Vol. 154, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1988 Pages 124-129

EFFECTS OF 3'-AZIDO-3'-DEOXYTHYMIDINE ON THE DEOXYNUCLEOTIDE TRIPHOSPHATE POOLS OF CULTURED HUMAN CELLS

Lloyd W. Frick*, Donald J. Nelson,* Marty H. St. Clair†, Phillip A. Furman†, and Thomas A. Krenitsky*

*Departments of Experiment Therapy and †Virology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Received June 1, 1988

Summary : The effects of 3'-azido-3'-deoxythymidine (AZT) on the deoxynucleotide pools of three human cell lines, HL-60, H-9, and K-562, were determined.The corresponding ED_{50}s for inhibition of cell growth were 670, 100, and 100 μM AZT. In all three lines, exposure to 200 μM AZT caused dTTP and dGTP initially to fall and then to return towards control levels. In contrast to a previous report [Furman et al., (1986) Proc. Nat. Acad. Sci. USA **83**, 8333-8337], dCTP levels increased.Pools of dATP were relatively unchanged. Qualitatively similar chages occurred in 10 μM AZT, but recovery was faster than at 200 μM AZT. After 24 hrs incubation with 200 μM AZT, AZT-5'-MP reached 2.8, 4.7, and 15.7 mM in the HL-60, H-9, and K-562 cells, respectively. When HL-60 and K-562 cells incubated in AZT were resuspended in fresh medium, AZT-5'-MP pools declined with respective $t_{1/2}$ values equal to 34 and 68 min. The concentration of thymidine, and to a lesser extent deoxyuridine, increased in the media of treated cells. AZT-5'-MP was found in the media of cells treated with AZT. © 1988 Academic Press, Inc.

3'-azido-3'-deoxythymidine (AZT, zidovudine, Retrovir®, 1-ß-D-(3'-azido-2',3'-dideoxy)ribofuranosylthymine, BW A509U) is useful for the treatment of human immunodeficiency virus (HIV) infections (1). However, AZT interferes with the maturation of bone marrow stem cells *in vitro* (2) and causes megaloblastic anemias in some patients (1). An earlier report from these laboratories noted that 50 μM AZT appeared to cause a marked decline of the dTTP and dCTP pools of the human T cell line, H-9 cells (3). The decline of dTTP was attributed to inhibition of thymidylate kinase, EC 2.7.4.9, by AZT-5'-MP, but the decline of dCTP was at variance with theories of the regulation of ribonucleotide reductase, EC 1.17.4.1 (4, 5). The latter enzyme is unaffected by AZT or its anabolites (6).

We have extended our investigation of the effects of AZT on deoxynucleotide metabolism to include HL-60 cells, which are derived from precursors of the granulocyte-macrophage lineage (7), and K-562 cells, which can synthesize hemoglobin and may be a model of the erythroblastic lineage (8). We have included an analysis of changes in the culture medium caused by AZT and investigated temporal aspects of AZT-induced perturbations of deoxynucleotide metabolism.

MATERIALS AND METHODS

Cells and Cell Culture. HL-60 and K-562 cells were obtained from the Tissue Culture Facility of the Lineberger Cancer Center at the University of North Carolina at Chapel Hill. H-9 cells were obtained from B. Hampar (National Cancer Institute, Frederick Cancer Center). Cells were cultured in RPMI-1640 (Gibco) supplemented with penicillin (50 U/ml), streptomycin (50

0006-291X/88 $1.50
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μg/ml), and 10% fetal calf serum (Hyclone). Cell numbers were determined by counting in a hemocytometer, using trypan blue or Erythrocin B as an indicator of viability. Cultures were free from mycoplasma (Gen-Probe, San Diego, California). Cell volumes of log-phase cells were determined using tritiated water and [^{14}C]sucrose obtained from New England Nuclear and dibutylphthalate from Fisher Chemical (9). The ED_{50} of AZT on growth was determined by incubation of cells ($1x10^5$ cells/ ml) with different concentrations of AZT for three days.

Preparation of Extracts. Cell cultures were split the day before drug treatment. The initial cell density was $150\text{-}450x10^3$ cells/ml. Cells were harvested by centrifugation and metabolites extracted overnight with 5 ml of 60% methanol at -20°C. After centrifugation to remove cell debris, the solvent was removed under reduced pressure and the dried extracts were resuspended in 200 μl of deionized water. Samples were stored frozen at -20°C before use. Samples of the culture media were obtained after centrifugation and were immediately ultrafiltered (Centrifree Micropartition System, Amicon) and stored frozen at -20°C.

Quantification of Metabolites . All metabolites were quantified by HPLC, using UV detectors set at 254 and 280 nm. Extracts were loaded on a SAX column (Whatman Partisil 4.6x250 mm, 10μ) and eluted with a gradient of ammonium phosphate (pH 3.5) in 7% ethanol. The gradient for AZT-5'-MP was 10 to 700 mM over 50 min and that for dNTPs was 300 to 700 mM over 50 min. AZT-5'-MP eluted at about 11 minutes. Deoxyribonucleotide triphosphates were measured using a modified periodate procedure (10, 11). The extract of 10 to 50 million cells was used to determine the dNTPs and one fifteenth of that amount to determine AZT-5'-MP. Cells from duplicate cultures were analyzed separately and the average values reported. Pools of dTTP and dCTP from H-9 cells treated with 50 μM AZT were also measured by the DNA polymerase method as described (12), except that synthetic template-primers were used (P. A. Sherman and J. A. Fyfe, manuscript in preparation). Media (200μl) were analyzed by reverse-phase HPLC (Rainin Microsorb, 4.6x250 mm, 5μ) using a gradient of acetonitrile (1-20% over 20 minutes) in ammonium acetate (pH 4.0, 100 mM). The peak areas of metabolites were digitized and integrated by computer (Digital Specialties, Chapel Hill, NC) and the concentrations determined by comparison to standards.

RESULTS

After 72 hrs, the average (n=2) ED_{50} values of AZT on growth were 670, 110, and 100 μM for the HL-60, H-9, and K-562 cells, respectively. The corresponding cell volumes were 0.55, 0.48, and 1.2 μl/million cells. Concentrations of dNTPs in control cells are presented in Table 1. The average coefficient of variation of the duplicate cultures was 7%, but when data obtained on different days were compared, the coefficient of variation was 27% (not shown).

Incubation in media containing 200 μM AZT caused substantial fluctuations in the dNTP pools. An example of the chromatographic data obtained for H-9 cells is shown in Figure 1. Figure 2 shows the changes in the dNTP pools for all three cell lines as a function of time after the addition of 200 μM AZT. Although there were differences among the cell lines in the degree to which they were affected by AZT, the qualitative changes were similar. Under these conditions, the concentrations of AZT-5'-MP after 24 hrs of incubation were 2.8, 5.7, and 15.7 mM in the HL-60, H-9, and K-562 cells, respectively. Equivalent results were obtained in at least two other similar experiments for each cell line. There was also an excellent correlation

Table 1. Deoxynucleotide Triphosphate Concentrations (μM) in Untreated Controls

Cell Line	dCTP	dTTP	dATP	dGTP
HL-60	16 (±0.8)	58 (±2.9)	50 (±1.7)	19 (±1.8)
H-9	44 (±2.1)	150 (±14)	71 (±1.3)	29 (±2.1)
K-562	24 (±0.2)	120 (±12)	57 (±6.2)	15 (±0.6)

Average of two determinations (±S.E.)

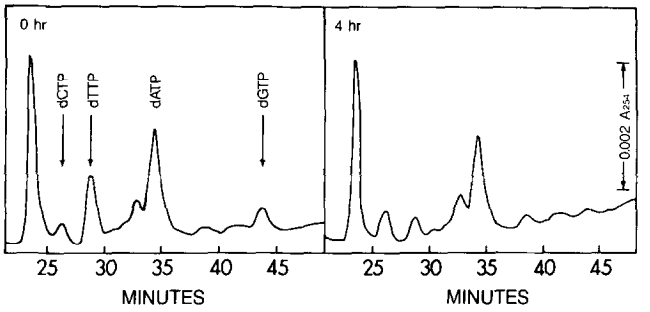

Figure 1. HPLC chromatograms of H-9 cell extracts.
a) Control culture b) Culture exposed to 200 μM AZT for 4 hrs. The figure shows the absorbance at 254 nm.

between data from the HPLC and DNA polymerase assays of the time-dependent effects of 50 μM AZT on the dTTP and dCTP pools of H-9 cells. The differences between the means for each assay were < 25%.

In each instance, pools of dTTP decreased on exposure to 200 μM AZT. In HL-60 cells, this decrease was slight and the lowest concentration of dTTP at 2 to 4 hr was equal to nearly 75% of the control value. Levels were close to normal by 8 hrs. H-9 cells experienced a more pronounced and more prolonged decline of dTTP to 30% of control values between 4 and 8 hr, with some recovery apparent by 24 hrs. In K-562 cells, dTTP dropped to less than 20% of control before returning to near-normal by 24 hrs.

AZT caused dCTP to increase in all cell lines. HL-60 cells experienced an initial increase of dCTP to 40% above control, followed by a return to control by 24 hrs. In H-9 cells, dCTP had increased nearly two-fold by 24 hrs. In K-562 cells, dCTP increased nearly six-fold by 8 hrs before declining slightly by 24 hrs.

Pools of dATP were hardly affected by AZT, but pools of dGTP were markedly decreased. After four hrs of incubation, dGTP in HL-60 cells dropped to about 50% of control values before returning to normal. In H-9 cells, the dGTP pool declined to less than 20% of control after 4 hrs of incubation and then rebounded to 40% of control by 24 hrs. In K-562 cells, dGTP became undetectable (< 2 μM) by 4 hrs, but returned to 40% of control by 24 hrs.

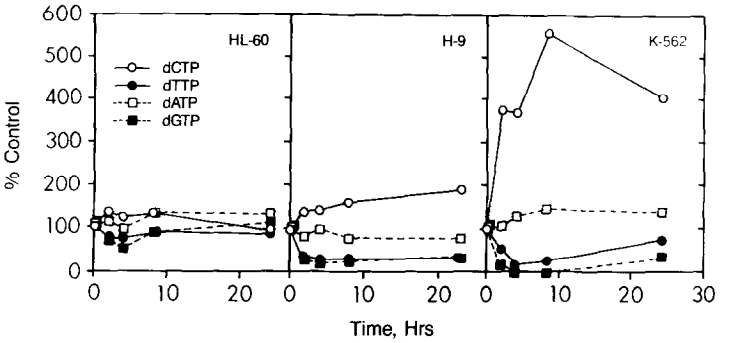

Figure 2. The effects of 200 μM AZT on dNTP pools.
Values are the averages of duplicate cultures and are given as per cent of the controls in Table 1.

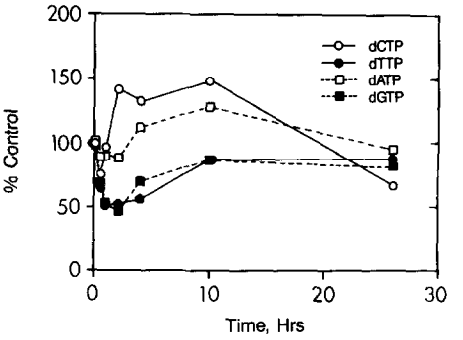

Figure 3. The effects of 10 μM AZT on the dNTP pools of K-562 cells

Incubation of K-562 cells with 10 μM AZT caused changes similar to those observed at 200 μM AZT, except that the effects were of lesser magnitude and duration (Figure 3). Pools of dTTP and dGTP fell to 50% of control by 2 hrs, but returned to control within 10 hrs. Pools of dCTP increased to 50% over control by 10 hrs, before declining to slightly less than control by 26 hrs. At this level of drug, AZT-5'-MP reached 1.6 mM at 8 hrs before declining to 0.8 mM at 26 hrs.

The media of cells treated with 200 μM AZT had higher levels of deoxyuridine and thymidine than did media from controls. At 24 hrs, thymidine was 7.5, 2.8, and 5 μM in media of HL-60, H-9, and K-562 cells, respectively. Without AZT, HL-60 cells excreted 1/3 as much thymidine, whereas H-9 cells did not excrete or consume thymidine and K-562 cells removed all detectable thymidine from the medium. When corrections were made for variation in cell number, little difference between the three cell lines was seen in the rate of thymidine secretion. The increase in deoxyuridine was much less than for thymidine, barely doubling to about 1 μM. AZT-5'-MP was a prominent feature of chromatograms of media from treated cells. All three cell lines excreted approximately 5-10% of their intracellular AZT-5'-MP per hr. No other intracellular metabolites were seen in the media, so that extracellular AZT-5'-MP did not result from cell lysis during sample preparation.

After transfer to fresh, drug-free medium,recovery from the imbalances in the dNTP pools caused by AZT was rapid in both HL-60 and K-562 cells The former took about 30 min, and the latter took between 100 and 150 min (Figure 4 a,b). Intracellular AZT-5'-MP concentrations declined in an apparent first order process, with respective $t_{1/2}$ values of 34 and 68 min (Figure 4c). Published data on H-9 cells allows calculation of a $t_{1/2}$ of 41 min (3).

DISCUSSION

The rise of dCTP pools in cells treated with AZT has not been noted before. We have not been able to repeat the observation (3) of a marked decline in the dCTP pool of H-9 cells, even under a variety of conditions. Since the HPLC assay used here (Figure 1) was validated using the DNA polymerase assay for dTTP and dCTP, we believe the earlier data to be in error on this point. Changes reported in the other pools were similar in both studies. Although differing in degree, the responses of the three cell lines were qualitatively the same, suggesting that they may

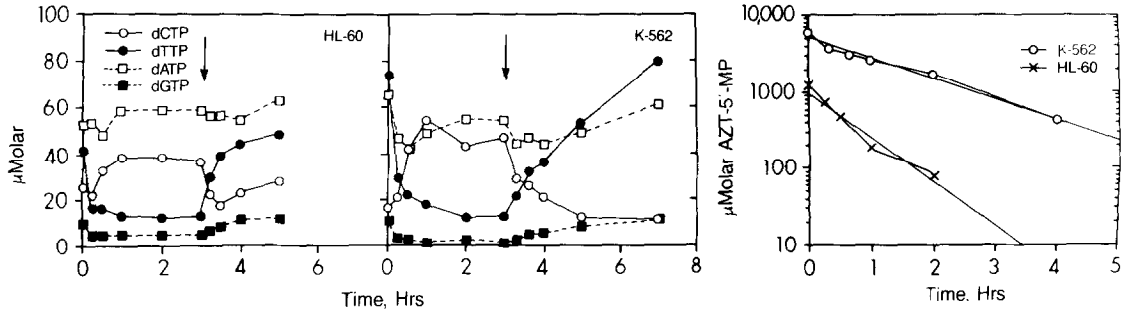

Figure 4. Recovery of the dNTP pools after removal of AZT.
a) HL-60 cells were incubated in 500 μM AZT for three hours and then centrifuged and resuspended in fresh medium. b) K-562 cells were treated with 200 μM AZT for three hours and then centrifuged and resuspended in fresh medium. Values are the averages of duplicate cultures. c) Clearance of intracellular AZT-5'-MP after removal of AZT.

occur in most human cells exposed to AZT. The dNTP pools recovered quickly at lower concentrations of AZT (Figure 3) making it imperative that studies of AZT and deoxynucleotide metabolism account for the possibility of changes occurring over time.

The K_M of thymidylate kinase for AZT-5'-MP is 8.6 μM (3). This value, together with the relatively high concentrations of AZT-5'-MP observed when cells are cultured with AZT, should result in significant inhibition, as has been suggested earlier (3). This inhibition probably accounts for the decline in the dTTP pools. The correlation between the rate of recovery of the dNTP pools when AZT is removed and the rate of clearance of intracellular AZT-5'-MP lends support to this idea, as does the somewhat looser correlation between the concentrations of AZT-5'-MP in the three cell lines at 24 hrs and the severity of the observed fluctuations in the dNTP pools. The increase in dCTP and decline in dGTP is consistent with models of the allosteric regulation of ribonucleotide reductase, in which low dTTP concentrations would enhance the reduction of both UDP and CDP and decrease the reduction of GDP (4, 5). Pools of dATP are less sensitive to fluctuations in dTTP and dGTP because of the ability of GTP to stimulate GDP reduction (13).

The $t_{1/2}$ for clearance of AZT-5'-MP may be an important determinant of the different steady state concentrations of the intracellular AZT-5'-MP seen in the three different cell lines, since there is a good correlation between the $t_{1/2}$ of the clearance of AZT-5'-MP from the cells and the concentration of intracellular AZT-5'-MP after 24 hrs incubation in 200 μM AZT.

The appearance of elevated levels of thymidine and deoxyuridine in the media of AZT-treated cells may be due to inhibition of thymidine kinase (EC 2.7.1.21) by AZT, which would disrupt the cycle between thymidine and dTMP mediated by thymidine kinase and 5'-nucleotidase (3, 14, 15). The ability of cells to excrete AZT-5'-MP is intriguing, but of limited significance *in vivo*, since it has a half-life of only 45 min in whole human blood (D. Nelson, unpublished).

The relevance of these pool imbalances, observed *in vitro*, to either the anti-HIV activity or bone marrow toxicity of AZT *in vivo* is not presently clear. Because of the relatively short duration of the effect and the high concentrations of drug required, it is possible that dNTP pool

perturbations do not play a primary role in either of these processes. However, it is clear that the effects of AZT on deoxynucleotide metabolism must be considered with *in vitro* systems used to study the mechanism of action of AZT.

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