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THYMIDINE AND ZIDOVUDINE METABOLISM IN CHRONICALLY ZIDOVUDINE-EXPOSED CELLS *IN VITRO*

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Abstract—Chronic exposure of H_9 cells to 25 μM zidovudine (H_9-AZT cells) causes a 2- to 3-fold increase in thymidine kinase (TK) activity (Agarwal RP, *Int J Purines Pyrimidine Res*, in press). The present study compared thymidine (TdR) and AZT anabolism in H_9 and H_9-AZT cells. After a 3.5-hr incubation with 10 μM TdR or AZT, the total intracellular accumulations of AZT (48.7 μM in H_9 cells and 32.8 μM in H_9-AZT cells) were 46.4% of TdR accumulation. Other major differences between TdR and AZT anabolism were: (i) the majority of TdR (84–87%) was incorporated into DNA compared to <1% of AZT; and (ii) whereas distribution of TdR in the nucleotides was TTP > TMP > TDP, zidovudine distributed was AZT-MP ⩾ AZT-TP ⩾ AZT-DP. Because of the poor substrate activity of AZT-MP for thymidylate kinase (TMP-kinase), most of the AZT (95–98%) remained as AZT-MP. TMP-kinase activities with TMP as substrate were 47.6 ± 20.3 and 91.4 ± 28.8 pmol/mg protein/min in H_9 and H_9-AZT cells, respectively. 5′-Nucleotidase activities with TMP as substrate were 428.9 ± 37.8 and 255.9 ± 28.7 pmol/mg protein/min in H_9 and H_9-AZT cells, respectively. Activities of these enzymes with AZT-MP as a substrate were very low. Despite an increase in TK and TMP-kinase, and a decrease in 5′-nucelotidase activities, the total intracellular accumulations of TdR and AZT were reduced significantly ($P < 0.05$) to 67.5% in H_9-AZT cells. Thymidine transport (0.66 to 0.68 pmol/sec/10^6 cells) was similar in both the cell lines. The severe reductions of TdR salvage caused by chronic exposure of cells to AZT, if it occurs in AIDS patients on AZT chemotherapy, may explain some of the long-term clinical toxicities of the drug.

Potent inhibition of *in vitro* replication and the cytopathic effects of human immunodeficiency virus (HIV) by AZT (zidovudine; 3′-azido-3′deoxythymidine) has led to its chronic use in patients with acquired immunodeficiency syndrome (AIDS) [1–3]. The drug in itself is inactive, but its phosphorylated metabolite, AZT-5′-trisphosphate (AZT-TP), selectively inhibits viral reverse transcriptase (RT), an enzyme essential for HIV replication [4–7], and is incorporated into viral DNA leading to chain termination [6, 8, 9]. It may also be incorporated into host cell DNA [10] and affect the intracellular nucleotide pool [11]. Thus, in general, the antiviral activity of AZT would depend on the ability of the host cells to accumulate AZT-TP.

The drug is anabolized as a thymidine (TdR) analog [4] through the action of thymidine kinase (TK; EC 2.7.1.21), thymidylate kinase (TMP-kinase; EC 2.7.4.9) and nucleoside diphosphokinase (NDPK; EC 2.7.4.7), which sequentially catalyze the reactions AZT → AZT-MP → AZT-DP → AZT-TP. Of these three kinases, TK has low cellular activity which is highly regulated [12]. Diminished deoxynucleoside kinase activities (including TK activity) with consequent reduction in their ability to phosphorylate dideoxynucleosides have been implicated in the failure of antiviral dideoxynucleosides to inhibit HIV replication [13]. We have reported recently that chronic exposure of cells to AZT causes a 2- to 3-fold increase in thymidine kinase activity in cells chronically exposed to AZT (H_9-AZT cells) [14]. If TK activity was the rate-limiting factor in the accumulation of the antiviral AZT metabolite AZT-TP, we postulated that increased TK activity in H_9-AZT cells should enhance AZT anabolism and allow high concentrations of AZT-TP to accumulate. The present investigation compares AZT and TdR salvage in H_9-AZT and unexposed H_9 cells.

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MATERIALS AND METHODS

Chemicals

[*methyl*-^{3}H]Thymidine (55 Ci/mmol) and [*methyl*-^{3}H]thymidine monophosphate were purchased from ICN Radiochemicals (Irvine, CA); [*methyl*-^{3}H]AZT (18 Ci/mmol) and [*methyl*-^{3}H]AZT monophosphate (AZT-MP) were obtained from Moravek Biochemicals Inc. (Brea, CA). AZT-MP was purified further as described below. Non-radioactive AZT was a gift from the Burroughs Wellcome Co., Research Triangle Park, NC. Other reagents were of the highest purity available from commercial sources.

Cell maintenance

H_9 and H_9-AZT cells have been described earlier [14]. In brief, the cells were grown in RPMI-1640 medium containing 20% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37° in a humidified 5% CO_2–95% air atmosphere; H_9-AZT cell growth medium also contained 25 μM AZT.

Enzyme assays

Exponentially growing cells were washed twice

with cold phosphate-buffered saline (PBS), suspended at a density of 1.0×10^7 cells/mL of 100 mM Tris–HCl buffer, pH 7.5, lyzed by sonication (8 bursts of 5 sec each at maximal output; Branson Sonifier), and centrifuged in a microfuge at the maximum speed for 10 min. The supernatant fluids were used for enzyme assays and protein determination [15].

Thymidylate kinase. TMP-kinase activity was determined by a radiochemical method using TMP and AZT-MP as substrates. The reaction mixture (500 μL) containing 100 mM Tris–HCl, (pH 7.5); 12.5 mM KCl, 5 mM NaF, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 10 μM tritiated TMP or AZT-MP (100 μCi/mmol) and the enzyme were incubated at 37° in a water bath. Following incubations for 0–120 min, 50-μL samples were withdrawn and added to 20 μL of ice-cold methanol and then chilled in ice. Twenty-five microliters of these samples was applied onto PEI-cellulose thin-layer plastic plates along with a mixture of carriers (TdR, TMP, TDP, and TTP). Chromatograms were developed with 50% methanol in H_2O, dried and then developed again with 0.5 M sodium formate to separate mono-, di-, and triphosphates. The metabolites were visualized by UV light, excised, extracted in 1.0 mL of 0.1 N HCl containing 0.1 M KCl, and counted for radioactivity. The TMP kinase activity was calculated as picomoles of di- and triphosphates formed per minute per milligram of protein.

5′-Nucleotidase. 5′-TMP or AZT-MP phosphohydrolase activity was determined by following the formation of thymidine or AZT from radiolabeled TMP and AZT-MP, respectively. A reaction mixture (500 μL) containing 100 mM Tris–HCl (pH 7.5); 5 mM $MgCl_2$, and 10 mM tritiated TMP or AZT-MP and the enzyme were incubated at 37° in a shaking water bath. Samples (50 μL) were withdrawn at 0–60 min and added to 20 μL of ice-cold methanol. Nucleosides and the nucleotides were separated by TLC in 50% methanol. The compounds were visualized, cut out, extracted, and counted, and the activities were calculated as picomoles of nucleoside formed per minute per milligram of protein.

Thymidine transport

Cells were washed and resuspended in fresh medium at a density of 2.5×10^6 cells/mL. Fifty-five microliters of 100 μM [^{3}H]thymidine (100 μCi/mmol) was placed in a 1.5-mL Eppendorf microcentrifuge tube. Cell suspension (0.4 mL containing 10^6 cells) was added to the tube, and the reaction was terminated at 0–10 sec at 1-sec intervals and at 20 and 30 sec by addition of 0.7 mL of an oil mixture (dibutylpthalate : dioctylpthalate, 4:1) and starting the centrifugation. All procedures were done at room temperature. The bottom portion of the tube was cut off, placed in a scintillation vial, dissolved in 0.5 mL NaOH, neutralized and counted. Zero time counts were subtracted from each subsequent time count. The picomoles of thymidine inside the cells were calculated assuming a volume of 1.16 μL/10^6 cells [16].

Purification of [methyl-^{3}H]*AZT-MP*

Radiolabeled AZT-MP obtained from Moravek was purified further by TLC. The samples were applied onto PEI-cellulose coated plastic TLC plates and developed in 50% methanol. The radioactive material remaining at the origin was extracted with 2 M ammonium carbonate solution. Extracts were evaporated and reconstituted in water.

Incorporation of thymidine and zidovudine in the nucleotide pools and macromolecules of H_9 and H_9-AZT cells

Exponentially growing cells were harvested by centrifugation at 1500 rpm for 15 min, washed twice with and suspended in AZT free growth medium at a density of 1×10^7 cells/mL. To 1.8 mL of cell suspension was added 0.2 mL of 100 μM tritiated TdR or AZT (100 μCi/μmol) (10 μM final concentration). Following incubation at 37° for 3.5 hr the cells were collected by centrifugation and washed twice with 5-mL of cold PBS. The pellets were extracted overnight in the freezer (−20°) with 1 mL of 65% methanol. The extracts were collected by centrifugation in a refrigerated centrifuge, the precipitate was washed once with 0.5 mL of cold 65% methanol, and the washings were collected with the extract. The combined extract and washings are referred to as methanol "soluble" fraction and the methanol-insoluble residue as "insoluble" fraction.

The "insoluble" fraction containing macromolecules was solubilized in 0.5 mL of 0.5 N NaOH, neutralized with 0.5 mL of 0.5 N HCl, and counted for radioactivity. The "soluble" fraction was dried under nitrogen gas, reconstituted in 100 μL of 50% methanol, and clarified by centrifugation. Fractions were counted for radioactivity and analyzed by HPLC. The concentrations were calculated from the specific activities of TdR and AZT and by assuming an intracellular water volume of 1.16 μL/10^6 cells [16].

Reconstituted extracts (50 μL) were mixed with 10 μL of markers (mixture of TdR, TMP, TDP, TTP, AZT, and AZT-MP), injected into an HPLC (Perkin–Elmers) equipped with a Partisil-10-SAX column (4.5 × 250 mm) and eluted at a flow rate of 1.0 mL/min with a gradient of solution B (0.75 M $NH_4H_2PO_4$) in solution A (15 mM $NH_4H_2PO_4$). The 100% gradient was attained in 60 min. The eluates were monitored at 254 nm, and 1-mL samples were collected and counted.

RESULTS

Incorporation of thymidine and zidovudine in H_9 and H_9-AZT cells

The distribution of incorporated TdR and AZT (in "soluble" and "insoluble" fractions and total) in H_9 and H_9-AZT cells after 3.5 hr of incubation is shown in Table 1. The data indicate that: (i) total AZT incorporation in H_9 cells (56.8 pmol/10^6 cells) and H_9-AZT cells (38.0 pmol/10^6 cells) was 46.4% of TdR incorporation (122.3 and 81.9 pmol/10^6 cells, respectively); (ii) in H_9-AZT cells the incorporation of both AZT and TdR was reduced to 66.9% of H_9 cells; (iii) TdR incorporation in "soluble" and "insoluble" fractions was reduced to 56 and 69%, respectively ($P < 0.05$); (iv) AZT incorporation in "soluble" fraction was reduced to 67% ($P < 0.05$);

Table 1. Distribution of thymidine and zidovudine in the intracellular "soluble" and "insoluble" fractions of H_9 and H_9-AZT cells

	Thymidine			Zidovudine		
	Total	Soluble	Insoluble	Total	Soluble	Insoluble
	Picomoles per 10^6 cells (Micromolar)					
H_9 cells						
Expt. 1	139.5 (117.7)	15.6 (13.4)	123.9 (——)	45.4 (39.1)	44.7 (38.5)	0.7 (——)
Expt. 2	97.0 (83.6)	17.5 (15.9)	79.5 (——)	61.2 (52.8)	60.5 (52.2)	0.7 (——)
Expt. 3	130.5 (112.5)	25.4 (21.9)	105.1 (——)	63.7 (54.9)	62.7 (54.0)	1.1 (——)
Mean ± SD*	122.3 ± 22.4 (104.6 ± 18.4)	19.5 ± 5.2 (17.1 ± 9.4)	102.8 ± 22.3 (——)	56.8 ± 9.9† (48.7 ± 8.5)	56.0 ± 9.8† (48.2 ± 8.5)	0.83 ± 0.23†
H_9-AZT cells						
Expt. 1	80.4 (69.3)	9.0 (7.8)	71.4 (——)	37.7 (32.4)	37.2 (32.1)	0.4 (——)
Expt. 2	71.8 (61.9)	13.0 (11.2)	58.8 (——)	45.0 (38.8)	44.3 (38.2)	0.7 (——)
Expt. 3	93.4 (80.5)	11.0 (9.5)	82.4 (——)	31.5 (27.2)	30.5 (26.3)	1.1 (——)
Mean ± SD*	81.9 ± 10.9‡ (70.6 ± 9.4)	11.0 ± 2.0‡ (9.5 ± 1.7)	70.9 ± 11.8‡ (——)	38.0 ± 6.8†‡ (32.8 ± 5.8)‡	37.3 ± 6.0†‡ (32.2 ± 6.0)‡	0.7 ± 0.4† (——)

Exponentially growing washed cells were incubated with 10 μM tritium-labeled TdR or AZT (100 μCi/μmol) for 3.5 hr. Following incubation, the cells were washed and extracted with 65% methanol overnight at −20°. Methanol "soluble" and "insoluble" fractions were separated and counted as described in Materials and Methods. Concentrations (μM) were calculated using values of intracellular water as 1.16 μL/10^6 cells [16].

* Mean ± SD of three different experiments.

† Significantly different from thymidine, $P < 0.05$.

‡ Significantly different from H_9 cells, $P < 0.05$.

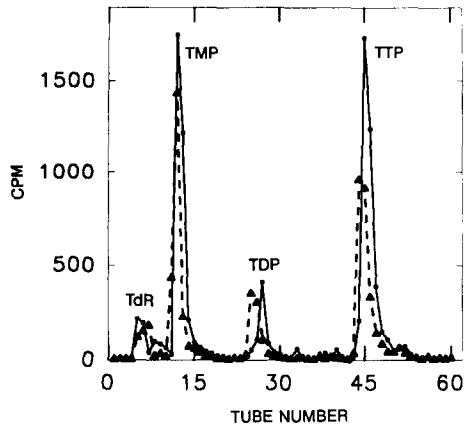

Fig. 1. HPLC profiles of incorporation of 10 μM [^{3}H] thymidine in the nucleotide pools of H_9 (——) and H_9-AZT (----) cells. Fifty microliters of reconstituted extracts was mixed with 10 μL of markers (mixture of TdR, TMP, TDP and TTP), injected into an HPLC (Perkin–Elmers) equipped with a Partisil-10-SAX column (4.5 × 250 mm), and eluted at a flow rate of 1.0 mL/min with a gradient of solution B (0.75 M $NH_4H_2PO_4$) in solution A (15 mM $NH_4H_2PO_4$). The 100% gradient was attained in 60 min. The eluates were monitored at 254 nm, and 1-mL samples were collected and counted.

and (v) the major fraction of the incorporated TdR (84–87%) appeared in the "insoluble" fraction, whereas the majority of AZT (>98%) remained in the "soluble" fraction in both cell lines.

Distribution of thymidine and its metabolites in H_9 and H_9-AZT cells

HPLC profiles and the distribution of the radiolabeled metabolites of thymidine in the intracellular pools of H_9 and H_9-AZT cells are shown in Fig. 1 and Table 2. More than 98% of the radioactivity loaded on the column was recovered and appeared as TdR, TMP, TDP, and TTP. Only an insignificant amount (<1.8%) was present as unidentified peaks. Although the percent distribution of radioactivity among the nucleosides and nucleotides was similar, the concentrations of labeled TMP (3.0 μM) and TTP (4.3 μM) in H_9-AZT cells were 44.8 and 49.4% of H_9 cells, respectively ($P < 0.05$).

Distribution of zidovudine and its metabolites in H_9 and H_9-AZT cells

It is clear from Fig. 2 and Table 2 that the anabolism of AZT was significantly different from that of TdR. A major metabolite of the AZT was AZT-MP (94–96%). AZT, AZT-DP and AZT-TP constituted the remaining 4–6% (note than the y-axis in Fig. 2 is presented as a log scale). AZT-TP concentrations were low (0.3 to 0.5 μM); however, they were high enough to inhibit viral RT which has a K_i value of about 0.04 μM [4]. Accumulation of AZT-MP and low concentrations of AZT-DP and AZT-TP suggested that the monophosphate was poorly anabolized to its di- and triphosphates and, in addition, might be poorly dephosphorylated to AZT.

Phosphorylation and dephosphorylation of TMP and AZT-MP in H_9 and H_9-AZT cell homogenates

Phosphorylation of AZT-MP and TMP to their diphosphates is catalyzed by TMP-kinase [4]. The TMP-kinase activities in H_9 and H_9-AZT cells, with TMP as a substrate, were 47.6 ± 20.3 and 91.4 ± 28.8 pmol/mg protein/min, respectively. However, the activities with AZT-MP as substrate were very low (<1% of TMP) in both cell lines (data not shown here). The activity of TMP-kinase was found to be quite variable and unstable on freezing overnight.

The 5′-nucleotidase activities with TMP as substrate were 428.8 ± 37.8 and 255.9 ± 28.7 (N = 3) pmol/mg protein/min in H_9 and H_9-AZT cells, respectively. The activity with AZT-MP as substrate was barely detectable.

Thymidine transport into H_9 and H_9-AZT cells

The time course of TdR transport in H_9 and H_9-AZT cells is shown in Fig. 3. Data were very similar in both cell lines. The rates of influx calculated from the initial linear velocities (1–9 sec) were 0.68 ± 0.17 and 0.66 ± 0.23 pmol/sec/10^6 cells in H_9 and H_9-AZT cells, respectively. Since other experiments were performed at 10 μM concentrations of TdR, transport was also studied at the same concentration. If there were any differences in the K_m values between the two cell lines, they were not investigated.

DISCUSSION

The dual aim of this investigation was to examine whether AZT anabolism differed from that of TdR and whether chronic exposure of cells to AZT altered the anabolism of these metabolites.

Several differences were apparent between AZT and TdR metabolism: (i) Intracellular accumulation of AZT (including its metabolites) was lower than the accumulation of TdR (46%; Table 1). This observation was consistent with our earlier findings and other reports that the V_{max} values for the phosphorylation of AZT were about 40% lower than TdR [4, 14]. (ii) Most of AZT (>98%) remained in the "soluble" fraction, whereas the majority of TdR (84–87%) was incorporated in the "insoluble" fraction. These observations were consistent with low cellular concentrations of AZT-TP and its poor substrate activity for cellular DNA polymerases [9]. (iii) AZT-MP was the major metabolite (94–96%) of AZT. AZT-DP and AZT-TP constituted only an insignificant fraction of the nucleotide pool. On the other hand, TdR was distributed in all the nucleotides (TTP > TMP > TDP). Extremely low concentrations of AZT-DP and AZT-TP compared to AZT-MP indicated a block at the TMP-kinase step, which was in agreement with the observations that AZT-MP was a poor substrate and a potent inhibitor of TMP-kinase [4].

Metabolic differences were also seen between H_9-AZT and H_9 cells. In addition to elevated TK activity, H_9-AZT cells had increased TMP-kinase and decreased 5′-nucleotidase activities. Taken together, these observations would suggest an increased anabolism of TdR and AZT. It was

Table 2. Distribution of thymidine and zidovudine in the "nucleotide pools" of H_9 and H_9-AZT cells

	Nucleoside	Nucleotides			
	TdR or AZT	Monophosphate	Diphosphate	Triphosphate	Total nucleotides
	Micromolar (% distribution)				
Thymidine					
H_9 cells	1.2 ± 0.6	6.7 ± 0.07	2.4 ± 1.3	8.7 ± 2.3	17.7 ± 3.5
	(6.0 ± 2.3)	(36.2 ± 8.3)	(11.9 ± 4.5)	(45.9 ± 1.4)	(94.0 ± 2.3)
H_9-AZT cells	0.7 ± 0.4	3.0 ± 1.4*	1.5 ± 0.06	4.3 ± 1.1*	8.8 ± 1.5†
	(7.2 ± 3.1)	(31.9 ± 12)	(16.2 ± 3.1)	(45.2 ± 8.6)	(92.8 ± 3.1)
Zidovudine					
H_9 cells	1.7 ± 0.3	45.4 ± 8.4	0.5 ± 0.1	0.5 ± 0.2	46.4 ± 8.5
	(3.6 ± 0.5)	(94.1 ± 1.0)	(1.1 ± 0.4)	(1.0 ± 0.2)	(96.4 ± 0.5)
H_9-AZT cells	0.8 ± 0.3	30.8 ± 5.5*	0.3 ± 0.2	0.3 ± 0.2	31.5 ± 5.7*
	(2.3 ± 0.5)	(95.8 ± 1.1)	(0.9 ± 0.3)	(1.0 ± 0.4)	(97.7 ± 0.5)

Methanol "soluble" fraction was dried, reconstituted, and analyzed by HPLC. Fractions (1.0 mL) were collected and counted. Percent distribution in the peaks corresponding to the nucleosides and nucleotides was calculated from the total counts recovered from HPLC. Concentrations (μM) in individual peaks were calculated based on the total concentration in the soluble fraction (Table 1). Except for the thymidine nucleoside and nucleotide values of H_9 cells (mean ± range of two different experiments), all other values are means ± SD of three experiments.

* Significantly different from H_9 cells, $P < 0.05$.

† Significantly different from H_9 cells, $P < 0.01$.

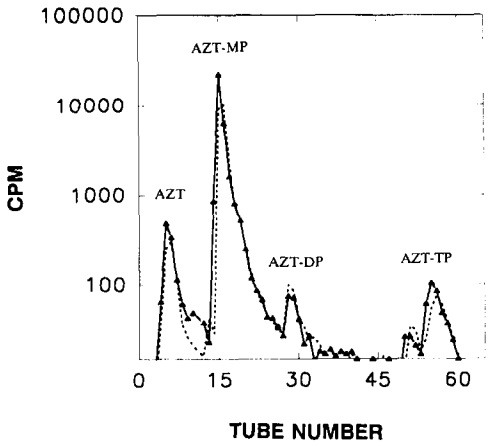

Fig. 2. HPLC profiles of incorporation of 10 μM [^{3}H]AZT in the nucleotide pools of H_9 (——) and H_9-AZT (----) cells as described in Fig. 1. The y-axis is presented as a log scale.

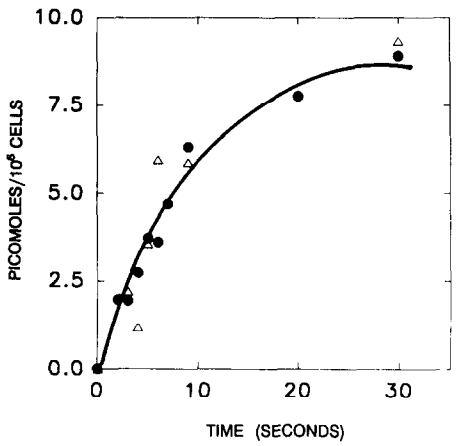

Fig. 3. Time dependence of influx of thymidine in H_9 and H_9-AZT cells. One million cells (0.4 mL) were incubated with 55 μL of 100 μM [^{3}H]thymidine (100 mCi/mmol) at room temperature. The reaction was stopped by the addition of an oil mixture (0.7 mL) and centrifugation. The cell pellet was solubilized and counted. The amount of thymidine inside the cells was calculated by assuming a volume of 1.16 μL/10^6 cells [16]. Key: H_9 cells (●); and H_9-AZT cells (△).

surprising to note that rather than increasing, the salvage of both the metabolites was decreased (53.6%) in H_9-AZT cells, causing reduced intracellular accumulation of TdR and AZT nucleotides. The mechanism(s) of this unexpected behavior is presently unclear. It is, possible, however, that chronic exposure of cells to AZT may have altered their nucleoside transport and/or that accumulated AZT metabolites in these cells caused inhibition of the salvage. TdR and AZT permeate the cell membrane by different mechanisms; whereas the former is transported by a facilitated carrier mediated nucleoside transport system, the latter permeates the cell membrane by non-facilitated diffusion [17].

Overlapping transport curves (Fig. 3) and similar rates of influx of TdR (0.66 to 0.68 pmol/sec/10^6 cells) in both cell lines ruled out altered influx as a cause of the difference. Cell membranes are considered impermeable to nucleotides, but a recent report has shown that AZT-MP is excreted from cells treated with AZT [11, 18]. Whether chronic exposure to AZT causes alteration in the excretion of AZT-MP and TMP, was not studied here, but it should be examined.

Although the cells were washed prior to the experiment, the H_9-AZT cells were maintained in a medium containing 25 μM AZT; therefore, they must have accumulated AZT metabolites, especially AZT-MP. Being a poor substrate and a potent inhibitor of cellular TMP-kinase ([4], this study), AZT-MP may have caused inhibition of the enzyme, thus depleting TTP concentrations.

AZT treatment has been shown to disturb the cellular deoxynucleotide pool and decrease TTP concentrations [4, 11, 18, 19]. Frick *et al.* [11] have reported a more pronounced and prolonged decline of the TTP pool which returned to a normal level by 24 hr. Another laboratory found that exposure of H_9, MOLT-4 and CEM cells to 50 and 200 μM AZT caused only a small (25%) decrease in TTP levels [18], but the decrease was significant in phytolhemagglutinin-stimulated human lymphoblasts [19]. These reports indicate that the effect of AZT on TTP varies among different cells. Although, in the present studies, we did not measure the TTP pool size, a decrease in TdR and AZT salvage in chronically AZT-exposed cells is consistent with the results of these reports.

Based on our present studies the following important conclusions may be drawn. First, in addition to thymidine kinase, TMP-kinase activity also appears to play a crucial role in the anabolism of AZT. Therefore, a detailed study of the structure–activity relationship of TMP-kinase is warranted which may help in the design of AZT analogs with improved antiviral activity.

Second, reduction of TTP concentrations, a competitor of AZT-TP, may enhance the inhibitory potential and/or incorporation into viral DNA of the latter, thus enhancing the chemotherapeutic efficacy of AZT. Another important issue that has not been explored yet is the role of AZT-MP [4], a metabolite which is accumulated at high concentrations.

Third, since chronic AZT exposure causes severe reduction in thymidine salvage and thymidine nucleotide pools, it is possible that the long-term toxicities seen in AIDS patients on chronic AZT therapy [20, 21] may have resulted from disturbances of thymidine metabolism in host tissues. This hypothesis needs further exploration by examining whether similar changes occur in patients who are on continuous AZT chemotherapy.

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