0026-895X/90/050665-06$02.00/0

MOLECULAR PHARMACOLOGY, 37:665-670

Relationship of Deoxynucleotide Changes to Inhibition of DNA Synthesis Induced by the Antiretroviral Agent 3′-Azido-3′-deoxythymidine and Release of its Monophosphate by Human Lymphoid Cells (CCRF-CEM)

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Received September 5, 1989; Accepted February 15, 1990

SUMMARY

The metabolism and cytostatic effects of 3′-azido-3′-deoxythymidine (AZT), one of the most effective agents being used in the treatment of acquired immunodeficiency syndrome, were investigated in the CCRF-CEM line of human T lymphoid cells. The concentration of drug required to inhibit cell growth by 50% (CD_{50}) was significantly lower when the cells were exposed to AZT for 24 hr (CD_{50} = 50 μM), as compared with 48 or 96 hr (CD_{50} = 225 and >300 μM, respectively). AZT at 25 μM blocked the progression of cells in S phase for about 12 hr, but this effect was reversed by 24 hr, despite the continued presence of drug in the medium. At this drug concentration, the level of dTTP decreased to about 75% of the control level by 4 hr but rebounded to 30% above normal by 8 hr of drug exposure. dGTP and dATP pool sizes were unchanged, whereas the dCTP pool increased 5-fold. The time course of these biochemical changes indicated that the onset of S phase arrest was not directly related to the decrease in deoxynucleoside triphosphate pools. CCRF-CEM cells incubated with 25 μM AZT accumulated about 0.9 mM 5′-monophosphate (AZTMP) after 4 hr whereas levels of the 5′-di- and 5′-triphosphates (AZTDP and AZTTP) plateaued at about 2 and 5 μM, respectively. After this period, there was a rapid decrease in AZTMP accumulation, to one third its initial level by 24 hr, whereas AZTDP and AZTTP pools decreased to only about 70%. The loss in AZT nucleotide formation with time of drug exposure was associated with a concomitant accumulation of AZTMP in the medium. Cellular excretion of AZTMP was not associated with any detectable cell lysis or leakage of other cellular metabolites. The ability of CCRF-CEM cells to excrete AZTMP may be an important factor limiting the biochemical and biological effects of the drug.

Analogs of nucleosides and nucleotides are currently receiving increased attention for the treatment of human immunodeficiency virus-induced diseases. The thymidine analog AZT is regarded as the major drug for the treatment of the acquired immunodeficiency syndrome (1–7). Although its antiviral effects and efficacy in patients with acquired immunodeficiency syndrome are undisputed, treatment with AZT is, however, associated with severe side effects. The major toxicity is bone marrow suppression, which leads to anemia and neutropenia (6, 7). These toxicities often require reduction or discontinuation of treatment with AZT. It is generally assumed that the antiviral effects of AZT are caused by the nucleoside analog triphosphate AZTTP, which inhibits the viral DNA polymerase (reverse transcriptase) and/or becomes incorporated into nascent viral DNA chains, terminating further viral DNA synthesis (8–10). The mechanism for the toxicity of AZT is less clear. It has been proposed to be mediated through accumulation of the nucleotide analog AZTMP, which at high concentration inhibits thymidylate kinase (EC 2.7.4.9), an enzyme responsible for the synthesis of dTTP, which is an essential precursor for DNA synthesis (9). This hypothesis gained support from studies demonstrating depletion of deoxynucleoside triphosphates in cultured human T lymphocytes exposed to AZT (9). However, more recent studies in a variety of human cell types have not shown any consistent dNTP pool size changes in AZT-treated cells (11–14). In addition, there is growing evidence that AZT can interfere with DNA synthesis by mechanisms that are independent of thymidylate kinase inhibition. Sommadosi *et al.* (14) showed recently that bone marrow cells exposed to 10 μM AZT incorporate some of the fraudulant analog into DNA over a 24-hr period. It is difficult to assess, on the basis of these results, how much AZT interference with

This research was supported by Grant CA43296, by National Cancer Institute Cancer Center Support (CORE) Grant P30 CA21765, and by the American Lebanese Syrian Associated Charities.

ABBREVIATIONS: AZT, AZTMP, AZTDP, and AZTTP, 3′-azido-3′-deoxythymidine and its mono-, di-, and triphosphates; dNTP, deoxynucleoside triphosphates; HPLC, high perforamnce liquid chromatography.

the action of DNA polymerase contributes to the cytotoxicity of the drug.

In the present study, we have investigated the effect of AZT on DNA synthesis of cultured human T cells (CCRF-CEM), using flow cytometric techniques. By correlating these effects with changes in deoxynucleotide pools in CCRF-CEM cells exposed to various concentrations of AZT, we were able to obtain evidence on the relative importance, in AZT action on cells, of thymidylate kinase inhibition versus direct interference on DNA synthesis. This report also investigated the relationship between extracellular AZTMP accumulation and the response of cells to the drug.

Experimental Procedures

Chemicals. Azido[5′-^{3}H]thymidine (3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Final purification of the [^{3}H]AZT (>99.9% homogeneous) was achieved by chromatography through a C_{18} reverse phase column. AZTTP was purchased from Pharmacia Biotechnology, Inc. (Piscataway, NJ). AZTMP standard was prepared by hydrolysis of AZTTP with venom phosphodiesterase (EC 3.1.4.1; Boehringer Mannheim Biochemicals, Indianapolis, IN). AZT was obtained from the Division of Cancer Treatment of the National Cancer Institute (Bethesda, MD). All other chemicals were of the highest quality available.

Cell culture and growth inhibition assay. Human lymphoid CCRF-CEM cells were maintained in logarithmic growth, at 37° in 5% CO_2, in Eagle's modified suspension culture medium supplemented with 10% heat-inactivated (55° for 30 min) newborn calf serum. For the experiments in this study, cells were incubated with medium supplemented with serum that was heat inactivated at 60° for 3 hr. This treatment almost completely eliminated phosphatase and nucleotidase activities that could contribute to nucleotide analog degradation. To measure growth inhibition, we incubated cells in the continuous presence of different concentrations of AZT for 24 or 48 hr and determined cell number and size on a Coulter counter. Cell membrane integrity was determined at various periods after drug exposure by erythrosine B exclusion. In addition to determining cell disruption, we investigated possible leakage of the intracellular enzymes deoxycytidine kinase and adenosine kinase into culture medium supernatants. The lack of detectable enzyme activity after 24 hr of incubation with 25 or 200 μM AZT indicated that neither protein was lost from cells to any appreciable degree.

Flow cytometric analysis. Cell samples containing 1×10^6 cells were centrifuged at 1000 rpm for 5 min at room temperature; the medium was discarded and the cell pellet was resuspended in 1 ml of a hypotonic solution of propidium iodide (0.05 mg/ml in 0.1% sodium citrate). Samples were treated with 2 μg of RNase (Calbiochem, La Jolla, CA) per ml of suspension for 30 min at room temperature, immediately before analysis by flow cytometry. Samples were filtered through 44-μM nylon mesh (Small Parts Inc., Miami, FL) and analyzed at a rate of approximately 1000 cells/sec on an EPICS 753 flow cytometer (Coulter Electronics Inc., Hialeah, FL), as described previously (15). The percentages of cells in the G_1, S, and G_2+M phases of the cell cycle were determined by computer analysis, using the program PEAK provided by D. P. Dean (16, 17).

HPLC analysis. Neutralized perchloric acid extracts of the CCRF-CEM cells or culture medium were analyzed by HPLC. Azido-nucleotides were separated on a Partisil SAX anion exchange column by elution with 0.005 M ammonium phosphate buffer (pH 4.0)/methanol (90:10, v/v) for 20 min, followed by a linear gradient to 0.7 M ammonium phosphate buffer over an additional 40 min, at a flow rate of 1.5 ml/min. Nucleosides were separated on a reverse phase HPLC column (Whatman Partisil ODS-3) and eluted with 0.010 M ammonium phosphate (pH 4.0)/methanol (75:25, v/v), at a flow rate of 0.25 ml/min. The radioactivity of separated nucleotides and nucleosides was measured in a Beckman LS8000 scintillation counter.

Identification of phosphorylated products. For alkaline phosphatase digestion, [^{3}H]AZTMP isolated by HPLC from cell extracts or incubation medium was pooled, desalted by adsorption onto charcoal, and eluted from the charcoal with 5% ammonium hydroxide in 50% ethanol. The eluate was evaporated to dryness and reconstituted with 10 mM Tris·HCl buffer, pH 7.5. This desalted extract was incubated with 10 μl (5 units) of calf intestinal alkaline phosphatase for 24 hr at 37°. The reaction was terminated by boiling for 2–3 min, followed by cooling on ice. The sample was centrifuged, and the supernatant was analyzed by reverse phase HPLC to confirm that the analog was, in fact, AZT.

Determination of deoxynucleotide pool sizes. dNTP pools were analyzed as described previously (18). The chromatography system comprised a Whatman Partisphere 5 SAX column with 0.4 M ammonium phosphate buffer (pH 3.0, 40 min at a flow rate of 2.0 ml/min) used to elute dNTP. Chemical detection was by UV absorption at 254 and 280 nm. The peak areas of metabolites were digitized and integrated by a Shimadzu integrator, and the concentration of deoxynucleotides was determined by comparison with standards.

Results

Effect of AZT on the growth of human lymphoid cells. Less AZT was required to inhibit the growth of CCRF-CEM cells in cultures incubated with the drug for a short period rather than for prolonged periods. Only 50 ± 15 μM AZT caused 50% inhibition of cell growth after a 24-hr incubation, as compared with 255 ± 48 and 350 ± 64 μM after 48 and 96 hr, respectively.

Cytokinetic effects. Flow cytometric studies delineated the effects of AZT on cell cycle progression. Approximately 41, 45, and 14% of exponentially growing CCRF-CEM cells were in the G_0/G_1, S, and G_2+M phases, respectively (Fig. 1A). Exposure of CCRF-CEM cells to either 25 μM AZT for 12 hr (Fig. 1B) or 200 μM AZT for 12 hr (Fig. 1D) or 24 hr (Fig. 1E) resulted in a marked accumulation of cells in S phase and a decrease in both the G_1 and G_2+M fractions. This S phase block was readily apparent after 6 hr (data not shown) but was almost completely reversed by 24 and 48 hr of exposure to 25 μM (Fig. 1C) and 200 μM (Fig. 1F) AZT, respectively.

Influence of AZT on deoxynucleotide pools. We next investigated the effect of AZT treatment on the deoxynucleotide pools of CCRF-CEM cells. Fig. 2 shows the relative changes in dNTP pools at various times after the addition of 25 or 200 μM AZT. When cells were treated with 25 μM AZT, the level of dTTP decreased to about 75% of control between 4 and 8 hr after incubation, but it then rebounded to 30% above the control level by 8 hr; DNA synthesis remained inhibited during these changes. Both dGTP and dATP levels were essentially unchanged at this drug concentration, whereas the dCTP level increased 5-fold after 2 hr and then progressively returned to the control level. Exposure to 200 μM AZT produced more pronounced effects on dNTP levels. This concentration of drug decreased the dTTP and dGTP levels to about 30 and 50% of the control level after 4 and 8 hr, respectively, whereas dATP levels remained unchanged and the dCTP pool increased about 3-fold. Even with 200 μM AZT, however, the dTTP and dGTP pools recovered by 24 hr.

Cellular accumulation of AZT metabolites. To assess the relationship between drug-induced alterations in DNA metabolism and cytotoxic nucleotide analog formation, we studied the formation of the latter in CCRF-CEM cells. Cells were incubated with either 25 or 200 μM [^{3}H]AZT and, at various times, intracellular ^{3}H was quantitated by HPLC. In agreement

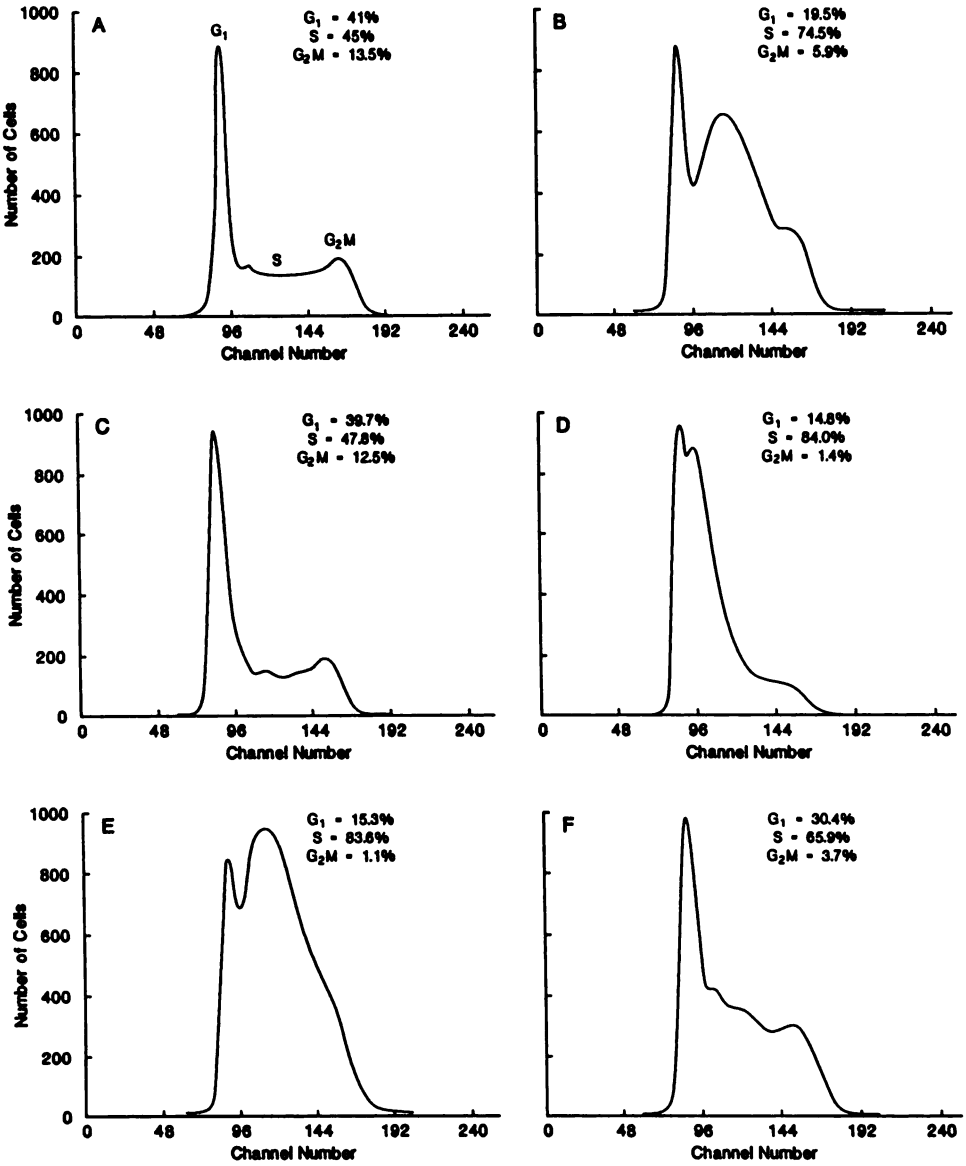

Fig. 1. DNA histograms of CCRF-CEM cells after AZT treatment. Cells were exposed to either 25 μM (B and C) or 200 μM (D–F) AZT and analyzed before treatment (A), after 12 hr (B and D), after 24 hr (C and E), and after 48 hr (F). Control cells (A) were untreated. Percentages of cells in different phases of the cell cycle are shown in the *upper right* of each *panel*.

with previous studies (9), AZT was metabolized predominantly to the monophosphate AZTMP rather than either AZTDP or AZTTP (Fig. 3). Upon incubation with 25 μM AZT, AZTMP, AZTDP, and AZTTP accumulated to their maximum levels within 4 hr. Then, the concentration of AZTMP decreased sharply, from about 1200 to 380 pmol/10^6 cells by 24 hr. AZTDP and AZTTP levels remained constant from 4 to 12 hr and then decreased by about 30% after 24 hr. When the same incubation was performed with 200 μM AZT, AZTMP accumulated to a peak concentration of about 2400 pmol/10^6 cells after 12 hr and then declined to about 1800 pmol/10^6 cells after 24 hr of drug exposure. It should be noted that, despite the substantial increase in the level of AZTMP produced with 200 μM AZT, no significant differences in levels of AZTDP or AZTTP were noted between the two different drug concentrations.

We also determined the concentration of AZT remaining in the culture medium during incubation with CCRF-CEM cells. As shown in Table 1, this concentration remained relatively stable for at least 24 hr. However, the analysis revealed the accumulation of significant levels of AZTMP in the culture medium with increased exposure time. More than 80% of the AZTMP formed was apparently excreted into the culture medium, almost from the start of the incubation with either 25 or 200 μM exogenous AZT (Fig. 4). The analog monophosphate was identified on the basis of its retention on ion exchange HPLC and recovery of the parent drug after hydrolysis of the nucleoside analog peak with alkaline phosphatase (data not shown). This release of AZTMP from cells was not accompanied by any loss of cell viability, as measured by vital dye exclusion or release of cytoplasmic enzyme (extracellular thymidine kinase and adenosine kinase activityies were <1% those of cell extracts). There was also no loss of endogenous adenine or guanine nucleotides from CCRF-CEM cells during the incubation with AZT, and the normal ratio of ATP to ADP in these cells was not significantly affected (data not shown). Thus, the cells could release AZTMP without the plasma membrane becoming permeable to different endogenous metabolites.

In addtion to AZT uptake, we also determined the excretion of radiolabeled AZT from CCRF-CEM cells. Cells were incubated with 25 μM [^{3}H]AZT for 4 hr to allow accumulation of radioactive nucleotide analog and were then resuspended in

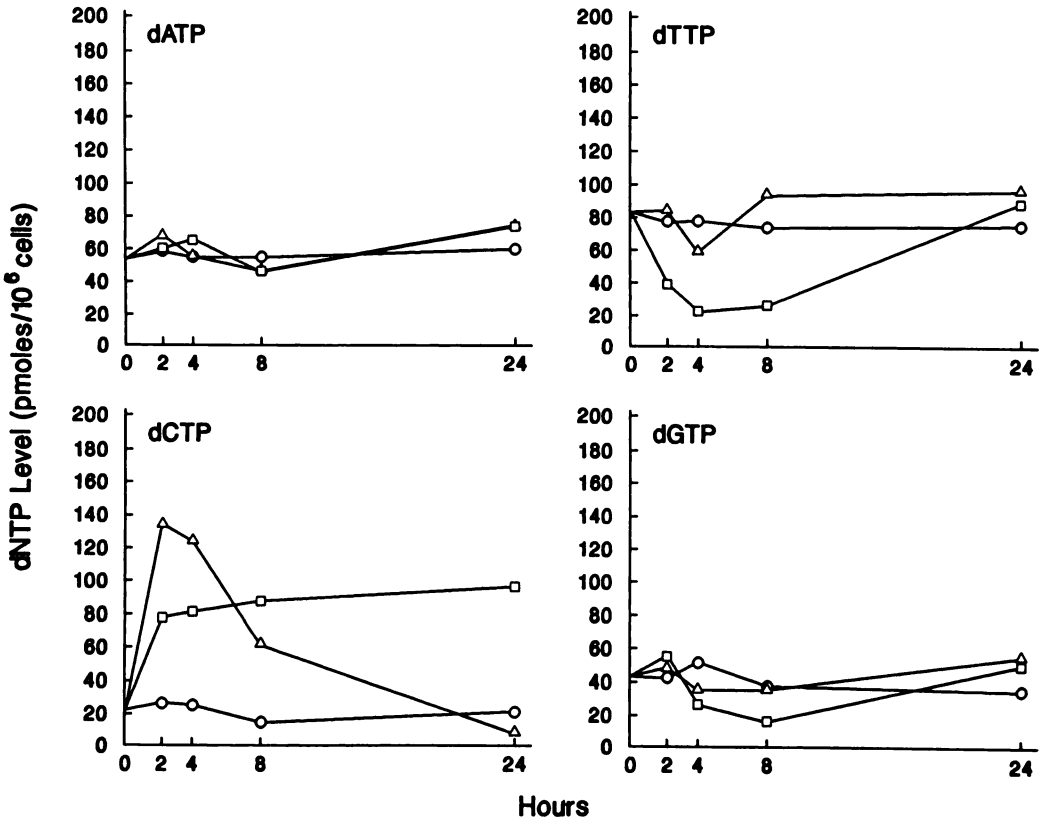

Fig. 2. Intracellular dNTT levels in CCRF-CEM cells exposed to AZT. Cells were exposed to AZT and the indicated levels were determined as described in Experimental Procedures. The values are from a typical experiment repeated at least twice. O, Untreated controls; △, cells exposed to 25 μM AZT; □, cells exposed to 200 μM AZT.

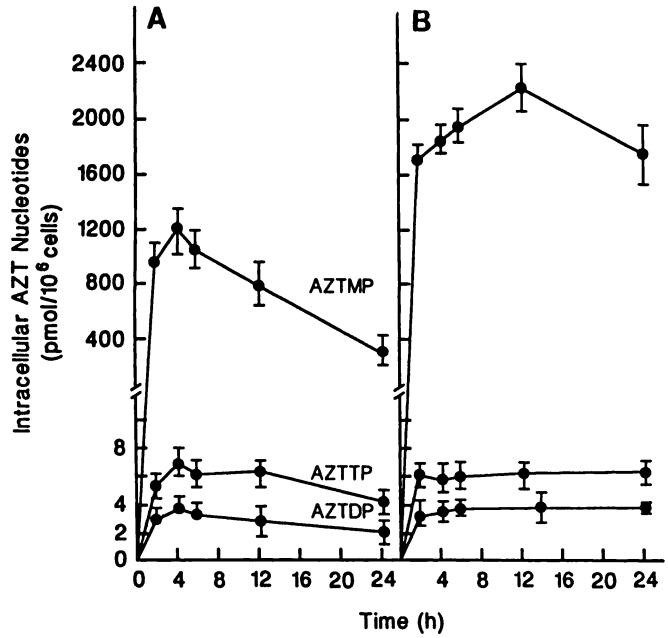

Fig. 3. Accumulation of AZT metabolites after incubation of CCRF-CEM cells with exogenous AZT. The cells were incubated at a density of 5 × 10^5/ml with either 25 μM (A) or 200 μM (B) [^{3}H]AZT (6 μCi/ml); the radioactivity in the intracellular nucleotide analogs was determined as described in Experimental Procedures. The results are the mean ± standard deviation of three separate experiments.

drug-free medium; the metabolites in these cells and in the media were analyzed at various times by HPLC. The elimination of AZTMP from the cells was quite rapid, with a 50-min half-life ($t_{1/2}$) at 37° for AZTMP (Fig. 5A). This result is very similar to that reported by others (13). By contrast, the intracellular levels of AZTTP and AZTDP that accumulated in the cells were stable for at least 4 hr of incubation. HPLC analysis of the radioactivity excreted into the medium indicated that about 40% of the radioactivity lost from the cells was recovered as AZTMP; the remainder was in the form of nucleoside analog (Fig. 5B). From these findings and the intracellular concentration of AZTMP that accumulated in cells, one can calculate that the excretion of AZTMP into the medium corresponded to a minimum rate of 2.25 pmol/10^6 cells/min.

Discussion

In the present investigation, we have demonstrated that the inhibitory effect of AZT on the growth of CCRF-CEM cells is highly dependent on both drug concentration and duration of exposure. Thus, at a given drug concentration, much greater suppression of growth was observed after a short term (24 hr) than after more prolonged exposure (48 hr) to AZT. Our estimate of the sensitivity (IC_{50} of 50 μM at 24 hr) of CCRF-CEM cells to AZT differs from those of other studies, which have reported that >200 μM inhibits growth of human lymphoid cells (9, 19). We attribute this discrepancy mainly to differences in observation times for determining drug effects; whereas we measured the CD_{50} in cultures incubated with AZT for as little as 1 day, others have generally waited for 3 to 5 days to determined cytotoxicity. Clinically, AZT is administered every 4 hr and, therefore, the present results would seem to approximate more closely *in vivo* drug therapy.

The exact mechanism by which AZT inhibits the growth of cells is still unclear. It has been proposed that it occurs mainly through inhibition of thymidylate kinase by accumulation of high concentrations of AZTMP (7, 9). This enzyme catalyzes the conversion of dTMP to its higher phosphates and ensures

TABLE 1

Extracellular levels of [^{3}H]AZT and [^{3}H]AZTMP with increasing incubation time

Cells (5 × 10^5/ml) were incubated with the indicated concentrations of [^{3}H]AZT, as described in Fig. 3. Aliquots (1.0 ml) of the media were analyzed by HPLC, as described in Experimental Procedures. Values represent the average of three separate experiments ± standard deviation.

AZT addition	AZT				AZTMP			
	2 hr	6 hr	12 hr	24 hr	2 hr	6 hr	12 hr	24 hr
μM	pmol/10^6 cells				pmol/10^6 cells			
25	17,160 ± 122	18,321 ± 193	17,456 ± 145	15,104 ± 119	236 ± 11	1,033 ± 96	2,069 ± 238	3,877 ± 409
200	141,094 ± 1607	114,720 ± 1017	127,008 ± 1121	128,284 ± 1301	512 ± 48	1,064 ± 114	3,207 ± 330	5,994 ± 602

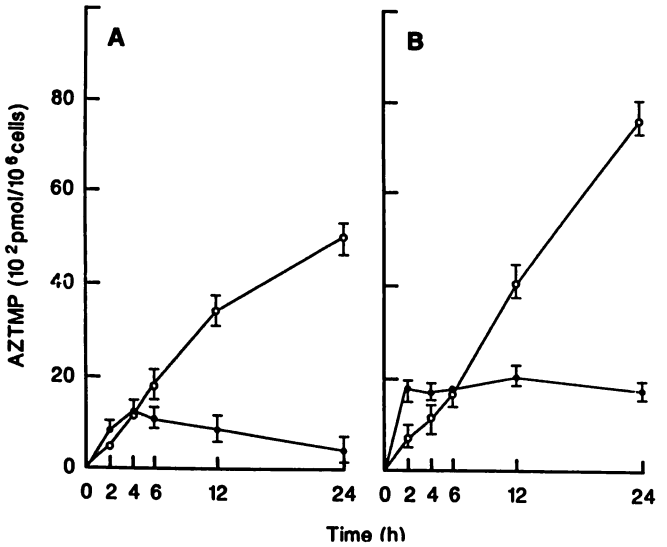

Fig. 4. Comparison of intracellular and extracellular accumulation of AZTMP by CCRF-CEM cells. The cells were incubated at a density of 5 × 10^5/ml with either 25 μM (A) or 200 μM (B) [^{3}H]AZT (6 μCi/ml); the intracellular (●) and extracellular (○) radioactivity was determined as described in Experimental Procedures. The results are the mean ± standard deviation of three separate experiments.

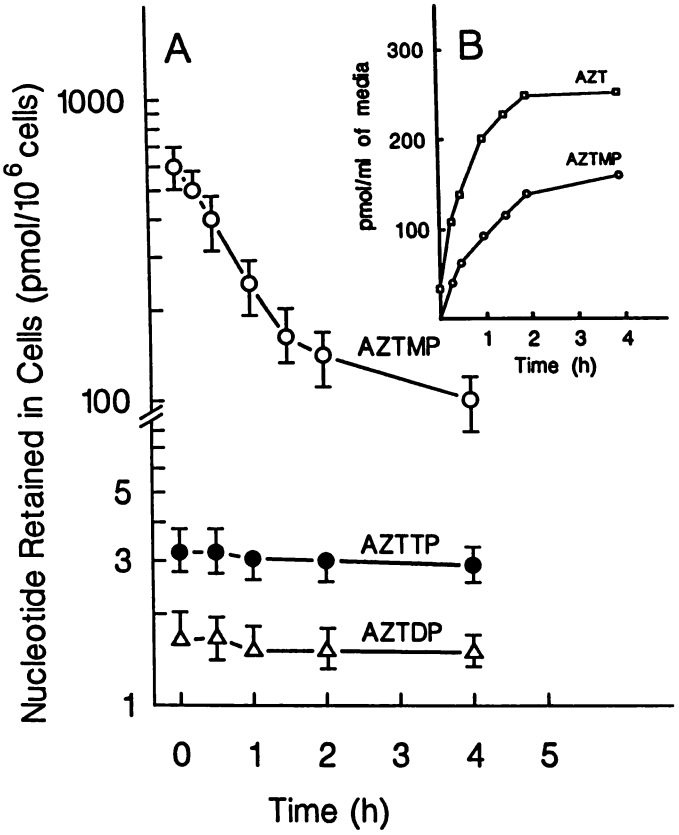

Fig. 5. Kinetics of elimination of AZT nucleotides in CCRF-CEM cells after drug removal. Cells (5 × 10^6/ml) were preincubated with 25 μM [^{3}H] AZT (7 μCi/ml) for 4 hr, centrifuged, washed in cold medium, and resuspended at the same concentration in drug-free medium at 37°. At the indicated times, nucleotides and nucleosides were analyzed separately in the medium and in cell extracts, as described in Experimental Procedures. The results are the mean ± standard deviation of three separate experiments.

an adequate supply of deoxynucleotides for DNA synthesis in replicating cells. According to the regulation of dNTPs *in vitro*, depletion of the dTTP level may interfere with the regulation of ribonucleotide reductase and suppress the synthesis of the other deoxynucleotides (20). In the present experiments, we did not observe a close relationship between dNTP pool depletion and S phase arrest. Thus, at a concentration of 25 μM, AZT caused S phase arrest for at least 12 hr but only a slight decrease in the dTTP pool, of about 25%, for a short period between 4 and 8 hr after drug addition. The other pools, dGTP and dATP, remained unchanged but dCTP increased about 5-fold after a 2-hr exposure to drug. Only after CCRF-CEM cells were exposed to concentration of AZT 8 times higher did decreases of >50% in the levels of dTP and dGTP result. In this case, the minimum pool size was reached after 4 and 8 hr of treatment, respectively, but unexpectedly showed considerable recovery after 24 hr of drug exposure. These results are in contrast to the earlier reports, in H-9 cells, that 50 μM AZT decreased dTTP, dGTP, and dCTP to 5, 20, and 5%, respectively, of control levels for at least 72 hr of treatment, but they are in good agreement with the recent studies of Frick *et al.* (13), who reported similar transient effects on dNTP pools in K562, HL-60, and H-9 cells treated with 200 μM AZT. While this manuscript was being prepared, Sommadosi *et al.* (14) reported a lack of correlation between decrease in dNTP level and inhibition of DNA synthesis induced by AZT in human bone marrow cells.

The inhibitory constant (K_d) of AZTMP for thymidylate kinase is about 8.6 μM (9). This low value relative to the high concentrations of AZTMP that accumulate in human cells has suggested that inhibition of this enzyme would result in drug-treated cells (9, 12, 13). In the present studies, AZTMP reached a maximum concentration of about 1200 and 2400 pmol of AZTMP/10^6 CCRF-CEM cells incubated with 25 and 200 μM, respectively. Based on a mean cell volume of about 1.6 μl/10^6 CCRF-CEM cells, this would correspond to a cellular concentration of about 0.9 and 1.8 mM, respectively. Assuming a uniform intracellular distribution of nucleotide analog, these levels of AZTMP are 150- and 300-fold greater than that reported for inhibition of thymidylate kinase. However, despite

these high concentrations of AZTMP, marked decreases in dNTP levels were observed only with the higher level of nucleotide analog accumulation in CEM cells. This suggests that concentrations well above millimolar range of AZTMP are necessary to significantly inhibit thymidylate kinase in intact cells.

An intriguing finding of the present study was that the efficacy of AZT decreased with length of exposure time. At least 5 times more AZT was needed at 48 hr than at 24 hr to inhibit the growth of CCRF-CEM cells. Balzarini *et al.* (21) also demonstrated a decrease in antiviral potency of AZT with prolonged exposure and attributed this to a chemical instability of the drug. However, no significant degradation of AZT was detected in the present study after 24 hr of incubation. On the other hand, we have found that the cells excrete AZTMP into the culture medium and accumulate progressively lower levels of intracellular AZTDP and AZTTP with continued drug exposure (Fig. 3). Thus, it seems likely that the decrease in growth inhibition of AZT with incubation time is the result of this loss of AZT metabolites from the cells. The mechanism of AZTMP release is not known; the observation is unusual, because the cell membrane is generally impermeable to nucleotides. This release of nucleotide analog is not due to a nonspecific increase in membrane permeability, because the drug-treated cells remained fully viable, and it is not accompanied by loss of other intracellular molecules such as ATP or GTP. This raises the possibility that the excretion of AZTMP may occur agaisnt a concentration gradient and may, therefore, involve an active process. Frick *et al.* (13) also reported, in their studies, finding excretion of AZTMP from HL-60, K562, and H-9 cells exposed to AZT but did not explore this further. Previous studies (22, 23) have demonstrated the export of certain adenine nucleotides from cells via a carrier-mediated system that is sensitive to inhibitors of energy production and distinct from the transport system for nucleosides. In preliminary studies, AZTMP export was insensitive to the inhibitor of nucleoside transport nitrobenzylthioinosine. It seems, therefore, possible to suggest that AZTMP may be an alternate substrate for a transport system that normally is involved in the excretion of certain nucleotides for cells or that represents a previously undefined transporter. More complete and detailed studies will be necessary to investigate the substrate specificity and physiological significance of this export system, as well as its importance for the selectivity of AZT or similarly acting dideoxynucleosides.

Acknowledgments

We thank Dolores Anderson for manuscript preparation and John Gilbert for editing. Grateful appreciation is also due Dr. Dave G. Johns for helpful discussions.

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