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3′-AZIDO-3′-DEOXYTHYMIDINE CYTOTOXICITY AND METABOLISM IN THE HUMAN COLON TUMOR CELL LINE HCT-8

JAMES W. DARNOWSKI* and FREDERICK A. GOULETTE

Department of Medicine, Brown University and the Roger Williams Hospital, Providence, RI 02908, U.S.A.

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Abstract—We have reported that 3′-azido-3′-deoxythymidine (AZT) possesses significant cytotoxicity in human tumor models when combined with agents that inhibit *de novo* thymidylate (dTMP) synthesis, such as 5-fluorouracil (FUra) and methotrexate (MTX). To aid in the further development of these and related cancer chemotherapeutic regimens, this study was undertaken to identify the biochemical processes relevant to the induction of AZT cytotoxicity in the model human colon tumor cell line HCT-8. The IC_{50} of AZT in this cell line after a 5-day exposure was 55 μM. In cells incubated for 5 days with various concentrations of [^{3}H]AZT alone, both [^{3}H]AZT nucleotide pools and [^{3}H]AZT incorporation into DNA increased as the concentration of AZT in the medium increased. In addition, a 5-day exposure to AZT, at medium concentrations ≤100 μM, resulted in a reduction in dTMP synthase (EC 2.1.1.45; methylene tetrahydrofolate:deoxyuridine-5′-monophosphate C methyltransferase) and dThd kinase (EC 2.7.1.27; ATP: thymidine phosphotransferase) activities, compared with cells incubated without drug. The IC_{50} of AZT was unchanged when the medium concentration of dThd was increased from 0.1 to 50 μM. Increasing the concentration of dThd to 50 μM also did not affect intracellular pools of [^{3}H]AZTDP and [^{3}H]AZTTP or the degree to which [^{3}H]AZT was incorporated into cellular DNA, but did reduce intracellular [^{3}H]AZTMP by ~75%. The degree to which 3′-amino-3′-deoxythymidine (AMT) was generated from AZT and incorporated into DNA also was not affected by varying the medium concentration of dThd. However, the amount of [^{3}H]-AMT detected in DNA, ≤3 pmol/10^6 cells at medium concentrations of [^{3}H]AZT ≤100 μM, was below that associated with significant cytotoxicity in these cells. These data support the notion that, in this model, AZT cytotoxicity is determined by the relative size of AZTTP pools and its utilization in DNA synthesis. Studies to verify this relationship assessed the effect of alterations in the concentration of dTTP and [^{3}H]AZTTP on [^{3}H]AZT incorporation into newly synthesized DNA *in vitro*, using DNA polymerases isolated from HCT-8 cells. The results of these studies confirmed that alterations in the concentration of either dTTP or AZTTP to reduce the dTTP/AZTTP ratio resulted in an increase in AZT incorporation into DNA. These findings are discussed in light of their biochemical implications and relevance to ongoing clinical trials.

Key words: AZT; AMT; cytotoxicity; nucleotides; DNA

AZT† is a dThd analogue that has clinical utility in the treatment of AIDS and AIDS-related complex [1–3]. Recent studies have suggested that AZT can possess anticancer activity when combined with agents that disrupt *de novo* dTMP synthesis. Studies by Brunetti *et al.* [4] demonstrated that AZT-induced cytotoxicity and incorporation into the nucleic acid fraction of HCT-8 human colon tumor cells are increased in the presence of FUra. Similarly, Tosi and coworkers [5] observed that the combination of MTX and AZT exerts significant *in vitro* and *in vivo* activity in the HCT-8 model. In their studies, Pressacco and Erlichman [6] reported that the combination of AZT and ICI D1694, a water-soluble inhibitor of *de novo* dTMP synthesis, causes enhanced cytotoxicity and increased AZT incorporation into DNA in both the HCT-8 and MGH-G1 human tumor lines. Recently, Tosi *et al.* [7] also reported synergystic cytotoxicity, associated with increased AZT incorporation into DNA, in selected human chronic myeloid leukemia models exposed to the combination of hydroxyurea and AZT.

As a result of our early findings, a comprehensive clinical analysis of AZT combined with FUra and LV was initiated [8–12]. Initial Phase I studies revealed that the combination of a 72-hr infusion of FUra with either oral or infused AZT (over the final 48 hr of FUra) was well tolerated but limited by the maximal dose that could be delivered [8, 9]. More recent Phase I studies, utilizing a new formulation of AZT, examined the combination of bolus FUra followed 1 hr later by a 2-hr infusion of high-dose AZT [10–12]. The observed maximally tolerated

* Corresponding author: Dr. James W. Darnowski, Department of Medicine, Brown University, Roger Williams Hospital, 825 Chalkstone Ave., Providence, RI 02908. Tel. (401) 456-2369; FAX (401) 456-2016.

† Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AMT, 3′-amino-3′-deoxythymidine; FUra, 5-fluorouracil; MTX, methotrexate; dThd, thymidine; AZTMP, 3′-azido-3′-deoxythymidine-5′-monophosphate; AZTDP, 3′-azido-3′-deoxythymidine-5′-diphosphate; AZTTP, 3′-azido-3′-deoxythymidine-5′-triphosphate; LV, leucovorin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PCA, perchloric acid; HCT-8, human colon tumor 8; and AIDS, acquired immunodeficiency syndrome.

dose of AZT by this route was 7–10 g/M^2, and the regimen possessed suggestive clinical activity. Phase II analysis of this regimen in patients with metastatic colon cancer is underway.

Clearly, the future development of AZT-containing antineoplastic combinations depends upon an understanding of the mechanism(s) by which AZT induces cytotoxicity in human tumor models. At the present time there is no consensus concerning the biochemical processes associated with AZT cytotoxicity. Studies by Frick *et al.* [13] and others [1] suggested that *in vitro*, in normal human fibroblasts and human leukemia cells, AZT cytotoxicity is related to its ability to reduce dThd nucleotide pools and generate large intracellular pools of AZTMP. In contrast, Sommadossi *et al.* [14] and Fridland and coworkers [15] demonstrated, in human bone marrow models, that AZT induces only a transient disruption of deoxyribonucleotide pools. Further, Sommadossi's group presented evidence that cytotoxicity is related to AZT incorporation into DNA [14]. More recent studies by this group suggest that the formation of AMT, an AZT metabolite, may be responsible for a significant portion of AZT-related cytotoxicity *in vitro* in human bone marrow cells [16, 17]. In our group, studies in human colon tumor cells exposed to [^{3}H]AZT ± FUra or MTX suggested that increased AZT cytotoxicity in the presence of these agents correlated with increased AZT incorporation into DNA and could be reversed by increasing the medium concentration of dThd [4, 5, 18–20]. Alternatively, Weber *et al.* [21, 22] have suggested that the enhanced cytotoxicity of AZT plus FUra or MTX reflects the ability of AZT to inhibit dThd kinase (EC 2.7.1.27; ATP: thymidine phosphotransferase) and complement the biochemical effects of inhibitors of *de novo* dTMP biosynthesis.

The present study was undertaken to examine the role of each of these mechanisms in the induction of AZT cytotoxicity in the human colon tumor cell line HCT-8. We found that cytotoxicity correlated most closely with the size of intracellular pools of AZTDP and AZTTP and the amount of AZT incorporated into total cellular DNA. These results indicate that AZT cytotoxicity can be manipulated by agents that alter intracellular pools of either AZTTP or dTTP, and are discussed in light of their relevance to cancer therapy. Preliminary aspects of these studies have been reported [18–20].

MATERIALS AND METHODS

Drugs. AZT was a gift of the Burroughs Wellcome Co. (Research Triangle Park, NC) and AMT was supplied by Dr. J. P. Sommadossi. [^{3}H]AZT (56 Ci/mmol), [^{3}H]AMT (14 Ci/mmol), [^{3}H]AZTTP (14 Ci/mmol) and non-labeled AZTTP were obtained from Moravek Biochemicals (Brea, CA). Thymidine was purchased from the Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium, FBS and dialyzed FBS (mol. wt cutoff = 12,000) were purchased from Gibco (Grand Island, NY). RNase-free DNase I and alkaline phosphatase were from Boehringer–Mannheim (Indianapolis, IN). Activated calf thymus DNA was purchased from Worthington Biochemicals (Freehold, NJ), and DEAE Sephadex (A25) and DNA-cellulose were obtained from Pharmacia (Piscataway, NJ). HPLC grade solvents, chemicals and disposable tissue culture supplies were obtained from Fisher Scientific (Medford, MA).

Cell line. Continuous cultures of HCT-8 human colon adenocarcinoma cells, obtained from the American Type Culture Collection, were used in these studies. The biochemical and histological characterization of this cell line has been reported [23]. Cells were cultured in sterile plastic tissue culture flasks as monolayers in RPMI 1640 medium supplemented with 10% FBS and subcultured twice weekly. Cell cultures were maintained in a humidified incubator at 37° in an atmosphere of 5% CO_2. Under these conditions, their doubling time was ~20 hr, and cells in logarithmic growth were used in all studies.

In vitro *evaluation of cytotoxicity.* HCT-8 cells (1×10^5) were added to 10 mL of RPMI 1640 medium containing 10% dialyzed FBS in 25 cm^2 culture flasks to which dThd had been added to achieve a final concentration of 0.1 μM. AZT, AMT, and additional dThd, previously dissolved in medium, were added at concentrations of 0.1 to 150 μM (AZT and AMT) and 50 μM (dThd), either alone or in various noted combinations. Control cultures received the same amount of medium without drug. After 5 days, cells were harvested, and growth inhibition was determined as described previously [4, 5]. Each experiment was performed in duplicate and repeated a minimum of four times.

Assessment of enzyme activities and cell cycle distribution. Thymidylate synthase (EC 2.1.1.45; methylene tetrahydrofolate:deoxyuridine-5′-monophosphate C methyltransferase) activity was measured in intact cells after a 5-day incubation in medium containing various concentrations of AZT by quantitation of ^3H_2O released from [5-^{3}H]dUrd, as previously described in detail [4, 24]. The effect of a 5-day exposure to AZT on cellular dThd kinase activity was assessed by TLC methods as we have reported elsewhere [25]. To assess the effect of a 5-day exposure to AZT on the percentage of cells in various stages of the cell cycle, cells were harvested, washed three times in PBS, and incubated in PBS containing 0.7 mg propidium iodide/mL for 45 min at 4° in the dark. Cellular DNA content was then quantitated on a FACScan (Becton–Dickinson) using the cellFIT analytical program.

Quantitation of intracellular AZT-nucleotide and dTTP pools. The effect of various concentrations of [^{3}H]AZT and dThd on the generation of [^{3}H]AZT nucleotides was quantitated by the incubation of ~2×10^6 cells in 15 mL medium containing 1–100 μM [^{3}H]AZT alone (0.5–2.0 mCi/mmol) or [^{3}H]AZT (1–100 μM) plus 50 μM dThd. After 5 days, the cells ($2–5 \times 10^7$) were harvested and homogenized in 1 mL of 0.2 M PCA. The PCA-insoluble material was saved to quantitate [^{3}H]AZT incorporation into DNA, as described below. The PCA-soluble material was analyzed to quantitate [^{3}H]AZT nucleotides by previously reported HPLC methods [5, 26].

The effect of AZT and exogenous dThd on dTTP pools was quantitated by a modification of the above

method in which ~2.0×10^6 cells were incubated in 50 mL of medium containing selected drug combinations. After 5 days the cells ($2–5 \times 10^7$) were harvested and homogenized in 0.5 mL of 0.2 M PCA. The acid-soluble material was removed and neutralized with KOH, and ribonucleotides were eliminated with sodium periodate [5, 27]. To assure complete removal of interfering ribonucleotides, standards containing 1–25 μM dTTP ± 100 μM UTP were also processed with each set of cell extracts. The dTTP content of cell extracts and standard solutions was quantitated by HPLC [5], and the dTTP peak areas for all standards were compared. The retention times for UTP, dTTP and dUTP were 40.5, 42.4 and 43.5 min, respectively, with peak area analysis and baseline placement aided by utilization of the Rainin (Woburn, MA) Dynamax HPLC data analysis program. Under these conditions, the lower level of detectability for dTTP was ~30 pmol.

Quantitation of [^{3}H]*AZT or* [^{3}H]*AMT incorporation into DNA.* The degree to which [^{3}H]AZT was incorporated into nuclear DNA after a 5-day exposure to [^{3}H]AZT, and the effect of exogenous dThd on this parameter, were quantitated in the PCA-insoluble material generated above, as previously described in detail [5].

[^{3}H]AMT incorporation was quantitated by this same method with the exception that cells were incubated for 5 days in 15 mL of medium containing 20 or 50 μM [^{3}H]AMT alone (2 μCi/mL).

Assessment of the relative incorporation of [^{3}H]-*AMT into DNA.* The relative percentages of AZT and AMT incorporated into DNA were assessed by a modification of the above method in which cells were incubated in medium containing 1–100 μM [^{3}H]AZT (1.5–8.0 μCi/mmol) and either 0.1 or 50 μM dThd. After 5 days, cells were harvested, washed three times in 1 mL of 0.2 M PCA, once in 350 μL of 0.1 M sodium acetate–5 mM $MgSO_4$ (pH 5.0), and incubated at 37° in 300 μL of 0.1 M sodium acetate–5 mM $MgSO_4$ (pH 5.0) containing 300 U of RNase-free DNase I [5]. After 1 hr, 300 μL of supernatant was removed, the pH adjusted to 8.5 with 10 N NaOH and to this was added 300 μL of 100 mM Tris (pH 8.1 at 37°) + 0.2 mM EDTA containing 20 U of alkaline phosphatase [28, 29]. After a 1-hr incubation at 37°, the pH was adjusted to ~5 with 14.7 N phosphoric acid, and the samples were stored at −20°. [^{3}H]AZT and [^{3}H]AMT were quantitated by a modification of previously reported HPLC methods [30] in which 450 μL samples were injected onto a computer-controlled Rainin modular HPLC system fitted with a Rainin Microsorb C18 analytical column (4.6 mm × 25.0 cm) maintained at 25° and eluted at 1 mL/min with a 30-min linear gradient of 0–35% CH_3CN in 25 mM phosphoric acid (pH 7.2). Column eluent was monitored by both an Gilson UV detector (254 nm) and an in-line Radiomatic scintillation detector (model A100). Under these conditions, the retention times of AMT and AZT were 14.8 and 26.4 min, respectively.

Isolation of cellular DNA polymerases and in vitro *assessment of AZT incorporation into newly synthesized DNA.* DNA polymerases were isolated and assayed by a modification of previously described methods [31]. Ten 150-cm^2 flasks containing HCT-8 cells were harvested and resuspended in 3 mL of 10 mM potassium phosphate (pH 7.5) + 2 mM DTT + 2 mM EDTA + 1 mM PMSF + 1 μg/mL pepstatin A + 1 μg/mL leupeptin + 10% glycerol (buffer A) containing 1 M KCl. Next, cells were lysed by freeze/thawing three times and then centrifuged at 4° for 30 min at 100,000 *g*; the supernatant was washed into a 50 mL DEAE column with 50 mL of buffer A. Adsorbed protein was eluted with 60 mL of 400 mM potassium phosphate (pH 7.5) + 2 mM DTT + 2 mM EDTA + 1 mM PMSF + 1 μg/mL pepstatin + 1 μg/mL leupeptin + 10% glycerol. Fractions containing protein, determined by the method of Bradford [32], were pooled and dialyzed overnight against buffer A. The dialyzed material was centrifuged at 4° for 30 min at 100,000 *g*, loaded onto an 8-mL DNA-cellulose Native DNA column, and eluted with 20 mL of buffer A containing 1 M KCl. The protein content of the eluted material was determined, and those fractions containing measurable protein were pooled and dialyzed overnight against 50 mM Tris (pH 8.0 at 37°); their volume was reduced to 1.5 mL using an Amicon concentrator.

The effect of alterations in the ratio of dTTP/[^{3}H]AZTTP on the incorporation of [^{3}H]AZTTP into newly synthesized DNA was determined [33] by adding from 10 to 50 μL of the above-generated material to 15 μg of activated calf thymus DNA, 100 μM (final concentration) dATP, dCTP and dGTP in 50 mM Tris (pH 8.0 at 37°) to achieve a final volume of 90 μL. The reaction was initiated by adding 10 μL of various concentrations of (dTTP + [^{3}H]AZTTP) and terminated at various times thereafter by the addition of 100 μL of ice-cold 15% TCA. Acid-insoluble material was collected on Whatman GF/A glass microfiber filters that were then washed with 30 mL of ice-cold 15% TCA. The degree to which AZT was incorporated into DNA, represented by cpm retained on the filters, was quantitated by liquid scintillation.

RESULTS

The IC_{50} of AZT in this HCT-8 cell line after a 5-day exposure to drug was ~55 μM. The lower cytotoxicity of AZT, compared with that found in our previous study [5], may reflect the fact that the cells used in the present study were obtained recently from ATCC. Nevertheless, the IC_{50} presently observed was unaffected when the medium concentration of dThd was increased from 0.1 to 50.0 μM (Fig. 1). This confirms our previous finding that increasing the medium concentration of dThd could reduce the cytotoxicity of AZT plus MTX but did not appear to affect the cytotoxicity of AZT alone [5]. Since AZT is anabolized as a dThd analogue [1], but its cytotoxicity was unaffected by increasing the medium concentration of dThd 500-fold, we postulated that analyzing selected aspects of AZT metabolism in cells incubated in medium containing either 0.1 or 50 μM dThd could reveal those AZT metabolites or metabolic processes most closely associated with cytotoxicity. As an extension of our previous study, and to more closely reflect the conditions employed in our cytotoxicity assays, we

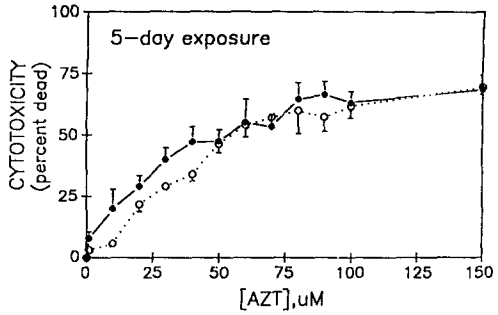

Fig. 1. Effect of dThd on the growth inhibitory effect of AZT in HCT-8 cells. Cells were incubated in various concentrations of AZT in medium containing dialyzed FBS plus either 0.1 μM (●) or 50 μM (○) dThd. After 5 days, cells were harvested, and the cell number was determined. Cytotoxicity is presented as the percentage of the control (no AZT) cell number, with each point representing the mean ± SEM of 10–14 determinations.

Table 1. Effect of various concentrations of AZT on cellular dTMP synthase and dThd kinase activities

AZT concn (μM)	dTMP synthase (pmol/hr/10^6 cells)	dThd kinase (nmol/hr/mg protein)
None	3.7 ± 0.5	143.5 ± 17.9
20	3.0 ± 0.6	113.7 ± 10.7
35	3.1 ± 0.5	111.4 ± 4.6
50	3.1 ± 0.6	106.6 ± 13.8
100	3.0 ± 0.7	102.3 ± 39.5

Cells (5×10^6) were incubated for 5 days in 50 mL of RPMI 1640 medium containing 10% dialyzed FBS, 0.1 μM dThd and the noted concentrations of AZT. To assess dTMP synthase activity, cells were harvested and resuspended at 1×10^7 cells/mL in RPMI 1640 medium; the assay was initiated by the addition of 200 μL of cell suspension to 30 μL of 8.3 μM [5-^{3}H]dUrd (30 μCi/mL) at 37°. Enzyme activity was quantitated by assessment of the formation of ^3H_2O as a function of time [5, 25]. To assess dThd kinase, cells were harvested, homogenized in 50 mM Tris (pH 7.4 at 37°) and centrifuged at 100,000 *g* for 1 hr. The enzyme reaction was initiated by incubating 10 to 30 μL aliquots of the resulting supernatant in a mixture of 200 μM [^{14}C]dThd (10 μCi/mL), 1 mM ATP and 50 mM Tris (pH 7.4 at 37°) in a final volume of 100 μL for various times (≤10 min). Activity was determined by quantitation of the formation of [^{14}C]dThd nucleotides by TLC methods [25]. Each value is the mean ± SEM of 6–9 (dTMP synthase) or 4 (dThd kinase) determinations, and activities following AZT exposure were not significantly different ($P \geq 0.1$, Student's *t*-test) than control cells.

chose to conduct these studies in cells exposed to AZT for 5 days.

Initial studies determined the effect of a 5-day exposure to various concentrations of AZT on the activities of key enzymes involved in dThd nucleotide metabolism, namely dTMP synthase and dThd kinase. In support of the findings of Brunetti *et al.* [4], these studies revealed (Table 1) that exposure to 20–100 μM AZT inhibited dTMP synthase activity by ~20%. In addition, and in agreement with the findings of Weber *et al.* [21], dThd kinase activity also was inhibited by up to 30%, but in an AZT concentration-dependent manner.

Studies next assessed the biochemical consequences of these effects by quantitation of intracellular pools of dTTP following incubation in various concentrations of AZT. In contrast to our previous findings in which we observed that dTTP pools were unaffected following a 1-day exposure to AZT, incubating cells for 5 days in concentrations of AZT ≤ 100 μM resulted in an AZT concentration-dependent decrease in dTTP pools, which were maximally reduced by 37% (Table 2). This finding may reflect the ability of AZT to inhibit both dTMP synthase and dThd kinase. Alternatively, reduced dTTP pools may reflect AZT-induced perturbations in the progression of cells through the cell cycle [34]. In this regard, however, cytofluorographic analysis of cells exposed to AZT for 5 days revealed no significant alterations in the percentage of HCT-8 cells in G_1, S, G_2 or M phase when compared with cells not exposed to AZT (data not shown). In any event, since increasing the medium concentration of dThd could increase intracellular dTTP (Table 2) without affecting AZT cytotoxicity, AZT-induced alterations in these pools appear not to be a major factor in AZT-induced cell death.

Studies next quantitated AZT nucleotide production under these conditions and revealed that, independent of the extracellular concentration of dThd, the sizes of intracellular AZTDP and AZTTP pools were nearly equal and increased as the medium concentration of AZT increased (Table 2). In contrast, intracellular AZTMP was from 6- to 14-fold greater than either the di- or triphosphate moiety (Table 2), but was reduced by ~75% when the concentration of dThd was increased. This relationship between the medium concentration of AZT and intracellular pools of AZTDP and AZTTP supported our earlier findings [5] and suggested that changes in AZT-related cytotoxicity may be related to changes in the size of intracellular pools of AZTDP and AZTTP. Unexpected, however, were the observations that AZTMP pools were significantly smaller than observed after a 1-day exposure and were reduced further when the medium concentration of dThd was increased. Presumably, the lower AZTMP pools observed after a 5-day exposure reflect the cumulative result of an AZT-mediated 5-day inhibition of dThd kinase. In this regard, exposing these ATCC-derived cells to AZT for only 1 day resulted in significantly larger AZTMP pools that were relatively insensitive to alterations in the medium concentration of dThd (data not shown). Nevertheless, since cytotoxicity was maintained in the presence of 50 μM dThd, in spite of large alterations in intracellular pools of AZTMP, these pools appear not to be associated with cytotoxicity in this model.

Next evaluated was the effect of alterations in the medium concentrations of dThd and AZT on the degree to which [^{3}H]AZT was incorporated into total cellular DNA. These studies revealed that, over a wide range of AZT concentrations and

Table 2. Effect of various concentrations of AZT alone or AZT plus 50 μM dThd on intracellular pools of AZT nucleotides and dTTP

Drug concn	Nucleotides (pmol/10^6 cells)				
	AZTMP	AZTDP	AZTTP	dTTP	TP-ratio*
None				35.6 ± 7.0	
+dThd, 50 μM				39.5 ± 2.8	
AZT, 1 μM	0.87 ± 0.13	0.15 ± 0.02	0.10 ± 0.02	32.6 ± 3.8	326:1
+dThd, 50 μM	0.19 ± 0.04†	0.13 ± 0.02	0.10 ± 0.01	38.4 ± 5.8	384:1
AZT, 20 μM	16.84 ± 2.89	1.79 ± 0.39	1.72 ± 0.35	26.7 ± 2.5	16:1
+dThd, 50 μM	4.46 ± 0.89†	2.08 ± 0.59	1.78 ± 0.29	31.0 ± 4.6	17:1
AZT, 50 μM	28.12 ± 1.33	2.64 ± 0.36	2.89 ± 0.48	22.0 ± 1.6	8:1
+dThd, 50 μM	6.86 ± 0.77†	4.22 ± 0.87	3.23 ± 0.72	25.7 ± 6.3	8:1
AZT, 100 μM	75.65 ± 9.17	5.42 ± 1.29	4.72 ± 0.28	22.1 ± 4.1	5:1
+dThd, 50 μM	16.66 ± 1.64†	6.40 ± 1.15	5.63 ± 0.43	26.2 ± 3.2‡	5:1

Cells (5–20 × 10^6) were incubated in 10–50 mL of RPMI 1640 medium containing 10% dialyzed FBS, 0.1 μM dThd and the noted concentrations of [^{3}H]AZT alone (2.0 to 3.3 μCi/mL), 50 μM dThd alone, or their various combinations. After 5 days, the cells were harvested and processed to quantitate AZT nucleotides and dTTP by HPLC methods, as described in the text. Each value is the mean ± SEM of 6 determinations and statistical analysis utilized Student's *t*-test with $P \leq 0.05$ considered significant.

* [dTTP]/[AZTTP].

† $P \leq 0.05$ vs same [AZT] + 0.1 μM dThd.

‡ $P \leq 0.05$ vs no AZT + 50 μM dThd.

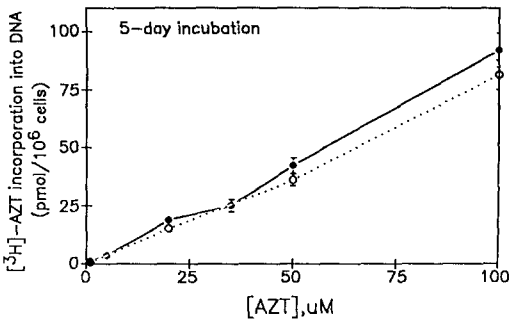

Fig. 2. Effect of dThd, 0.1 μM (●) or 50 μM (○), on the incorporation of [^{3}H]AZT into the DNA fraction of HCT-8 cells. HCT-8 cells (2 × 10^6) were incubated in 15 mL of RPMI 1640 medium containing 10% dialyzed FBS, the stated concentration of dThd, and [^{3}H]AZT (1–100 μM, 0.5–2.0 μCi/mmol). After 5 days, the cells were harvested and homogenized in 1 mL of 0.2 M PCA. The acid-insoluble material was removed, washed and incubated three times in 300 U of RNase-free DNase I, as described in the text. The [^{3}H]-content of the pooled DNase I hydrolyzable material was determined by liquid scintillation techniques and normalized to harvested cell number. Each point is the mean ± SEM of 6 determinations.

independent of the medium concentration of dThd, there appeared to be a near linear relationship between the extracellular concentration of [^{3}H]AZT and the degree to which it was incorporated into cellular DNA (Fig. 2). Incubation in medium containing 20, 50 or 100 μM [^{3}H]AZT resulted in ~20, ~40 or ~90 pmol [^{3}H]AZT incorporated/10^6 cells.

These findings suggest that as intracellular AZTTP increases, AZT incorporation into DNA also increases. We also have observed that as the intracellular concentration of dTTP decreases AZT incorporation into DNA increases [5]. To assess further the relationship between AZT incorporation into DNA and pools of dTTP and AZTTP, total cellular DNA polymerases were isolated from these cells and used to monitor the effect that alterations in the dTTP/[^{3}H]AZTTP ratio had on [^{3}H]AZT incorporation into newly synthesized DNA *in vitro*, using calf thymus DNA as a template. The results of these studies clearly indicated that as the dTTP/[^{3}H]AZTTP ratio was decreased from 20:1 to 1:1, reflecting either a decrease in the concentration of dTTP or an increase in AZTTP, AZT incorporation into newly synthesized DNA increased proportionally (Table 3).

Finally, Sommadossi and coworkers [16, 17] have suggested that the intracellular conversion of AZT to AMT also may be partially responsible for the induction of cytotoxicity. As with AZT, the exact mechanism(s) involved in the induction of AMT cytotoxicity is yet to be defined [17, 35]. Nevertheless, one potential mechanism is its ability to be incorporated into DNA and disrupt chain elongation. Studies were conducted, therefore, to assess the relative percentages of AMT and AZT incorporated into DNA after a 5-day incubation in medium containing 0.1 or 50 μM dThd and various concentrations of [^{3}H]AZT. The results revealed that, independent of the concentration of dThd, at medium concentrations of AZT > 35 μM, a relatively constant amount of AMT, ~3 pmol/10^6 cells, was incorporated into DNA (Fig. 3). Since it has been reported that, in human marrow cells, AMT is more toxic than AZT [16, 17], this relatively small amount

Table 3. Effect of alterations in the concentration of dTTP and [^{3}H]AZTTP on the *in vitro* incorporation of AZT into newly synthesized DNA

[^{3}H]AZTTP (μM)	dTTP (μM)	TP-ratio*	AZT incorporated into DNA (fmol/15 μg DNA/hr)
1.0	20.0	20/1	1.44 ± 0.64
5.0	20.0	4/1	7.42 ± 1.92
10.0	20.0	2/1	20.04 ± 7.34
20.0	20.0	1/1	37.34 ± 16.20
20.0	50.0	2.5/1	16.16 ± 3.80
20.0	100.0	5/1	8.30 ± 1.92

Total cellular DNA polymerases were partially purified from HCT-8 cells contained in ten 150-cm^2 tissue culture flasks, as described in Materials and Methods. The effect of alterations in the concentration of dTTP and [^{3}H]AZTTP on [^{3}H]AZT incorporation into newly synthesized DNA was then assayed by adding aliquots of the above-generated polymerases to 15 μg of activated calf thymus DNA, dATP, dCTP and dGTP (final concentration = 100 μM) and the noted concentrations of ([^{3}H]-AZTTP + dTTP), all contained in a final volume of 100 μL in 50 mM Tris (pH 8.0 at 37°). The reaction was terminated by the addition of ice-cold 15% TCA; acid-insoluble material was collected on Whatman GF/A filters and [^{3}H]AZT incorporation into DNA was quantitated by liquid scintillation techniques. Incorporation values are the means ± SEM of 3–8 determinations.

* [dTTP]/[AZTTP].

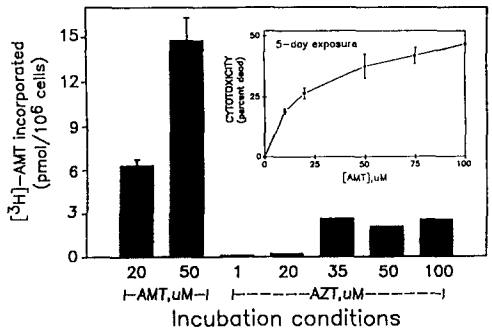

Fig. 3. Assessment of [^{3}H]AMT incorporation into DNA in HCT-8 cells. HCT-8 cells (2×10^6) were incubated in 15 mL of RPMI 1640 medium containing 10% dialyzed FBS and either [^{3}H]AMT (20 or 50 μM, 3 μCi/mL) + 0.1 μM dThd, or the stated concentrations of [^{3}H]AZT (0.5–2.0 μCi/mmol) + 0.1 or 50 μM dThd. After 5 days, all cells were harvested and homogenized in 1 mL of 0.2 M PCA. For cells incubated in [^{3}H]AMT, the acid-insoluble material was assessed to quantitate tritiated material in DNA, as described in the legend of Fig. 2. Each bar represents the mean ± SEM of 6 determinations. For cells incubated in [^{3}H]AZT, the acid-insoluble material was removed, washed and incubated in 300 U of RNase-free DNase I. After 1 hr, the insoluble material was removed, the pH of the supernatant was adjusted to 8.1, and the supernatant was incubated in the presence of 20 U of alkaline phosphatase for 1 hr at 37°. Hydrolyzed [^{3}H]AZT and [^{3}H]AMT were quantitated by HPLC methods, as described in the text. Each bar represents the pooled data (N = 4–8) from cells incubated in either 0.1 or 50 μM dThd. Inset: Growth inhibitory effect of AMT in HCT-8 cells. Cells were incubated in various concentrations of AMT in medium containing dialyzed FBS plus 0.1 μM dThd. After 5 days, cells were harvested, and the cell number was determined. Cytotoxicity is presented as the percentage of the control (no AMT) cell number, with each point representing the mean ± SEM of 4–8 determinations.

of AMT may indeed induce cytotoxicity in these cells. To assess this possibility, an examination of the relationship between AMT cytotoxicity and incorporation into DNA in this cell line was also undertaken. The IC_{50} of AMT after a 5-day incubation was found to be ~100 μM (Fig. 3, inset). Incubating cells for 5 days in medium containing either 20 μM [^{3}H]AMT or 50 μM [^{3}H]AMT resulted in 6.1 or 15.3 pmol AMT incorporated into cellular DNA/10^6 cells (Fig. 3). Thus, the amount of AMT generated from AZT and incorporated into DNA ($\leq$3 pmol/10^6 cells) was well below that associated with the induction of significant cytotoxicity in this model.

DISCUSSION

Using a variety of *in vitro* model systems, several biochemical mechanisms have been invoked as responsible for the induction of AZT cytotoxicity [1, 4, 5, 13–22]. We have examined four proposed mechanisms and have determined that only the size of intracellular AZTDP and AZTTP pools and the degree to which AZT is incorporated into DNA correlated with cytotoxicity in the HCT-8 human colon tumor cell line. As with other antimetabolites, however, the precise mechanism(s) involved in the induction of cytotoxicity may be different in different cell lines and governed by factors such as duration of exposure and drug concentration [34]. Clearly, subtle pharmacological effects, apparent after chronic exposure to AZT, while relevant to the antiviral efficacy and long-term toxicity of this agent, appear to be less important when HCT-8 cells are exposed for a relatively short duration to high-dose AZT, conditions that are more appropriate for antimetabolite cancer chemotherapy.

The present findings generally support the conclusions drawn from our previous examination of the cytotoxic effects of AZT plus MTX after a 1-day exposure to these agents [5]. Results from that

study indicated that alterations in the intracellular ratio of dTTP and AZTTP could predict changes in both AZT cytotoxicity and incorporation into DNA. In our present study, as the medium concentration of AZT increased, the intracellular concentration of AZTTP also increased and dTTP pools were reduced slightly. The result was that the ratio of dTTP/AZTTP was reduced linearly over a wide range of medium concentrations of AZT (Table 2). This change in nucleotide ratios closely correlated with the near linear increase in the degree to which AZT was incorporated into DNA. Indeed, in our *in vitro* analysis of DNA synthesis (Table 3), a similar relationship was observed in that AZT incorporation into newly synthesized DNA was altered by manipulations in the ratio of dTTP/AZTTP. It is noteworthy that following a relatively short-term exposure to AZT (≤5 days), the dTTP/AZTTP ratio, AZT incorporation into DNA, and AZT-induced cytotoxicity were all relatively unaffected by large alterations in the medium concentration of dThd.

We were surprised to observe that after a 5-day exposure to AZT, AZTMP pools were relatively small and further reduced as the medium concentration of dThd was increased. Several mechanisms can be invoked to explain this observation. Lower AZTMP pools in the presence of elevated medium dThd may reflect competition between AZT and dThd at the level of membrane transport. Indeed, it has been reported that AZT competitively inhibits dThd transport in human red blood cells with a K_i of 1 mM [36]. This K_i, and the reported K_m for dThd (~230 μM) [36], are well above the medium concentrations of AZT and dThd presently employed and thus significant interactions at the level of transport in these 5-day studies seem unlikely. Also a possibility is that elevated medium dThd indirectly affected intracellular AZTMP pools via feedback inhibition of dThd kinase as dTTP pools were maintained at more normal levels [37–39]. Finally, it is possible that the relatively low AZTMP and dTTP pools observed after a 5-day exposure reflect the transient nature of the effect of AZT on these pools, as suggested by Sommadossi *et al.* [14] and Fridland *et al.* [15].

Our data also reveal that, unlike that observed in human marrow cells [16, 17], the conversion of AZT to AMT was not a major factor in the induction of cytotoxicity in this model. It must be stressed, however, that the exact mechanism of AMT cytotoxicity is yet to be defined and may involve mechanisms such as a direct effect on DNA polymerase activity [35, 40] or cellular protein synthesis [17].

Clinically, our laboratory findings to date support the concept that the potential antineoplastic activity of AZT, when administered either as a 2-hr bolus or a 48-hr infusion, is related to the degree to which AZT is incorporated into DNA. Our results also suggest that one way to modulate the cytotoxic and potential antineoplastic activity of AZT is by manipulating tumor pools of either AZTTP or dTTP. Our biochemical analysis of several AZT-based combinations [4, 5, 7] support this notion and suggest that there are several biochemical loci, such as dTMP synthase inhibition (with FUra or ICI D1694 [6]), dihydrofolate reductase inhibition (with MTX) and the inhibition of dThd transport and uptake (with aIFN [41–43] or dipyridamole [26, 44]), that can be exploited to enhance the therapeutic activity of AZT. Indeed, it is this rationale that has guided our clinical evaluation of AZT combined with FUra and LV [8–12] in patients with metastatic colon cancer. Clearly, further biochemical and clinical studies of these and related combinations are warranted to fully evaluate and exploit the antineoplastic potential of AZT.

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