Vol. 165, No. 1, 1989
November 30, 1989

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 401-407

# INCREASED THERAPEUTIC EFFICACY OF ZIDOVUDINE IN COMBINATION WITH VITAMIN E

Sudhir R. Gogu, Barbara S. Beckman, S.R.S. Rangan, and Krishna C. Agrawal*

Department of Pharmacology, and Delta Primate Research Center,
Tulane University School of Medicine, New Orleans, LA

Received October 9, 1989

Antiviral activity and bone marrow toxicity of 3'-azido-3'deoxythymidine (Zidovudine; AZT) was evaluated in the presence of α-D-tocopherol acid succinate (ATS) in the MT4 cell line and in murine hematopoietic progenitor cells, respectively. At varying concentrations (.016 to .125 μM) of AZT, addition of ATS (5 to 15 μg/ml) showed a dose-dependent increase in anti-HIV activity. The $ED_{90}$ of AZT in this test system was 0.37 μM, whereas in the presence of ATS (15 μg/ml) it was 0.06 μM, thus producing an approximately 6-fold increase in anti-HIV activity. In contrast, in murine bone marrow cells, ATS (4 μg/ml) showed significant protection($p<0.05$) against AZT-induced toxicity as measured by CFU-E and CFU-GM assays. The $IC_{50}$ values in the presence and absence of ATS for CFU-E were 3.7 and 1.5 μM, whereas for CFU-GM were 6.0 and 2.7 μM, respectively. Overall, these data suggest that AZT in combination with ATS has greater therapeutic efficacy against HIV-1. 

Zidovudine (AZT), an inhibitor of the activity of reverse transcriptase of human immunodefiency virus, is at the present time the only FDA approved drug available for the treatment of acquired immunodeficiency syndrome (AIDS) (1). Significant dose-related toxicity has been associated with the administration of AZT to patients with AIDS (2-5). Anemia and neutropenia were the most common toxicities observed in patients receiving various doses of AZT (6-8). AZT has been reported to inhibit human erythroid and granulocyte-macrophage colony formation in vitro (9-11).

A recent review on nutrition suggests that megadoses of vitamin E (α-D-tocopherol), selenium and zinc seem to restore cell mediated immunity by increasing T-cell number and their activity (12). Vitamin E, a fat soluble vitamin, has been demonstrated to have multiple effects on the body including effects on the hematopoietic and immune systems (13,14). Furthermore, vitamin E has been shown to increase the half-life of erythrocytes and to cause an erythropoietic response in anemic patients (15-16). In addition, because of its antioxidant properties,

*To whom correspondence should be addressed.

The abbreviations used are: AZT, Zidovudine; ATS, α-D-tocopherol acid succinate; CFU-E, colony forming units-erythroid; CFU-GM, colony forming units granulocyte-macrophage; HIV, human immunodeficiency virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

0006-291X/89 $1.50

vitamin E has a significant role in protecting a wide variety of cells from chemically induced injuries (17-18).

We have initiated studies with vitamin E in an effort to explore its interaction with cell membrane phospholipids that may lead to changes in cell permeability, thereby allowing increased diffusion of drugs such as AZT into the target cells (19). In this report, we describe the ability of ATS, a relatively more soluble derivative of vitamin E to increase anti-HIV activity of AZT in a human lymphocytic cell line, MT4 (20) and to protect AZT-induced bone marrow toxicity in murine hematopoietic progenitor cells in vitro as measured by clonogenic assays.

## MATERIAL AND METHODS

Cells. MT4 cells susceptible to cell-killing by HIV-1 were maintained in RPMI-1640 supplemented with 15% fetal bovine serum (heat inactivated), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2.0 mM). Cells were kept in logarithmic growth phase by sub-passaging at intervals of 3 to 4 days at a concentration of $2x10^5$ cells/ml. All cultures were incubated at 37°C in 5% $CO_2$ and 95% humidified air in an incubator.

Virus. HTLV-III 3b isolate of HIV, designated as HIV-1, was grown in H9 cells. Cell free virus in culture supernatant was harvested at low-speed (1000 g for 10 min) centrifugation 5 to 7 days following infection when the virus-induced syncytial cytopathic effect was maximal. Aliquots of the virus preparation were kept at -70°C.

Mice. Male CD-1 white mice (Charles River) 6-8 weeks old were used and all the mice were caged under viral free condition. Femurs were obtained from these mice.

Drugs. Zidovudine (AZT) was synthesized in our laboratory. Human recombinant erythropoietin was supplied by AmGen Corp. (Thousand Oaks, CA.). L-cell conditioned medium was a kind gift of Dr. Richard Shadduck of Montefiore Hospital (Pittsburg, PA). ATS was obtained from Sigma Chemical Co. (St. Louis, MO). ATS solution was prepared by dissolving in ethanol at a concentration of 10 mg/ml. This solution was diluted directly in RPMI medium to produce the final desired concentration. Vehicle control had the same concentration of ethanol in RPMI medium.

Cytotoxicity. Cytotoxic studies were initially carried out in the MT4 cell line by employing conditions identical to the antiviral assay. Approximately $2x10^5$ cells/ml in RPMI-1640 were plated in 10 ml test tubes in the presence of various concentrations of ATS (5,7.5,10 and 20 µg/ml) incubated at 37°C for 7 days in 5% $CO_2$ and 95% humidified air. The medium was changed at day 3 and 5 containing respective concentrations of ATS. Viability was determined at day 7 by trypan blue dye exclusion and percent viability was calculated.

In Vitro Inhibition of HIV-1 Replication. MT4 cells ($2x10^5$ cells/ml) were incubated in growth medium in the presence and absence of various concentrations (5, 10, and 15 µg/ml) of ATS. The cells were harvested 48 hr later and resuspended at $2x10^5$ cells/ml in medium containing twice the initial concentration of ATS. Each cell suspension was then distributed in 100 µl in 96 well plates in triplicates containing 100 µl of twice the concentration of AZT under study. Following a 4 hr incubation at 37°C the microtiter plate was centrifuged at 1000 x g for 10 min. The supernatant fluid from each well was carefully removed by vacuum aspiration and replaced with 50 µl of medium containing 4 times the original con-

centration of ATS, 50 µl of medium containing 4 times the concentration of AZT, followed by 100 µl of HIV containing $10^4$ infectious units (21). One set of triplicate control wells received 50 µl AZT-free medium followed by 100 µl of HIV and another set 150 µl medium. One-half of the culture fluid from the wells was removed on day 3 post-infection and replaced with an equal volume of the respective medium. The anti-HIV activity was assessed by measuring cell viability in the control and experimental wells by a colorimetric assay (22). On day 7, 100 µl of culture supernatant was removed from all wells and 10 µl of filtered stock of MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide} in phosphate buffered saline (5 mg/ml) was added. After 4 hr at 37°C the supernatant fluids were carefully removed and replaced with 150 µl of acid-isopropanol (0.04 N HCL in isopropanol). Following thorough mixing of the dark blue formazan crystals formed and their complete dissolution at room temperature the optical densities (OD) in each well was recorded at a wavelength of 540 nm with a Titertek-Multiskan plate reader. The percent viability of cells in each group was obtained from the ratio of the mean OD to that of control cells maintained in the presence and absence of ATS and AZT.

Preparation of Murine Bone Marrow Cells. Femurs were aseptically removed after anesthetizing CD-1 mice with ether, and marrow was flushed with a 25x5/8 gauge needle. A single cell suspension was made with a pipette after repeated aspirations. The cells were resuspended in RPMI-1640 at a final concentration of $1x10^6$ cells/ml.

CFU-E Assay. Methylcellulose solutions (1.3%) were prepared in RPMI-1640 containing L-glutamine (2.0 mM), fetal bovine serum (20%), 2-mercaptoethanol (0.1 mM), penicillin and streptomycin (100 U/ml and 100 µg/ml) and erythropoietin (200 mU/ml). Bone marrow cells ($1x10^6$/ml) were diluted 1:10 in the methylcellulose mixture. One milliliter of cell suspension ($1x10^5$ cells/ml) was plated in duplicate in 35 mm petri dishes and incubated in a 5% $CO_2$ and 95% humidified air at 37°C for 48 hr. The plates were stained with 3,3'-diaminobenzidine and colonies were counted containing 8 or more benzidine positive cells under an inverted microscope (Olympus, Tokyo).

CFU-GM Assay. Methylcellulose solutions (3%) were prepared in RPMI-1640 containing L-glutamine (2.0 mM), fetal bovine serum (15%), 2-mercaptoethanol (0.1 mM), penicillin and streptomycin (100 U/ml and 100 µg/ml) and L-cell condition medium (15%). Bone marrow cells ($1x10^6$/ml) were diluted 1:10 in the methylcellulose mixture. One milliliter of cell suspension ($1x10^5$cells/ml) was plated in duplicate in 35 mm petri dishes incubated in a 5% $CO_2$ and 95% humidified air at 37°C for 7 days. Colonies which contained 50 or more cells were counted under an inverted microscope.

## RESULTS AND DISCUSSION

The cytotoxicity of ATS was initially determined in MT4 cells under the conditions employed for the antiviral assay. The data indicate that ATS at the maximum concentration of 20 µg/ml was relatively toxic to MT4 cells since the number of viable cells declined at each time interval of 3,5 and 7-days (Fig. 1). The lower concentrations of ATS at 5 to 10 µg/ml seemed to increase the proliferative rate of MT4 cells especially at 5 and 7-day intervals.

The anti-HIV-1 activity in MT4 cells was determined by measuring the viability of the survived cells from HIV-1 induced cytolysis after a 7-day incubation in the presence and absence of AZT and ATS. For combination studies, the MT4 cells were pretreated with ATS at various concentrations for 48 hr before the infection with HIV-1. The viability was assessed by a colorimetric assay which

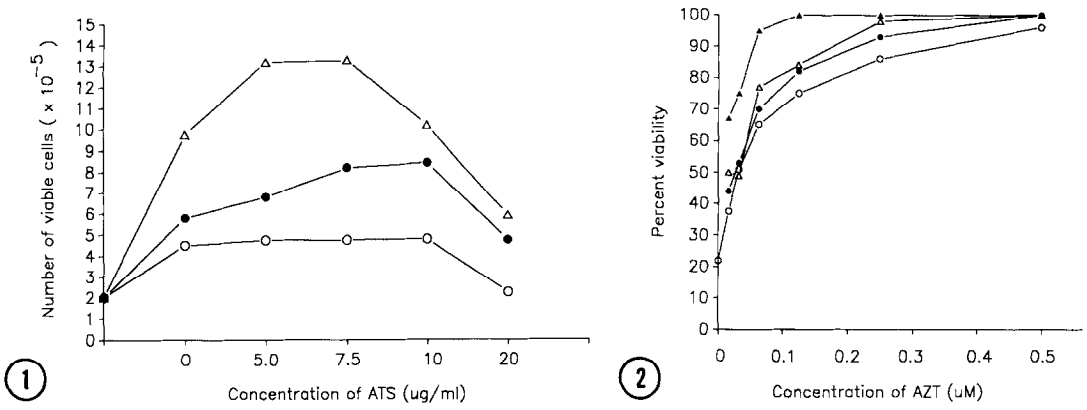


Fig. 1 Cytotoxicity in MT4 cells in the absence and presence of various concentrations of ATS. Number of viable cells were counted at day 3 (○–○) day 5 (●–●), and day 7 (△–△) by trypan blue dye exclusion.

Fig. 2 Anti-HIV-1 activity in MT4 cells as measured by the viability of the survived cells determined by metabolic reduction of MTT: AZT (○–○); AZT (0.0-0.5 μM) in the presence of various concentrations of ATS, 5 μg/ml (●–●), 10 μg/ml (△–△) 15 μg/ml (▲–▲). All points were in triplicate and repeated at least twice.

is based upon the metabolic reduction of MTT to yield a blue colored formazan product. ATS in combination with AZT significantly increased the anti-HIV-1 activity in a dose-dependent manner under these conditions (Fig. 2). However, ATS by itself had no significant antiviral effect except that at 15 μg/ml it showed minimal anti-HIV activity (data not shown). The $ED_{90}$ of AZT in this test system was 0.37 μM, whereas in the presence of ATS (15 μg/ml), it was .06 μM, an approximately 6-fold increase in the anti-HIV-1 potency of AZT. These data suggest that AZT in combination with ATS enhances the inhibition of HIV-1 synergistically. To our knowledge, this is the first report to indicate a synergistic enhancement of anti-HIV activity of AZT which may lead to a substantial therapeutic gain in combating AIDS.

The effects of AZT in combination with ATS on murine bone marrow progenitor cells were measured by CFU-E and CFU-GM assays (Figs. 3 and 4). The data indicate that the formation of CFU-E and CFU-GM was significantly higher ($p<0.05$) in the cells treated at various concentrations of AZT in the presence of ATS (4 μg/ml) than in those cells which were treated with AZT alone. The $IC_{50}$ values of AZT in this system were 1.5 and 2.7 μM, whereas in the presence of ATS the values were increased significantly to 3.7 and 6.0 μM, respectively. These data demonstrate that ATS can also protect the bone marrow from AZT-induced toxicity to a limited extent.

Although several reports have been published on the toxicity of AZT to hematopoietic progenitor cells (6,9-11,23) the amelioration of bone marrow toxi-

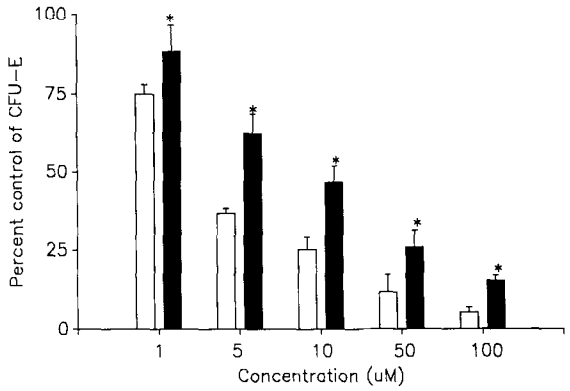


Fig. 3 Effect of AZT (1-100 μM) on murine hematopoietic progenitor cell colony formation by CFU-E in the absence ( □ ) and presence ( ■ ) of ATS (4 μg/ml). Columns represent the mean percentage control of CFU-E colonies in four separate experiments and each experiment was performed in duplicate; bars represent the standard error (P <0.05) when compared to control. Statistical analysis was done by Student's "t" test.

city with ATS has not been reported previously. Sommadossi et al. (11) have recently reported that uridine reverses the AZT-induced toxicity in human granulocyte-macrophage progenitor cells without impairment of anti-viral activity. Our results, however, suggest that vitamin E in combination with AZT not only reduces the bone marrow toxicity approximately two fold but also increases anti-HIV activity 6-fold in MT4 cells.

It is likely that the synergistic increase in anti-HIV activity by ATS may not be specific for AZT, but the other 2',3'-dideoxynucleosides and non-

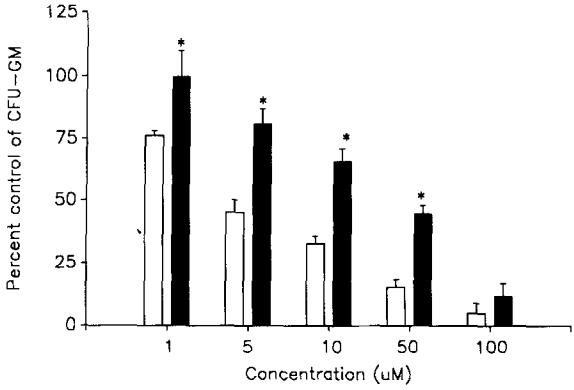


Fig. 4 Effect of AZT (1-100 μM) on murine hematopoietic progenitor cell colony formation by CFU-GM in the absence ( □ ) and presence ( ■ ) of ATS (4 μg/ml). Columns represent the mean percentage control of CFU-GM colonies in four separate experiments and each experiment was in duplicate; bars represent the standard error (P <0.05) when compared to control. Statistical analysis was done by Students "t" test.

nucleoside anti-HIV agents may also demonstrate similar response. We have, therefore, initiated a systematic study to determine the role of ATS in increasing the antiviral activity of other anti-HIV drugs. The mechanism(s) associated with the synergistic increase in anti-HIV activity may involve either interactions of ATS with cellular membrane phospholipids thereby increasing diffusion of the drugs into the target cells (19,24) and/or modulation of glycosylation resulting in altered glyconjugate biosynthesis and expression of glycoproteins (25). Thus, a combination of AZT and ATS may cause a sequential blockade in HIV replication due to the inhibition of reverse transcriptase activity by AZT and modulation of glycosylation of viral proteins by ATS during the assembly of virus particles. In contrast, the mechanism(s) associated with the reversal of AZT-induced bone marrow toxicity may involve either increased rate of cellular proliferation or activation of erythropoietin. The results in this report demonstrate that the combination of AZT with vitamin E (ATS) might provide a drug regimen with a significant increase in therapeutic efficacy of AZT for the treatment of patients with AIDS. The precise mechanism(s) involved in achieving higher anti-HIV-1 activity and in the amelioration of AZT-induced bone marrow toxicity by this combination of AZT with ATS will be further investigated.

ACKNOWLEDGMENTS: This work was supported by a PHS grant AI-25909 and in part by AI-27674 awarded by the National Institute of Allergy and Infectious Diseases, DHHS. We thank Ms. Ann Impey for technical assistance in conducting antiviral activity assays.

REFERENCES

1. Yarchoan, R., Weinhold, K.J., Lyerly, H.K., Glemann, E., Blum, R.M., Shearer, G.M., Mitsuya, H., Collins, J.M., Myers C.E., Klecker, R.W. Merkham, P.D., Durack, D.T., Lehrman, S.N., Barry, D.W., Fischl, M.A., Gallow, R.C., Bolognesi, D.P., Border, S. (1986) Lancet 1:575-580.

2. Eickhoff, T.C. (1988). Ann. Intern. Med. 108:460-469.

3. Fischl, M.M., Richman, D.D., Grieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E. Mildvan, D., Schooley, R.T., Jackson, G.G., Durack, D.T., King, D. (1987) N. Engl. J. Med. 317: 185-191.

4. Richman, D.D., Fischl, M.A., Grieco, M.H., Gottlieb, M.S. Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E. Mildvan, D., Hirsch, M.S., Jackson, G.G., Durack, D.T., Lehrman, S.N. (1987) N. Engl. J. Med. 317:192-197.

5. Jackson, G.G., Paul, D.A., Falk, L.A., Rubenis, M., Despotes, J.C., Mack, D., Knigge, M., Enison, E.E. (1988) Ann. Intern. Med. 108:175-180.

6. Ruprecht, R.M., O'Brien, L.G., Rossoni, L.D., Lehrman, S.N. (1987) Nature 325:467-469.

7. Mitsuya, H. and Border, S. (1987) Nature 325:773-778.

8. De Vita, V.T., Border, S., Fauci, A.S., Kovacs, J.A., Chabner, B.A. (1987) Ann. Intern. Med. 106:568-581.

9. Sommadossi, J.P., Zhu, Z., Carlisle, R. (1987) Pro. Am. Assoc. Cancer Res. 28:329-331.

10. Sommadossi, J.P., and Carlisle, R. (1987) Antimicrob. Agents Chemother 31:452-454.

11. Sommadossi, J.P., Carlisle, R., Schinazi, R.F., Zhou, Z. (1988) Antimicrobial Agents Chemother 32:997-1001.

12. Dwyer, J.T., Bye, R.L., Holt, P.L. Lauze, S.R. (1988) Nutrition Today. March/April, p. 30.

13. Corash, L., Spielberg, S., Barsocas, C., Boxer, L., Steinberg, R., Sheetz, M., Eagan, M., Schlesseleman, J. and Schulman, J.D. (1980) N. Engl. J. Med. 303:416-420.

14. Barker, M.O., and Brin, M. (1975) Archiv. Biochem. Biophys. 166:32-40

15. Spielberg, S.P., Boxer, L.A., Corash, L.M., Schulman, J.D. (1979) Ann. Intl. Med. 90:53-54.

16. Drake, J.R., Fitch, C.D. (1980) Am. J. Clin. Nutr. 33:2386-2393.

17. Summerfield, F.W., and Tappel, A.L. (1984) Mutat. Res. 126:113-116.

18. Pascoe, G.A., Olafsdottir, K. and Reed, D.J. (1987) Arch. Biochem. Biophys. 256:150-158.

19. Gogu, S.R., Aggarwal, S.K., Rangan, S.R.S. Agrawal, K.C. (1988) Blood 72:1319.

20. Harada, S., Kovanagi, Y., and Yamamoto, N. (1985) Science 229:563-566.

21. Mitsuya, H., Weinhold, K.S., Furman, P.A., clair, St. M.H., Lehrman, S.N., Gallow, R.C., Bolognesi, D., Barry, D.W., Broder, S. (1985) Proc. Natl. Acad. Sci. USA 82:7096-7100.

22. Schwartz, O., Henin, Y., Marechal, V., and Montagnier, L. (1988) Aids Res. & Human Retrovirus 4:441-448.

23. Gogu, S.R., Beckman, B.S., Agrawal, K.C. (1989). Life Sciences 45:iii-vii.

24. Pascoe, G.A., and Reed, D.J. (1987). Arch. Biochem. Biophys. 256:159-166.

25. Yogeeswaran, G., and Gogu, S.R. (1988) Fed. Proc. 72:A638.