft, J., Nielsen, C.M. and Vestergaard, B.F. (1992) Trend towards decreased survival in patients infected with HIV resistant to zidovudine. Scand. J. Infect. Dis. 24, 563–565.

Harmenberg, J., Akesson-Johansson, J., Vrang, L. and Cox, S. (1990) Synergistic inhibition of human immunodeficiency virus replication in vitro by combinations of 3′-azido-3′-deoxythymidine and 3′-fluoro-3′-deoxythymidine. AIDS Res. Hum. Retroviruses 6, 1197–1202.

Japour, A.J., Chatis, P.A., Eigenrauch, H.A. and Crumpacker, C.S. (1991) Detection of human immunodeficiency virus type 1 clinical isolates with reduced sensitivity to zidovudine and dideoxyinosine by RNA · RNA hybridization. Proc. Natl. Acad. Sci. USA 88, 3092–3096.

Lacey, S.F., Reardon, J.E., Furfine, E.S., Kunkel, T.A., Bebenek, K., Eckert, K.A., Kemp, S.D. and Larder, B.A. (1992) Biochemical studies on the reverse transcriptase and RNase-H activities from human immunodeficiency virus strains resistant to 3′-azido-3′-deoxythymidine. J. Biol. Chem. 267, 15789–15794.

Larder, B.A. (1992) 3′-Azido-3′-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 36, 2664–2669.

Mitsuya, H., Yarchoan, R. and Broder, S. (1990) Molecular targets for AIDS therapy. Science 249, 1533–1544.

Parker, W.B., White, E.L., Shaddix, S.C., Ross, L.J., Buckheit Jr., R.W., Qualls, K.J. and Shannon, W.M. (1991) Mechanism of inhibition of human immunodeficiency virus reverse transcriptase and human DNA polymereses α, β and γ by the 5′-triphosphates of carbovir, 3′-azido-3′deoxythymidine, 2′3′-dideoxyguanosine, and 3′-deoxythymidine. J. Biol. Chem. 266, 1754–1762.

Parker, W.B., Shaddix, S.C., Bowdon, B.J., Rose, L.M., Vince, R., Shannon, W.M. and Bennett Jr., L.L. (1993) Metabolism of carbovir, a potent inhibitor of human immunodeficiency virus type 1, and its effects on cellular metabolism. Antimicrob. Agents Chemother. 37, 1004–1009.

Reardon, J.E. (1992) Human immunodeficiency virus reverse transcriptase: Steady-state and pre-steady-state kinetics of nucleotide incorporation. Biochem. 31, 4473–4479.

Reardon, J.E. and Miller, W.H. (1990) Human immunodeficiency virus reverse transcriptase. Substrate and inhibitor kinetics with thymidine 5′-triphosphate and 3′-azido-3′-deoxythymidine 5′-triphosphate. J. Biol. Chem. 265, 20302–20307.

Richman, D.D. (1992) HIV drug resistance. AIDS Res. Hum. Retroviruses 8, 1065–1071.

Schinazi, R.F. (1992) Combined chemotherapeutic modalities for viral infections: rationale and clinical potential. In: T.-C. Chou and D.R. Rideout (Eds), Synergism and Antagonism in chemotherapy, pp. 109–181. Academic Press, San Diego, CA.

Schinazi, R.F., Mead, J.R. and Feorino, P.M. (1992) Insights into HIV chemotherapy. AIDS Res. Hum. Retroviruses 8, 963–990.

Segel, I.H. (1975) Multiple inhibition analysis. In: Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems. pp. 465–505. John Wiley & Sons, New York.

Shannon, W.M. (1990) Antiretroviral activity of carbocyclic nucleoside analogs. In: R.B. Diasio and J.-P. Sommadossi (Eds), Advances in Chemotherapy of AIDS, pp. 75–95. Pergamon Press, New York.

Shirasaka, T., Yarchoan, R., Obrien, M.C., Husson, R.N., Anderson, B.D., Kojima, E., Shimada, T., Broder, S. and Mitsuya, H. (1993) Changes in drug sensitivity of human immunodeficiency virus type-1 during therapy with azidothymidine, dideoxycytidine, and dideoxyinosine: An in vitro comparative study. Proc. Natl. Acad. Sci. USA 90, 562–566.

Smith, M.S., Kessler, J.A., Rankin, C.D., Pagano, J.S., Kurtzberg, J. and Carter, S.G. (1993) Evaluation of synergy between carbovir and 3′-azido-2′,3′-deoxythymidine for inhibition of human immunodeficiency virus type-1. Antimicrob. Agents Chemother. 37, 144–147.

Tudor-Williams, G. and Emery, V.C. (1992) Development of in vitro resistance to zidovudine is likely to be clinically significant. Rev. Med. Virol. 2, 123–129.

White, E.L., Parker, W.B., Macy, L.J., Shaddix, S.C., McCaleb, G., Secrist III, J.A., Vince, R. and Shannon, W.M. (1989) Comparison of the effect of carbovir, AZT, and dideoxynucleoside triphosphates on the activity of human immunodeficiency virus reverse transcriptase and selected human polymerases. Biochem. Biophys. Res. Commun. 161, 393–398.

Yarchoan, R., Klecker, R.W., Weinhold, K.J., Markham, P.D., Lyerly, H.K., Durack, D.T., Gelmann, E., Nusinoff-Lehrman, S., Blum, R.M., Barry, D.W., Shearer, G.M., Fischl, M.A., Mitsuya, H., Gallo, R.C., Collins, J.M., Bolognesi, D.P., Myers, C.M. and Broder, S. (1986) Administration of 3′azido-3′-deoxythymidine, an inhibitor of HTLV-III replication, to patients with AIDS and AIDS-related complex. Lancet 1, 575–580.

Yarchoan, R., Perno, C.F., Thomas, R.V., Klecker, R.W., Allain, J.P., Wills, R.J., McAtee, N., Fischl, M.A., Dubinsky, R., McNeely, M.C., Mitsuya, H., Pluda, J.M., Lawley, T.J., Leuther, M., Safai, B., Collins, J.M., Myers, C.E. and Broder, S. (1988) Phase I studies of 2′,3′-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). Lancet 1, 76–81.