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Comparative Effects of 3′-Azido-3′-deoxythymidine and Its Metabolite 3′-Amino-3′-deoxythymidine on Hemoglobin Synthesis in K-562 Human Leukemia Cells

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SUMMARY

We previously demonstrated that 3′-azido-3′-deoxythymidine (AZT) inhibits hemoglobin (Hb) synthesis and globin gene transcription in butyric acid-induced K-562 leukemia cells, suggesting that these effects may play a role in the AZT-induced anemia observed in patients [*Mol. Pharmacol.* **38**:797–804 (1990)]. The recent discovery by our group of a novel metabolite of AZT, 3′-amino-3′-deoxythymidine (AMT), which exhibits a high degree of toxicity toward human hemopoietic cells [*Mol. Pharmacol.* **39**:258–266 (1991); *Antimicrob. Agents Chemother.* **35**:801–807 (1991)], has led us to explore potential effects of this AZT metabolite on Hb production, globin mRNA expression, and heme synthesis in butyric acid-induced K-562 human erythroleukemia cells. AMT inhibited Hb synthesis by approximately 21%, as measured by benzidine staining, at concentrations as low as 25 μM, with slightly increased inhibition at higher AMT concentrations. The inhibition of Hb production by AMT was substantially lower, compared with that of AZT. AMT inhibited globin mRNA steady state levels in a dose-dependent manner to a similar extent as did the parent drug, with approximately 50% inhibition by each compound at a concentration of 100 μM. Nuclear run-on transcription assays demonstrated that inhibition by AMT of globin mRNA synthesis was associated with a decreased rate of globin-specific gene transcription. Globin mRNA stability was not affected by either 100 μM AZT or AMT, as measured after blockage of transcription with actinomycin D. To gain insight into potential mechanism(s) responsible for the different quantitative effects of AZT and AMT on Hb synthesis, the effect of each compound on induction of heme synthesis in K-562 cells was determined. Although heme induction was not affected by AMT, a significant inhibition approximating 20% was observed in the presence of 100 μM AZT. In addition, AZT down-regulated mRNA steady state levels under conditions where heme synthesis was inhibited by succinylacetone. These data suggest that inhibition by AZT of globin gene expression is a direct effect and is not secondary to inhibition of heme synthesis. This study emphasizes the role of AMT in the pharmacodynamic properties of AZT, in relation to its toxicity, and suggest that both AMT and AZT may be involved in the inhibition of erythroid differentiation observed *in vivo*, through changes in gene expression.

AZT, the first clinically approved drug for the treatment of AIDS, is therapeutically limited by its toxic side effects, primarily on bone marrow cells (1). A major side effect of AZT is anemia, which occurs in approximately 25% of AZT recipients and requires blood transfusions in a substantial number of patients (2). Although decreased AZT dosage has recently been evaluated, the percentage of patients developing these toxicities remained approximately the same (3, 4). In a recent study, our group evaluated the potential genetic mechanism(s) involved in AZT toxicity toward hemopoietic cells and demonstrated that AZT inhibits both Hb synthesis and globin gene transcription in butyric acid-induced K-562 leukemia cells (5). In contrast, DDC, which had a significantly greater cell-growth inhibition, had no measurable effect on Hb synthesis, consistent with the absence of DDC-induced anemia in patients (6). The inhibition of globin gene expression by AZT was associated with a decreased rate of globin gene transcription and was not a result of a general inhibition of mRNA transcription. These data suggest that inhibition of globin gene expression in erythroid cells may play a role in the anemia observed in AZT recipients. *In vivo* studies in a murine model of AIDS (7) and in cats infected with feline leukemia virus (8) demonstrated that administration of AZT resulted in a progressive anemia. Of importance, the number of immature cells of the erythroid lineage was increased and abnormalities were observed in their

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ABBREVIATIONS: AZT, 3′-azido-3′-deoxythymidine; AMT, 3′-amino-3′-deoxythymidine; DDC, 2′,3′-dideoxycytidine; CFU-GM, granulocyte-macrophage colony-forming unit; BFU-E, erythroid burst-forming unit; Hb, hemoglobin; MOPS, 3-*N*-morpholinopropanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

maturation, suggesting a blockage in terminal differentiation. These data are, thus, consistent with our *in vitro* results, which suggest important effects of AZT on the Hb production machinery.

AMT has recently been discovered by our laboratory as a metabolite of AZT in both human liver microsomes and hepatocytes, and its formation presumably occurs through intracellular reduction of the 3′-azido group by cytochrome P-450 isoenzyme(s) (9). Studies using human hemopoietic progenitor cells demonstrated that AMT was 5–7-fold more toxic for human CFU-GM and BFU-E, compared with AZT (9). Formation of AMT was also demonstrated *in vivo* both in rhesus monkeys (10) and in humans[1] after AZT administration, with the area under the curve values of AMT accounting for as much as 10–30% of the area under the curve values of the unchanged drug. The substantial AMT plasma levels *in vivo* suggest that this metabolite may contribute to the hemopoietic toxicity of AZT. The purpose of the present study was to assess the effects of AMT, compared with AZT, on Hb production in butyric acid-induced K-562 leukemia cells. In particular, effects of AMT on steady state levels, stability, and rate of synthesis of globin mRNA, total heme levels, and Hb production were assessed.

Experimental Procedures

Chemicals. AMT and AZT were generous gifts of Dr. Raymond F. Schinazi (Veterans Administration Medical Center and Emory University, Atlanta, GA). [α-^{32}P]GTP and [α-^{32}P]dATP were obtained from ICN Biochemicals, Inc., Radiochemicals Division (Irvine, CA). ATP, CTP, GTP, and UTP were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). DNase I, proteinase K, and yeast tRNA were obtained from Bethesda Research Laboratories (Gaithersberg, MD). Restriction endonucleases *Eco*RI, *Hind*III, and *Pvu*II were purchased from International Biotechnologies Inc. (New Haven, CT). DDC, benzidine dihydrochloride, dithiothreitol, α-amanitin, polyvinylpyrrolidone, hemin, MOPS, HEPES, and salmon sperm DNA were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll was from Pharmacia (Piscataway, NJ). RPMI 1640 medium, dialyzed fetal bovine serum, L-glutamine, penicillin, and streptomycin were obtained from GIBCO Laboratories (Grand Island, NY).

Cells and culture conditions. K-562 human leukemia cells were kindly provided by Dr. Denise Shaw, University of Alabama at Birmingham (Birmingham, AL), and were maintained in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum (heat inactivated at 56° for 30 min), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells (1.6×10^5 cells/ml, unless specified otherwise) were incubated at 37° in a humidified atmosphere of 5% CO_2 in the presence or absence (control) of 1.4 mM butyric acid. Various concentrations of dideoxynucleoside (AZT, AMT, or DDC) were added to cells 25 hr after onset of induction, to allow the early phases of the induction process to occur in the absence of drug but sufficiently early so that the nucleoside was present during increasing globin transcription. Cultures were harvested at 72 hr (nuclei isolation) or 96 hr (RNA analysis) after initiation of the experiments. Cell growth and viability were assessed using a hemacytometer and trypan blue exclusion methodology, respectively. Cell viability was >95% for all concentrations of AZT and AMT tested.

Hb synthesis. Hb synthesis was monitored by a benzidine cell staining assay, as previously described by our group (5).

Plasmids. The plasmids used in these experiments were as follows. 1) PγIVS(−)SP3, a human Gγ-globin cDNA inserted into SP64, was a generous gift from Dr. Jeffrey Ross, McArdle Laboratory, University of Wisconsin (Madison, WI). 2) −200 Aγ, a human Aγ-globin genomic sequence inserted into pUC19, was kindly provided by Dr. Tim M. Townes, University of Alabama at Birmingham. 3) pRβA-1, a rat β-actin cDNA inserted into the Okayama-Berg vector, was a gift from Dr. Peter E. Barker, University of Alabama at Birmingham. 4) pUC19, used as a vector control in nuclear run-on experiments, was purchased from Bethesda Research Laboratories. Plasmid DNA was purified as described previously (5).

RNA analysis. Total cellular RNA was purified by a modified acid-guanidinium-phenol-chloroform method previously described (11). Briefly, cells were harvested and washed with sterile phosphate-buffered saline. Cells (3×10^6 cells) were then lysed in 300 μl of denaturing buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol. To this lysate were sequentially added 1/10 volume of 3 M sodium acetate (pH 5.2), ½ volume of water-saturated redistilled phenol, and 1/5 volume of chloroform, with thorough mixing after each addition. The lysate was cooled on ice for 15 min and then centrifuged at 10,000 × *g* for 20 min at 4°. The RNA present in the aqueous phase was precipitated with an equal volume of cold isopropanol and pelleted at 10,000 × *g* for 20 min at 4°. The RNA pellet was washed with 4 M LiCl to solubilize polysaccharides (12), resuspended in 100 μl of denaturing buffer, and precipitated with an equal volume of isopropanol. The final RNA pellet was washed with 70% cold ethanol and dissolved in RNase-free diethylpyrocarbonate-treated water. Quantitation of RNA was performed by determining absorbance at 260 nm (A_{260} of 1 = 40 μg/ml RNA).

Northern blot analysis was performed as described previously (5). For RNA blot studies, the γ-globin probe was a gel-purified 1.35-kilobase *Pvu*II fragment of the human γ-globin cDNA plasmid pγIVS(−)SP3, containing the first exon and part of the second exon of the Gγ-globin gene. The actin probe was a gel-purified 735-base pair *Ava*I fragment of the rat β-actin cDNA plasmid pRβA-1.

For RNA dot blot analysis, RNA (1-, 2-, and 4-μg aliquots from each sample) was dissolved in 50 μl of 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, to which were added 30 μl of 20× standard saline citrate (1× standard saline citrate = 0.15 M NaCl, 0.015 M sodium citrate) and 20 μl of formaldehyde. After incubation at 60° for 15 min, samples were applied to 0.45-μm nitrocellulose filters, using a Minifold I apparatus (Schleicher and Schuell, Inc., Keene, NH). RNA was fixed to the filters by heating for 1 hr at 80°, and dot blots were hybridized to globin or actin probes as previously described for Northern blot analysis (5).

RNA stability analysis. K-562 cells were induced, as described above, and exposed to 100 μM AMT, 100 μM AZT, or no compound (control). At 72 hr, 2.5 μg/ml actinomycin D was added to inhibit further RNA synthesis. Aliquots of cells were removed every 12 hr until 120-hr final culture time, and RNA was isolated, as described above. A similar amount of total RNA (5 μg) from each time point was electrophoresed on an agarose/formaldehyde gel, as described previously (5), except that 0.3 μg/ml ethidium bromide was added to samples to allow visualization of ribosomal RNA bands on the gel before transfer to the Nylon filter (see Fig. 2). The filter was then hybridized sequentially with the γ-globin and β-actin probes, as described previously (5). Hybridization was performed in a siliconized glass tube using a hybridization incubator (model 310; Robbins Scientific Corp., Sunnyvale, CA).

Nuclear run-on transcription. K-562 cells induced by 1.4 mM butyric acid were exposed to AMT concentrations of 0, 25, or 100 μM, or 100 μM AZT, using the same conditions described above. Nuclei were isolated after 72 hr, using methods previously described (5). For *in vitro* transcription assays, 10^7 nuclei were incubated at 25° for 30 min, in a total volume of 200 μl containing 1.5 μM [α-^{32}P]GTP (200 μCi), 0.5 mM each of ATP, CTP, and UTP, 0.12 M KCl, 5 mM magnesium acetate, 0.05 mM EDTA, 2.5 mM dithiothreitol, and 25 mM Tris·HCl (pH 8). The reaction was terminated by the addition of 13 μg of RNase-free DNase I and further incubation for 5 min at 25°.

[1] M. P. Stagg, E. M. Cretton, L. Kidd, R. B. Diasio, and J. P. Sommadossi. Clinical pharmacokinetics of 3′-azido-3′-deoxythymidine (AZT) and catabolites with formation of a toxic catabolite, 3′-amino-3′-deoxythymidine (AMT). Submitted for publication.

Highly purified RNA was isolated from nuclei as described previously (5). Total labeled RNA recovered ranged from 2.2 to 2.9 × 10^7 cpm, except in a control reaction, where RNA incorporation was inhibited approximately 60% in the presence of 1 μg/ml α-amanitin. Dot blot hybridizations were performed to quantitate the amount of globin nuclear RNA transcripts, as previously described (5). The hybridized filters were exposed to X-ray film at −80°, using an intensifying screen, and radioactivity was determined by scintillation counting of the dots.

Densitometric scan analysis. Autoradiograms were scanned using a Shimadzu CS9000 U flying-spot densitomer.

Fluorometric assay of heme. Total intracellular concentration of heme was determined fluorometrically, using a method described by Sassa (13). K-562 cells were induced as described above, except at an initial cell density of 2.0 × 10^5 cells/ml. The cell suspension (1.5 × 10^6 cells) was pelleted at 1,000 × *g* for 5 min and washed with phosphate-buffered saline. Cell pellets were resuspended in 500 μl of 2 M oxalic acid and heated for 30 min in a boiling water bath. Aliquots of a 10 mM hemin stock solution prepared in 10 mM KOH were used as standards and heated in 2 M oxalic acid, as described above. After cooling, fluorescence was determined in a Perkin-Elmer model 650-10S fluorescence spectrophotometer (Perkin-Elmer, Norcross, GA), using exciting light of 400 nm and emission at 662 nm. The fluorescence generated by hemin standards was linear between 10^{-8} and 10^{-6} M.

Results

Effect of AMT on Hb production. AMT inhibited Hb synthesis at concentrations between 10 and 250 μM (Table 1). Of note, whereas a concentration of 25 μM AMT inhibited benzidine-positive cells by approximately 21%, a 10-fold increase of extracellular AMT resulted in a minimal additional reduction of Hb synthesis. These findings contrast with those obtained with AZT, in which minimal inhibitory effects on Hb production were observed at a concentration of 25 μM and the magnitude of that inhibition was much more pronounced at 100 and 250 μM (5). In the present study, a degree of inhibition of Hb synthesis similar to that of the previous study (5) was obtained when 100 μM AZT was used as a control (Table 1). Additive effects on inhibition of Hb synthesis were observed when cells were exposed simultaneously to 100 μM concentrations of each nucleoside analog (Table 1).

Inhibition of globin mRNA expression by AMT. To determine whether the observed inhibition of Hb synthesis by AMT was related to a decrease in steady state globin mRNA level, total RNA was isolated from butyric acid-induced cells exposed to various concentrations of AMT. Effects of AMT on globin mRNA level, as assessed by RNA blot analysis, are illustrated in Table 2 and Fig. 1. RNA samples from cultures exposed to 100 μM AZT or no drug were simultaneously evaluated, as positive and negative controls, respectively, as was RNA from uninduced cells. The globin mRNA steady state level decreased in a dose-dependent fashion with increasing concentrations of AMT between 10 and 250 μM. Of note, inhibition of globin mRNA steady state level at 100 μM AMT, which averaged about 45%, approximated that measured in the presence of 100 μM AZT, when both compounds were evaluated in parallel experiments (see experiments 2 and 3 of Table 2). No substantial difference was detected in the steady state actin mRNA level after exposure of cells to either compound, consistent with our previous data on AZT (5).

Nuclear run-on transcription analysis. In order to determine whether the inhibition of steady state levels of globin mRNA by AMT was associated with a decreased rate of globin mRNA synthesis, as previously demonstrated for AZT (5), *in vitro* nuclear run-on assays were performed. Nuclei from 10^7 cells were isolated at 72 hr after initiation of cultures and were incubated in the presence of 200 μCi of [α-^{32}P]GTP. The labeled

TABLE 1

Effect of AMT and AZT on Hb synthesis in butyric acid-induced K-562 cells after 96 hr

Data represent the mean values ± standard deviation of at least two separate experiments.

Treatment	Concentration	Hb synthesis efficiency[a]
	μM	*ratio relative to control*
AMT	10	0.94 ± 0.03
	25	0.79 ± 0.01
	100	0.77 ± 0.03
	250	0.71 ± 0.05
AZT	100	0.55 ± 0.01
AMT + AZT	100 + 100	0.46 ± 0.03

[a] Ratio of percentage of benzidine-positive cells in test culture to percentage of benzidine-positive cells in control culture. Benzidine-positive cells in control cultures ranged from 39 to 50% and in uninduced cultures were <5%.

TABLE 2

Effect of AMT and AZT on globin mRNA steady state level at 96 hr

For experiments 1 and 2, RNA samples were analyzed by Northern blot and, for experiment 3, by RNA dot blot (see Experimental Procedures). Densitometric scan areas for the globin hybridization signal were corrected for variations in the actin signal. The autoradiogram for experiment 2 is shown in Fig. 1.

Treatment	Concentration	Relative globin mRNA level		
		Expt. 1	Expt. 2	Expt. 3
	μM			
Control		1.000	1.000	1.000
AMT	10	ND[a]	ND	0.797
	25	0.683	0.542	0.676
	100	0.635	0.441	0.577
	250	0.428	ND	ND
AZT	100	ND	0.491	0.513
Uninduced		ND	0.019	0.031

[a] ND, not determined.

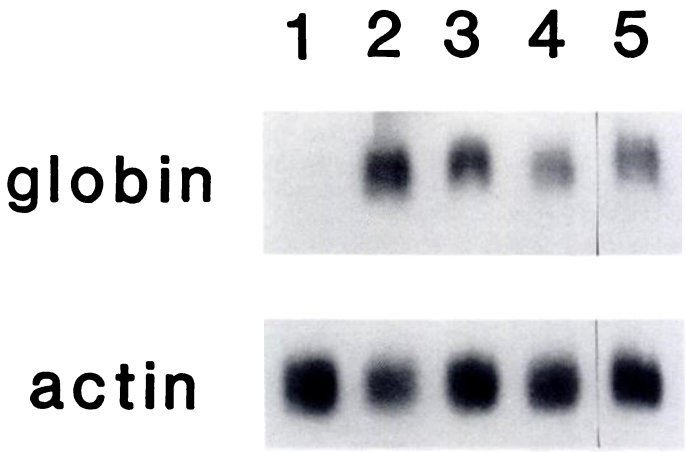


Fig. 1. Effect of AMT and AZT on globin mRNA steady state levels at 96 hr. Total mRNA was purified by an acid-guanidinium-phenol-chloroform method, and 10 μg of RNA from each sample were electrophoresed on a denaturing 2.2 M formaldehyde/1% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled γ-globin cDNA probe (see Experimental Procedures). After autoradiography, the γ-globin probe was removed from the blot in boiling distilled water and the blot was hybridized with a ^{32}P-labeled actin probe. *Lane 1*, uninduced cells; *lane 2*, butyric acid-induced cells; *lanes 3* and *4*, butyric acid-induced cells exposed to AMT concentrations of 25 and 100 μM, respectively; *lane 5*, butyric acid-induced cells exposed to 100 μM AZT.

RNA was hybridized to γ-globin and pUC19 vector control DNAs immobilized on nitrocellulose filters, by a dot-blot method. As shown in Table 3, K-562 cells exposed to 25 or 100 μM AMT had a substantially decreased rate of globin transcription. This decrease was approximately equal in nuclei from cells treated with 100 μM AMT and in those treated with 100 μM AZT. The absolute content of globin hybrids was 125 ppm in control nuclei. Mean incorporation per nucleus of [γ-^{32}P] GTP into *in vitro* synthesized RNA was not inhibited by AMT or AZT, indicating a lack of general inhibition of RNA transcription rate by AMT or AZT in these cells (Table 3).

Effects of AZT and AMT on globin mRNA stability. The stability of specific mRNA transcripts in eukaryotic cells is known to play a significant role in gene expression (14). Globin mRNA is known to be extremely stable, with a half-life reported in excess of 50 hr in differentiating murine erythroleukemia cells (15). To evaluate whether down-regulation of globin mRNA expression by AZT or AMT may also be associated with instability of cytoplasmic RNA, induced K-562 cells were exposed to 100 μM AZT or 100 μM AMT for 48 hr, and then 2.5 μg/ml actinomycin D, a nonspecific transcription inhibitor, was added. Total RNA was isolated from aliquots of cells every 12 hr during the next 48 hr. Globin and actin mRNA decay was determined by Northern blot analysis. As shown in Fig. 2, globin mRNA was as stable as rRNA in control cells and in cells exposed to either AZT or AMT. Of note, globin mRNA was more stable than actin mRNA in control and drug-treated cells. These data demonstrate that neither AZT nor AMT affects globin mRNA stability in differentiating K-562 cells. Therefore, the observed decrease of globin mRNA steady state levels in the presence of AZT or AMT is not associated with decreased globin mRNA stability.

Effects of AMT and AZT on total heme levels. As shown in Table 2, AZT and AMT at a concentration of 100 μM inhibited globin gene expression to a similar degree, approximating 45%, compared with control. In contrast, whereas 100 μM AZT inhibited Hb synthesis by 45%, only a 23% inhibition was observed in the presence of 100 μM AMT (Table 1). In order to gain insight into potential mechanism(s) responsible for the rather small inhibition of Hb synthesis by AMT, compared with AZT, we investigated whether changes in heme biosynthesis produced by these nucleoside analogs may also play a role in the observed effects.

TABLE 3

Effect of AMT and AZT on rate of globin transcription in isolated nuclei

Nuclei isolated from butyric acid-induced K-562 cells at 72 hr after initiation of the experiment (47-hr exposure to AMT or AZT) were labeled with [α-^{32}P]GTP for 30 min, and nuclear RNA was isolated as described in Experimental Procedures. Globin hybrid RNA cpm values were determined by scintillation counting of hybrids binding to ~200 Aγ-globin plasmid DNA, with correction for nonspecific hybridization to pUC-19 vector control DNA, which ranged from 125 to 184 cpm. Globin hybrids were inhibited by >90% in RNA synthesized in control nuclei in the presence of 1 μg/ml α-amanitin, which selectively inhibits RNA polymerase II transcription.

Treatment	Concentration	Radioactivity in total RNA/10^7 nuclei	Hybridization input	Globin hybrid RNA	
	μM	cpm × 10^7	cpm × 10^7	cpm	relative level
Control		2.4	2.2	2724	1.000
AMT	25	2.4	2.2	2248	0.825
	100	2.9	2.2	1994	0.732
AZT	100	2.2	2.2	1933	0.710

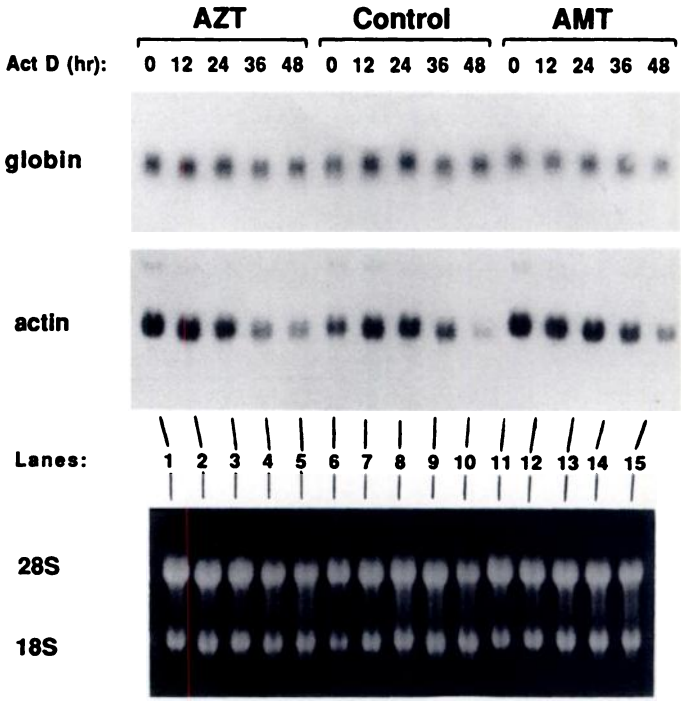
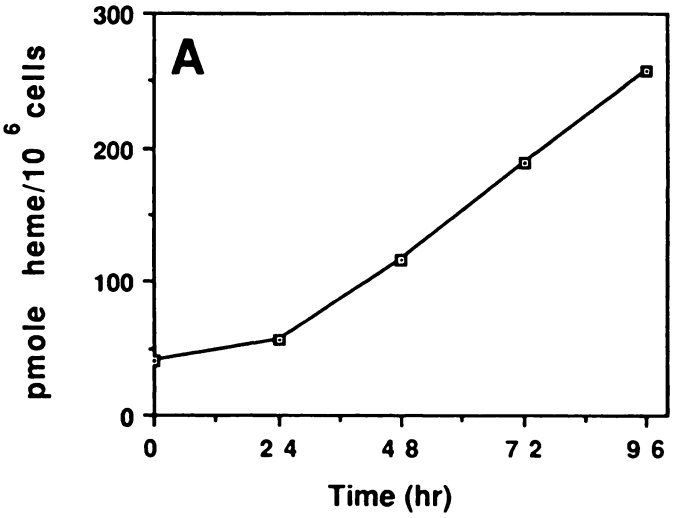


Fig. 2. Effect of 100 μM AZT or 100 μM AMT on RNA stability. Butyric acid-induced cells were exposed to 100 μM AZT, 100 μM AMT, or no drug (control) at 24 hr. At 72 hr, actinomycin D was added to block transcription. RNA was then isolated from cell aliquots every 12 hr, and 5 μg of each were electrophoresed and transferred to nylon membranes, as described in Experimental Procedures. *Top* and *Center*, autoradiograms of the filter sequentially hybridized to the γ-globin and actin probes, respectively. *Bottom*, UV fluorescence pattern in the gel before transfer to the membrane.

As shown in Fig. 3A, total heme content increased by approximately 4–5-fold during the 4-day induction period in K-562 cells induced with butyric acid in the absence of nucleoside analogs, consistent with a similar previous report (16). In contrast, total heme content in uninduced cells remained unchanged (data not shown). Heme synthesis was determined in K-562 cells after exposure to 100 μM concentrations of each nucleoside analog for various time periods (Fig. 3B). After 24 hr of exposure to AZT (48 hr from initiation of the experiment) total heme represented 88% of the control values, and that subtle inhibition further increased to approximately 80% heme, compared with control, by 72 hr (96 hr from initiation of the experiment). That inhibition of heme production by AZT was demonstrated to be significant, as judged by *t* test analysis, by 72 hr from initiation of the experiment ($p < 0.001$).

In contrast, under similar experimental conditions, no effect on heme synthesis was observed in the presence of 100 μM AMT over the same time interval. Of note, 100 μM DDC had, similar to AMT, no effect on heme synthesis, consistent with our previous data demonstrating that DDC at similar and higher concentrations produced no substantial inhibition of benzidine-positive cells (5). These findings suggest that the marked inhibition of Hb synthesis by AZT, compared with AMT, may reflect the differential inhibition by AMT and AZT of heme induction.

Hb synthesis involves close coordination between the globin and heme pathways (17). Therefore, the down-regulation of heme synthesis by AZT described above might result in a down-regulation of globin mRNA synthesis. To explore that possibil-



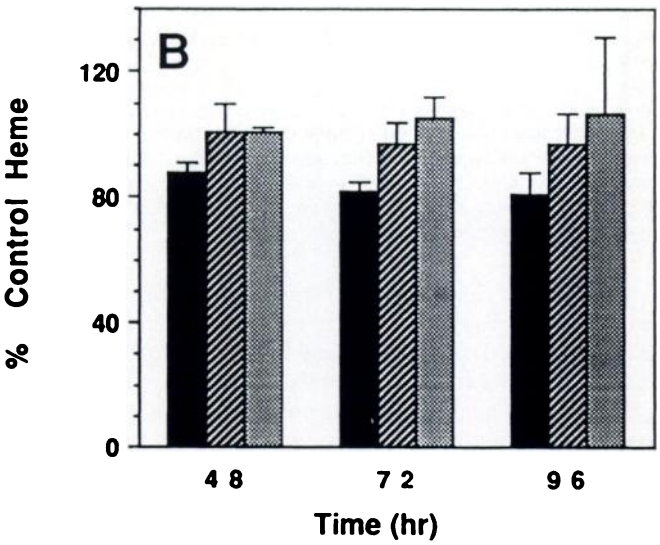


Fig. 3. A, Kinetics of intracellular heme induction in butyric acid-differentiated K-562 cells. The heme concentration was determined fluorometrically, as described in Experimental Procedures. Data are from a single representative experiment. B, Effect of 100 μM AZT, AMT, or DDC on intracellular heme concentration during butyric acid induction of K-562 cells. Cells were induced with 1.4 mM butyric acid and exposed to 100 μM AZT (■), 100 μM AMT (▨), or 100 μM DDC (▩) 24 hr later. Data represent the relative ratios of heme in test cultures, compared with control cultures not exposed to dideoxynucleoside, and are the mean of three separate experiments. *Error bars*, standard deviation.

ity, steady state globin mRNA levels were determined after incubation of cells in the presence of 100 μM AZT and in the presence or absence of 0.5 mM succinylacetone, a potent and selective inhibitor of heme synthesis (18). Under these conditions, steady state globin mRNA levels were inhibited by approximately 50% in the presence of either AZT or succinylacetone (Table 4). Of importance, whereas induction of benzidine-positive cells was completely inhibited by succinylacetone, the globin mRNA level was further inhibited by AZT to a degree similar to that measured in the absence of succinylacetone

TABLE 4

Effect of AZT on globin mRNA steady state level during inhibition of heme synthesis by succinylacetone (SA)

Cells were induced by butyric acid in the presence or absence of 0.5 mM succinylacetone, added at initiation of the experiment. AZT was added 25 hr later, and at 96 hr benzidine staining was performed and RNA was isolated for dot blot analysis. Relative globin mRNA levels were determined by RNA dot blot analysis. Densitometric scan areas for the globin hybridization signal were corrected for variations in the actin signal.

Treatment	Benzidine-positive cells	Relative globin mRNA level
	%	
Control	39	1.000
100μM AZT	22	0.513
0.5 mM SA	<1	0.450
0.5 mM SA + 100 μM AZT	<1	0.200

(Table 4). These data suggest that, although inhibition of heme induction by AZT may be an important mechanism in the observed decrease of Hb synthesis, inhibition by AZT of globin mRNA synthesis is a primary effect, rather than secondary to inhibition of heme synthesis.

Discussion

Studies from our group and others have attempted to elucidate the mechanism(s) involved in AZT-induced toxicity toward human hematopoietic cells, with demonstration that inhibition of host DNA synthesis by AZT-produced chain termination may play a role in that observed toxicity (19–21). Our recent hypothesis that AZT-induced anemia may also result from specific inhibition of key proteins involved in erythroid maturation has led to the demonstration that AZT inhibits Hb synthesis in butyric acid-induced K-562 cells, used as an *in vitro* cellular model (5). In that study, Hb inhibition by AZT was demonstrated to be associated with inhibition of globin gene transcription, and not a result of general inhibition of gene transcription through decreased mRNA synthesis. Recently, our laboratory has reported that AZT is metabolized to AMT in liver systems *in vitro*, through a NADPH-dependent enzymatic reduction. Relevant to potential mechanism(s) of AZT-induced host cell toxicity, AMT was demonstrated to be about 5–7-fold more toxic than AZT toward human hemopoietic cells (9). The substantial *in vivo* formation of AMT that was demonstrated by our group both in monkeys (10) and, in particular, in humans,[1] where the AMT area under the curve accounted for as much as 30%, compared with that of AZT, suggests that AMT probably participates in the overall toxic effects observed in patients undergoing AZT treatment.

In light of these findings, we have investigated whether AMT might also affect the Hb synthesis machinery during K-562 cell differentiation, as demonstrated previously with the parent drug. The present study demonstrates that AMT and AZT exhibit similar inhibition patterns of globin mRNA expression, with values approximating 50%, compared with control, in the presence of 100 μM levels of each compound. Neither AZT nor AMT caused a decrease in the stability of globin mRNA after blockage of transcription with actinomycin D. Using *in vitro* nuclear run-on assays, the inhibition of globin mRNA steady state level by AMT was shown to be associated with a decreased rate of globin gene transcription, similar to results obtained with AZT (5). In contrast, AMT inhibited Hb production in cells to a lesser degree than did AZT. Although the relative importance of heme and globin biosynthesis in the regulation

of Hb production is poorly understood, studies have suggested that the heme pathway may have a predominant role in Hb synthesis regulation (17). Therefore, differential effects of AZT and AMT on heme biosynthesis in butyric acid-induced K-562 cells may account for the differential inhibition of Hb production by the parent drug and its metabolite. Indeed, whereas AZT was demonstrated to inhibit heme synthesis by about 20% during the 4-day induction period (Fig. 3), AMT had no effect on heme synthesis. Of note, although inhibition of heme synthesis by AZT may appear limited, the combination of this effect with its inhibition of globin mRNA synthesis could account for the larger inhibition of benzidine-positive cells by AZT, compared with AMT.

Enhanced cell-growth inhibition of K-562 cells was observed with AMT, compared with AZT, with approximately 40% inhibition of cell proliferation in the presence of 25 μM AMT (data not shown), whereas no substantial inhibition was detected with a similar AZT concentration (5). Similarly, we previously found that AMT was 5–7-fold more toxic than AZT in human bone marrow erythroid progenitor cells, as determined by using a clonogenic assay (9). Of note, the clonogenic assay used in that study involves both growth and differentiation of a "clonal" progenitor cell into an erythroid colony. Therefore, the higher level of cell growth inhibition by AMT, when coupled with its toxic effects on erythroid cell maturation, including globin gene expression as shown in the present study, could account for the more potent toxicity toward erythroid progenitor cells of AMT, compared with AZT, using the clonogenic assay (9).

The present demonstration of inhibition of heme biosynthesis by AZT is consistent with the observations of Lutton *et al.* (22), who reported that the activity of a regulating enzyme of the heme synthetic pathway, 5-aminolevulinate synthetase, was inhibited in bone marrow cells isolated from rats treated with AZT (22). In addition, the protective effects of hemin on AZT toxicity to bone marrow CFU-E and BFU-E colonies in both humans and mice (23) further confirm that inhibition of heme, in addition to our demonstrated effects on globin expression, is probably an important mechanism in the AZT inhibition of Hb synthesis.

Recent studies have suggested that AZT inhibits mitochondrial DNA synthesis, which may affect hemopoietic cell functions (24, 25). However, it should be noted that these effects were observed at higher concentrations than those required to inhibit nuclear DNA synthesis, as recently reported by Cheng and co-workers (26). Relevant to the present study, it is important to emphasize that four of the enzymatic steps of the heme pathway occur in the mitochondria (17), including that catalyzed by 5-aminolevulinate synthase, an enzyme that has been shown to be inhibited by AZT (22). Thus, it is conceivable that AZT may decrease the number of mitochondria per cell, affecting the 5-aminolevulinate synthase activity, which, in turn, would result in a decreased level of heme synthesis. However, DDC, an anti-human immunodeficiency virus agent that has been shown to be a more potent inhibitor of mitochondrial DNA synthesis than AZT (27), had no effect on heme synthesis (Fig. 2), suggesting that the inhibition of Hb production machinery by AZT is independent of its potential effects on mitochondrial DNA synthesis.

As stated above, regulation of heme and globin biosynthetic pathways are coordinated, consistent with the observed decreased translation of globin chains after depletion of free heme (17). Furthermore, succinylacetone, a potent inhibitor of heme synthesis, has been shown also to decrease globin mRNA expression in murine erythroleukemia cells (28), suggesting a dual feedback regulation of heme on globin synthesis, at the levels of both globin mRNA and protein synthesis. These findings raise the question of whether our demonstrated inhibition of globin gene expression by AZT may be related to a direct effect on globin genes or an indirect effect through inhibition of the heme pathway. The present data showing that AZT was equally potent in inhibiting globin mRNA steady state levels under conditions in which heme synthesis was inhibited by succinylacetone (Table 4) unambiguously demonstrate that inhibition of globin mRNA level by AZT is a direct effect rather than an effect secondary to inhibition of heme synthesis. In addition, these results demonstrate that AZT interacts with the Hb production machinery at multiple sites.

Evaluation of RNA stability (Fig. 2) showed that actin mRNA was less stable than globin mRNA in these cells. These data support our previous hypothesis that AZT inhibits globin mRNA transcription without affecting mRNA transcription in general (5). Indeed, if AZT (or AMT) were inhibiting mRNA transcription in general, one would observe a more pronounced decrease of actin mRNA steady state levels, compared with globin mRNA, due to the shorter half-life of that transcript. However, our results clearly demonstrate that this does not occur (see Fig. 1 and Ref. 5).

In summary, the present study demonstrates that AMT, a recently identified metabolite of AZT, inhibits globin gene expression in K-562 erythroleukemia cells to a degree similar to that previously demonstrated for the parent drug (5). Although effects of AMT on Hb production may be subtle, compared with those obtained with AZT, probably resulting from the differential effects of each compound on heme synthesis, both nucleoside analogs may be involved in the inhibition of erythroid differentiation observed *in vivo*, through changes in gene expression.

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