0026-895X/90/060797-08$03.00/0

MOLECULAR PHARMACOLOGY, 38:797-804

3′-Azido-3′-deoxythymidine Inhibits Globin Gene Transcription in Butyric Acid-Induced K-562 Human Leukemia Cells

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Received June 17, 1990; Accepted August 29, 1990

SUMMARY

We previously demonstrated that 3′-azido-3′-deoxythymidine (AZT) inhibits proliferation of human bone marrow progenitor cells *in vitro* and that incorporation of AZT into nuclear DNA may be one mechanism responsible for AZT-induced bone marrow toxicity [*Antimicrob. Agents Chemother.* **31**:452–454 (1987); *Mol. Pharmacol.* **36**:9–14 (1989)]. The present study explores possible genetic mechanisms involved in AZT-induced anemia by evaluating the effects of AZT on globin gene expression at both the transcriptional and the translational levels in butyric acid-induced K-562 human erythroleukemia cells. AZT, at concentrations ranging from 10 to 250 μM, was added to cells 25 hr after initiation of induction of hemoglobin (Hb) synthesis with 1.4 mM butyric acid. Hb synthesis, as measured by benzidine staining, was inhibited by AZT in a dose- and time-dependent manner in these cells. AZT inhibition of cell growth was not the major contributing factor in the net inhibition of Hb synthesis in K-562 cells. As assessed by Northern blot analysis, AZT inhibition of Hb synthesis was associated with a decrease in globin mRNA steady state levels without inhibition of total RNA synthesis or actin mRNA steady state levels. In particular, a decrease of globin mRNA levels of 23% by 25 μM AZT was observed, reaching a maximum inhibition of 59% in the presence of 250 μM AZT. *In vitro* translation experiments demonstrated that essentially all nonglobin translatable mRNAs were not inhibited by AZT concentrations as high as 250 μM, whereas globin mRNAs coding for ε, ζ, Aγ, Gγ, and α chains were substantially inhibited to similar levels by AZT, in a dose-dependent manner. Transcriptional run-on studies with isolated nuclei from AZT-treated K-562 cells demonstrated a 20 and 50% inhibition of *in vitro* synthesized globin transcripts from cells exposed to 25 and 100 μM AZT, respectively. 2′,3′-Dideoxycytidine also inhibited K-562 cell growth in the same concentration range as AZT but, of importance, had no effects on Hb production. These data suggest that inhibition of globin gene expression may play a role in the cytotoxicity of AZT to the erythroid cell.

AZT is the first clinically approved drug for the treatment of AIDS. AZT has shown clinical benefit in some patients with AIDS and AIDS-related complex (1, 2). The effects of this antiretroviral agent are probably due to the inhibition of human immunodeficiency virus reverse transcriptase, as demonstrated *in vitro* (3). Unfortunately, AZT treatment is limited by its toxic side effects to bone marrow cells, manifested as anemia and neutropenia (4). In general, Hb levels drop below 7.5 g/dl in approximately 25% of AZT recipients. This toxicity is consistent with *in vitro* studies from our laboratory, which demonstrated a dose-dependent inhibition by AZT of both human CFU-GM and BFU-E at clinically achievable AZT concentrations (5). Additional studies by several groups have confirmed our findings on the toxic effects of AZT on human hemopoietic progenitor cells (6–8).

This work was supported by Public Health Service Grants HL-42125 and AI25784. J.-P.S. is a recipient of a Junior Faculty Research Award from the American Cancer Society.

In a recent paper (9), we demonstrated that AZT was incorporated into nuclear DNA of human bone marrow cells. This biochemical event correlated to some degree with inhibition of CFU-GM colony formation, suggesting that incorporation of AZT into nuclear DNA may be one mechanism responsible for AZT-induced bone marrow toxicity. In contrast to initial studies (10), which suggested that depletion of TTP pools through inhibition of thymidylate kinase by AZT monophosphate may be a mechanism responsible for "host" toxicity, our study demonstrated that imbalance of deoxyribonucleotide pools by AZT was not a critical factor in AZT inhibition of DNA synthesis of human bone marrow cells. Consistent with these findings, we previously showed that thymidine was unable to reverse AZT toxicity in human bone marrow cells (11).

Additional studies from our group (12) and others (6–8) have demonstrated that nucleoside analogs are substantially more toxic toward human BFU-E, as compared with CFU-GM, which may result from a different degree of maturation or different

ABBREVIATIONS: AZT, 3′-azido-3′-deoxythymidine; AIDS, acquired immunodeficiency syndrome; CFU-GM, granulocyte-macrophage colony-forming unit; BFU-E, erythroid burst-forming unit; SDS, sodium dodecyl sulfate; DDC, 2′,3′-dideoxycytidine; Hb, hemoglobin; MOPS, 3-*N*-morpholinopropanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SSC, standard saline citrate.

cell proliferation pattern, because BFU-E are earlier progenitors than CFU-GM (13). However, other processing mechanisms, in addition to cell growth inhibition and specific to the erythroid lineage, may be affected by AZT, including modulation of Hb synthesis machinery. Potential mechanisms include transcriptional effects on globin genes or genes that regulate heme biosynthesis, effects on translation of globin messenger RNA, and/or effects on enzyme activity involved in heme biosynthesis.

In this report, potential genetic mechanism(s) involved in AZT toxicity toward hemopoietic precursor cells were explored. In particular, the effects of AZT on total RNA synthesis, mRNA synthesis, globin gene expression, and Hb production in butyric acid-induced K-562 human leukemia cells were evaluated. This cell line was originally established from the pleural effusion of a patient with chronic myeloid leukemia in acute phase (14) and it can be induced to synthesize embryonic and fetal Hb by various agents including butyric acid (15, 16). To determine whether the observed effects on Hb production were specific to AZT or dideoxynucleosides in general, DDC was simultaneously evaluated. In the experiments reported herein, AZT inhibited both Hb production and globin gene expression in a time- and dose-dependent manner, with minimal effect on nonglobin mRNA synthesis or total RNA synthesis. In contrast, DDC had no effect on Hb production, despite an increased inhibition of cell growth, as compared with AZT. The inhibition of globin gene expression by AZT was associated with a decreased rate of globin mRNA transcription, as assessed by nuclear run-on assays.

Experimental Procedures

Chemicals L-[3,4,5-^{3}H] Leucine was purchased from Dupont, NEN Division (Boston, MA). [α-^{32}P] GTP was 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 (Gaithersburg, MD). Restriction endonucleases *Eco*RI, *Hind*III, and *Pvu*II were purchased from International Biotechnologies, Inc. (New Haven, CT). AZT, DDC, benzidine dihydrochloride, β-mercaptoethylamine, pyronin Y, dithiothreitol, α-amanitin, polyvinylpyrollidone, MOPS, HEPES, and salmon sperm DNA were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll was from Pharmacia (Piscataway, NJ). Globin chain markers Hb A/F were a gift from Isolab Inc. (Akron, OH). 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 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 (uninduced control) of 1.4 mM butyric acid. Various concentrations of dideoxynucleoside (AZT 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 AZT (or DDC) was present during increasing globin transcription. Reactions were stopped at 72 or 96 hr following initiation of the experiments. Cell growth and viability were assessed using a hemacytometer and a trypan blue exclusion methodology, respectively.

Benzidine staining of cells. The synthesis of heme or Hb was determined by a benzidine staining methodology (17). After the culture flasks were gently shaken for a few seconds to ensure homogeneous distribution of cells, an aliquot (250 μl) was placed in a 1.5-ml Eppendorf microcentrifuge tube to which was added 25 μl of 0.2% (w/v) benzidine dihydrochloride in 0.5 M acetic acid solution containing 0.4% of a 30% hydrogen peroxide solution. After 10 min, cells (≥300) were scored for Hb production using a hemacytometer.

Plasmids. The plasmids were obtained as purified DNA. 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 (Birmingham, AL). 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 (Birmingham, AL). 4) pUC19, used as a vector control, was purchased from Bethesda Research Laboratories. Plasmids were transformed into *Escherichia coli* strain JM109 (Promega Biotech, Madison, WI) using a calcium chloride method (18). Plasmid DNA was purified from saturated bacterial cultures using an alkaline denaturation/neutral renaturation method, as described previously (19).

RNA isolation and Northern blot analysis. Total cellular RNA was purified by a guanidine thiocyanate-cesium chloride method (20). Briefly, cells were harvested and washed with sterile phosphate-buffered saline. Cells were subsequently lysed in 2.7 ml of 4 M guanidinium thiocyanate, 20 mM sodium acetate (pH 5.2), 0.1 mM dithiothreitol, 0.5% *N*-lauryl sarcosine. DNA was sheared by drawing the suspension four times through a 20-gauge needle. The suspension was layered on a cushion of 5.7 M CsCl, in a siliconized tube, and centrifuged at 18° and 35,000 rpm for 18 hr, using a Beckman SW-60 rotor. The RNA was suspended in 10 mM Tris·HCl (pH 7.4), 5 mM EDTA, 1% SDS, and precipitated in 0.3 M sodium acetate and ice-cold absolute ethanol. RNA was recovered by centrifugation at 9,500 rpm and dissolved in distilled water. The ethanol precipitation procedure was then repeated. Quantification of RNA was performed by determining absorbance at 260 nm (A_{260} of 1 = 40 μg of RNA/ml).

For Northern blot analysis of globin mRNA, total RNA equivalent to 4×10^5 cells (approximately 8 μg) was denatured at 60° for 5 min in 50% deionized formamide, 2.2 M formaldehyde, in MOPS buffer (20 mM MOPS, 5 mM-sodium acetate, 1 mM EDTA, pH 7.0). The RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde in MOPS buffer, followed by Northern blot transfer to a 0.45-μm nitrocellulose filter. The location and integrity of 28 S and 18 S ribosomal RNA bands were assessed from duplicate lanes of the gel stained with ethidium bromide. Transfer of RNA to the blot was complete, as evidenced by ethidium bromide staining of the gel. RNA was fixed to the filter by heating for 2 hr at 80°. The blots were prehybridized for at least 2 hr at 40° in 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, 1 mM EDTA, 2.25× Denhardt's solution (1× Denhardt's = 0.02% polyvinylpyrollidone, Ficoll, bovine serum albumin), 50% formamide, 5 mM sodium phosphate (pH 6.5), 50 μg/ml salmon sperm DNA (sonicated and denatured). Hybridization was carried out at 40° overnight in the same composition buffer, using approximately 1×10^6 cpm/ml ^{32}P-labeled DNA probe labeled by nick translation (specific activity $2–6 \times 10^8$ cpm/μg of DNA). The blots were washed twice in 2× SSC, 0.1% SDS, at room temperature for 10 min and then three times in 0.1× SSC, 0.1% SDS, for 10 min, with the final wash at 40°. The blots were exposed to X-ray film with an intensifying screen at −80°. Following film exposure for globin hybridization, the γ-globin probe was released from the blot in boiling water. The blot was subsequently hybridized with the actin probe under the same conditions as described above.

For these Northern 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 and part of the second exon of the Gγ-globin gene. The actin probe was a 770-base pair *Nco*I/*Taq*I fragment of the chicken β-actin gene (Oncor, Inc., Gaithersburg, MD).

***In vitro* translation of RNA and gel analysis.** Total RNA at a concentration of 200 μg/ml was translated *in vitro* with a wheat germ

translation system (Boehringer Mannheim) at 30° for 60 min, in the presence of 15 μCi of [^{3}H]leucine (specific activity, 144 Ci/mmol), in a total volume of 50 μl. To determine [^{3}H]leucine incorporation, aliquots (3 μl) were precipitated in duplicate with 5% trichloroacetic acid on Whatman 3MM cellulose filters, which were then counted in a Beckman LS 5000 TA scintillation counter.

For gel analysis of translation products, 20 μl of the translation sample were mixed with 20 μl of gel loading buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 20% glycerol), heated for 5 min at 100°, and electrophoresed in 0.1 × 14 × 16-cm 10% polyacrylamide, 0.1% SDS gel for 3 hr at 150 V. Protein molecular weight markers (Pharmacia) were run in parallel lanes and were identified by staining in 0.05% Coomassie brilliant blue. The gel was processed for fluorography in a glacial acetic acid solution containing 20% 2,5-diphenyloxazole, as described by Waterborg and Matthews (21). The film was then exposed for 6 days at −80°.

For analysis of specific globin chains, 20 μl of the translation sample were electrophoresed in a 0.1 × 14 × 16-cm 2% Triton X-100, 6 M urea, 5% acetic acid, 12% polyacrylamide gel, using a modification of a technique previously described by Alter *et al.* (22). Preelectrophoresis was performed in one step with gel wells overlaid with 1 M β-mercaptoethylamine for 90 min at a constant voltage of 150V, using a 5% acetic acid running buffer with the anode at the top. β-mercaptoethylamine was washed from wells and the anode buffer was replaced with fresh buffer. Samples (20 μl) were mixed with 30 μl of a mixture containing 10.2 M urea, 8.5% acetic acid, 1.4 M mercaptoethanol, and 0.7 mg/ml pyronin Y and were applied to the gel. Electrophoresis was carried out for 13 hr at a constant current of 8 mA. The gel was stained in 0.05% Coomassie brilliant blue to identify globin chain markers and then processed for fluorography as described above. The film was then exposed for 6 days at −80°.

Nuclear run-on transcription. K-562 cells, induced by 1.4 mM butyric acid, were exposed to AZT concentrations of 0, 25, and 100 μM under the same conditions as described above, except that the initial cell density was 1.8 × 10^5 cells/ml. Nuclei were isolated after 72 hr using the method of Marzluff and Huang (23). Cells (3–5 × 10^6/ml) were suspended in 5 ml of buffer I (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM Tris·HCl, pH 8) and homogenized with 15 strokes in a Dounce homogenizer fitted with a B pestle. The homogenate was diluted with 7 ml of buffer II (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 8) and layered over a 7-ml cushion of buffer II. Centrifugation was performed at 30,000 × *g* and 4° for 45 min. The nuclear pellet was suspended in 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM dithiothreitol, 5 mM Tris-HCl, pH 8, at a final concentration of 10^8 nuclei/ml. Nuclei were either used extemporaneously or stored at −80° in 100-μl aliquots.

For *in vitro* transcription assays, 10^7 nuclei were incubated at 25° for 30 min in a total volume of 200 μl containing 1.7 μM [α-^{32}P] GTP (70 μCi), 0.5 mM levels 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°.

In preliminary experiments, the hybridization background of RNA isolated from nuclei by a phenol/chloroform extraction method described by Marzluff and Huang (23) was too high to permit accurate determination of globin transcription rates. Therefore, highly purified RNA was isolated from nuclei using a method described by Groudine *et al.* (24). Nuclei in a 200-μl volume were lysed by the addition of 200 μl of 2% SDS, 10 mM EDTA, 20 mM Tris·HCl, pH 7.4. The nuclear lysate was deproteinized with 100 μg/ml proteinase K for 30 min at 42°, followed by an extraction with phenol/chloroform. After addition of 100 μg/ml yeast tRNA, nucleic acids were precipitated by addition of 1 volume of ice-cold 10% trichloroacetic acid containing 60 mM $Na_4P_2O_7$. After incubation for 30 min at 4°, the precipitate was collected on a 0.45-μm nitrocellulose disk and then washed three times with 3% trichloroacetic acid containing 30 mM $Na_4P_2O_7$. The filters were subsequently digested with 25 μg of DNase I in 0.9 ml of 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$ in a glass scintillation vial at 37° for 30 min. DNase digestions were terminated by addition of EDTA to 15 mM and SDS to 1%. RNA was eluted from filters by incubation at 65° for 10 min and the eluant was transferred to a 15-ml Corex centrifuge tube. The filter was incubated for an additional 10 min at 65° in 0.5 ml of 1% SDS, 10 mM Tris·HCl (pH 7.5), 5 mM EDTA. The two eluant fractions were combined, incubated in the presence of 25 μg/ml proteinase K for 30 min at 37°, and extracted twice with equal volumes of phenol/chloroform and chloroform alone. The aqueous phase was precipitated at −20° overnight with 0.1 M NaCl and 2.5 volumes of ethanol. The RNA pellet was suspended in 50 μl of 10 mM Tris·HCl (pH 7.5), 2 mM EDTA, and the radioactivity of a 2-μl aliquot was determined in a Beckman 5801 liquid scintillation counter. Total radioactivity recovered ranged from 6.25 × 10^6 to 9.75 × 10^6 cpm.

Dot-blot hybridizations were performed to quantitate the amount of globin nuclear RNA transcripts. Plasmids were linearized with restriction enzymes and then heat denatured at 65° for 5 min in 0.1 M NaOH. Plasmid DNA (5 μg of each) was then immobilized on 0.45-μm nitrocellulose filters in 6-mm-diameter dots, using a Minifold I apparatus (Schleicher and Schuell Inc., Keene, NH). The nitrocellulose strips containing plasmid DNA were prehybridized at 65° for 5 hr with 1.2 ml of hybridization buffer (7% SDS, 0.25 M sodium phosphate, pH 7.2, 0.25 M NaCl, 10 μg/ml molecular biology grade bovine serum albumin, 1 mM EDTA) in heat-sealed bags (Kapak Corp., Minneapolis, MN). Equal amounts of radioactivity (6.25 × 10^6 cpm) of each RNA sample were denatured at 90° for 3 min in 600 μl of hybridization buffer and then hybridized to the filters at 65° for 46 hr. Filters were washed twice in 2× SSC, 0.1% SDS, and once in 0.5× SSC, 0.1% SDS, at room temperature and once in 0.5× SSC, 0.1% SDS, at 50°. The filters were then exposed to X-ray film at −80° using an intensifying screen.

Densitometric scan analysis. Autoradiograms were scanned using either a BioRad model 620 videodensitometer on a Shimadzu CS9000 U flying-spot densitomer.

Results

Effects of AZT on cell growth and Hb production. Growth of butyric acid-induced K-562 cells was partially slowed by AZT, in a dose-dependent manner (Fig. 1). AZT at concentrations of ≤25 μM had minimal effects on cell growth, whereas higher AZT concentrations of 100 and 250 μM inhibited cell growth by approximately 50%. Of note, viability of cells, as assessed by trypan blue exclusion, was greater than 95% at any given AZT concentration. In addition, inhibition of growth of induced K-562 cells with a concentration of 100 μM AZT was observed at all time points between 48 and 96 hr (data not shown). Under similar conditions, DDC exhibited an increased dose-dependent inhibition of growth for induced K-562 cells, with significant inhibition at concentrations as low as 25 μM and with a 50% inhibitory concentration of approximately 30 μM (Fig. 1). Effects of AZT on Hb synthesis were monitored by a benzidine staining method (Table 1). The percentage of cells synthesizing Hb dramatically increased with butyric acid induction, with values ranging from 28.9 to 51.6%, as compared with only 0.6 to 4.2% in uninduced K-562 cells. No substantial inhibition of benzidine-positive cells was observed in the presence of 10 or 25 μM AZT. In contrast, exposure of cells to AZT concentrations of 100 or 250 μM resulted in a marked inhibition of benzidine-positive cells, with average ratios of 0.63 and 0.45, respectively, as compared with control. These data demonstrate that AZT inhibits Hb synthesis in a dose-dependent fashion. DDC had minimal effects on Hb synthesis at concentrations as high as 250 μM, with a ratio of 0.93 for benzidine-positive

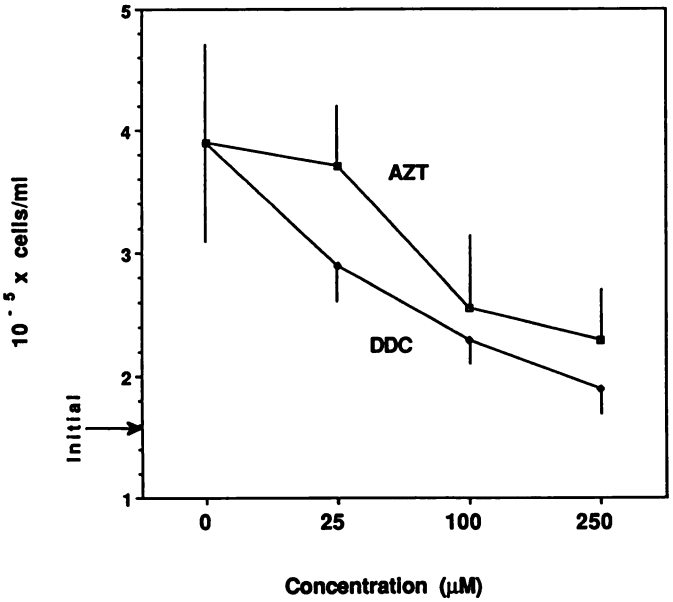

Fig. 1. Effect of increasing AZT and DDC concentrations on induced K-562 cell growth. Cells were cultured in the presence of 1.4 mM butyric acid and exposed to AZT (□) or DDC (♦) at 25 hr, under the same conditions as described in Experimental Procedures. Cell number was determined after 96 hr. Each *point* represents the mean density of six experiments ± standard deviation.

cells at this concentration, as compared with control (Table 2). These data are consistent with the absence of DDC-induced anemia in clinical trials and demonstrate that inhibition of Hb production is not a general mechanism for dideoxynucleoside toxicity.

Inhibition of globin mRNA expression by AZT. Northern blot analysis was performed to determine whether the observed inhibition of Hb synthesis by AZT was related to a decrease in globin gene expression. Previous studies have demonstrated that K-562 cells express embryonic and fetal globin but minimal adult β-globin (25). Therefore, globin gene expression was monitored using a cDNA probe for the human γ-globin gene. Medium stringency hybridization and washing conditions leading to potential detection of ε-globin transcripts were used. Transcripts detected with this probe will be referred to as globin transcripts.

AZT had no substantial effect on total RNA synthesis in induced K-562 cells. RNA recovered per 10^6 cells averaged 21.8 μg for control and 21.4, 21.3, and 18.9 μg for cells exposed to 25, 100, and 250 μM AZT, respectively. In contrast, exposure of cells to AZT concentrations between 25 and 250 μM decreased steady state globin mRNA levels in a dose-dependent manner (Fig. 2). In particular, a decrease in globin mRNA levels of 23% by 25 μM AZT was observed, reaching a maximum inhibition of 59% in the presence of 250 μM AZT, as determined by densitometric scan analysis. When the same blot was rehybridized with an actin-specific DNA probe, no substantial difference was detected in the steady state actin mRNA level, even at a concentration of AZT as high as 250 μM. These data suggest that inhibition of globin gene expression by AZT is not associated with a general inhibition of mRNA or total RNA synthesis.

Time course experiments on inhibition of globin mRNA expression were performed using cells exposed to 100 μM AZT (Fig. 3). Steady state globin mRNA levels were determined after incubation of cells for 24, 48, 72, and 96 hr. Inhibition of globin mRNA synthesis was demonstrated as early as 23 hr after addition of AZT (48-hr time point), reaching a maximum value of approximately 35% at 96 hr, as determined by densitometric scan analysis. In control cultures, globin mRNA expression increased throughout the period of induction, reaching a maximum level by 72 hr.

***In vitro* translation analysis.** In order to assess potential inhibition of other mRNAs by AZT, *in vitro* translation analysis was performed using wheat germ extracts. This method also permits the determination of AZT effects on specific globin transcripts, using an electrophoresis system that allows separation of the translation products of the various globin genes. As shown in Table 3, AZT has little effect on the level of total translatable mRNA, as measured by [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material. In contrast, total globin RNA showed a dose-dependent inhibition by AZT when analyzed on a 10% polyacrylamide, 0.1% SDS gel (Fig. 4A). Most of the other bands showed no substantial change with AZT exposure, as assessed by densitometric scan analysis of representative bands (Table 3). This demonstrated that AZT does not inhibit mRNA synthesis in general but is rather specific for globin mRNA.

Effects of AZT on each globin mRNA were assessed by a different gel system, which separates the individual globin chains (Fig. 4B). Levels of ζ-, ε-, Aγ, Gγ, and α-globin transcripts were all inhibited by AZT at approximately similar

TABLE 1

Inhibition of Hb synthesis by AZT in butyric acid-induced K-562 Cells after 96 hr

Experiment		Control	10 μM AZT	25 μM AZT	100 μM AZT	250 μM AZT
1	B+ cells[a] (%)	29.0	23.5	ND[b]	18.7	9.1
	Ratio of B+ cells compared with control	1.000	0.810		0.645	0.314
2	B+ cells (%)	28.9	30.8	ND	20.5	15.1
	Ratio of B+ cells compared with control	1.000	1.066		0.709	0.522
3	B+ cells (%)	51.6	ND	52.3	30.7	28.0
	Ratio of B+ cells compared with control	1.000		1.014	0.595	0.543
4	B+ cells (%)	49.8	ND	45.6	27.9	21.8
	Ratio of B+ cells compared with control	1.000		0.916	0.560	0.438
	Average ratio of B+ cells compared with control	1.00	0.94 ± 0.13[c]	0.96 ± 0.05	0.63 ± 0.06	0.45 ± 0.09

[a] Percent of benzidine-positive cells. Percentage values of benzidine-positive cells in uninduced culture ranged from 0.6 (experiment 1) to 4.2 (experiment 3).

[b] ND, not determined.

[c] Values are mean ± standard deviation.

TABLE 2
Effect of DDC on Hb synthesis in butyric acid-induced K-562 cells after 96 hr

Experiment		Control	25 μM DDC	100 μM DDC	250 μM DDC
1	B+ cells[a] (%)	34.3	34.2	35.2	35.8
	Ratio of B+ cells compared with control	1.000	0.997	1.026	1.044
2	B+ cells (%)	49.1	47.2	39.5	45.2
	Ratio of B+ cells compared with control	1.000	0.959	0.798	0.921
3	B+ cells (%)	58.2	52.6	48.1	48.4
	Ratio of B+ cells compared with control	1.000	0.904	0.826	0.832
	Average ratio of B+ cells compared with control	1.00	0.95 ± 0.04[b]	0.88 ± 0.10	0.93 ± 0.09

[a] Benzidine-positive cells.
[b] Values are mean ± standard deviation.

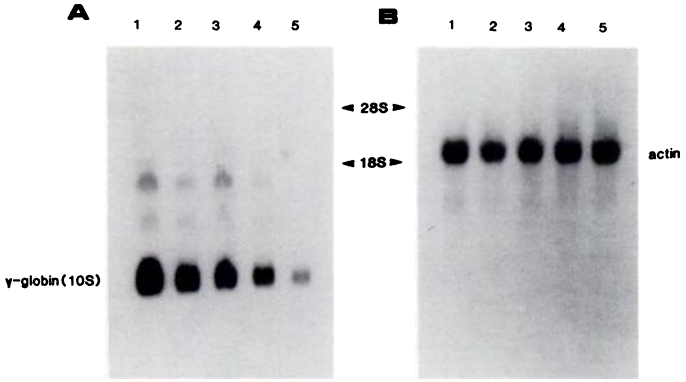

Fig. 2. Inhibition of globin mRNA synthesis by AZT. Cells were induced with 1.4 mM butyric acid and, 25 hr after initiation of the experiment, various concentrations of AZT were added for 71 hr. Total mRNA was purified by a guanidine thiocyanate-cesium chloride method. RNA equivalent to 4×10^5 cells (approximately 8 μg) was applied to each lane, electrophoresed on a denaturing 2.2 M formaldehyde/1% agarose gel, transferred to nitrocellulose, and hybridized to the ^{32}P-labeled γ-globin cDNA probe (see Experimental Procedures). Following autoradiography, the γ-globin probe was removed from the blot in boiling distilled water, and the blot was rehybridized with the ^{32}P-labeled actin probe. A, γ-Globin probe; B, actin probe. *Lanes 1–4*, butyric acid-induced cells exposed to AZT concentrations of 0, 25, 100, and 250 μM, respectively. *Lane 5*, uninduced cells. Densitometric scan analysis showed inhibition of globin levels of 23, 39, and 59% at AZT concentrations of 25, 100, and 250 μM, respectively (adjusted for differences in actin areas). The globin band in uninduced cells was 0.13 of control.

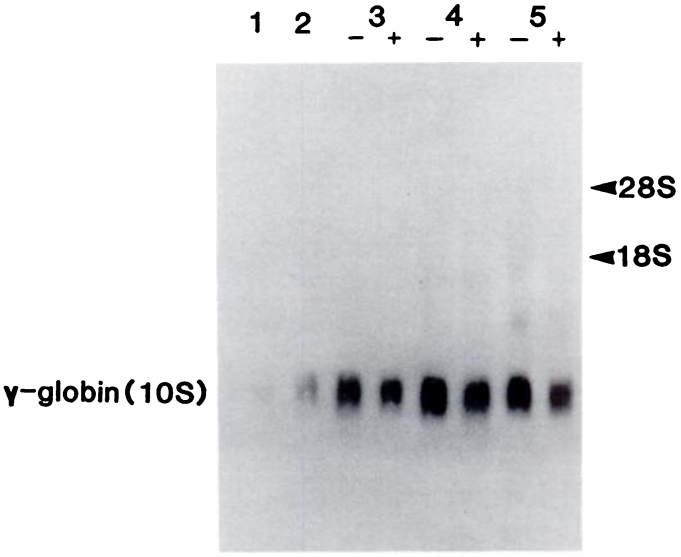

Fig. 3. Time course of globin mRNA expression in cells exposed to 100 μM AZT. Cells were cultured as described in Experimental Procedures. Cell aliquots (10 ml) were removed every 24 hr and total RNA was isolated. RNA (8 μg) from each time point was subjected to Northern blot analysis and hybridized with the γ-globin cDNA probe. −, RNA from control culture; +, RNA from culture exposed to 100 μM AZT. *Lane 1*, 0 hr; *lane 2*, 24 hr; *lane 3*, 48 hr; *lane 4*, 72 hr; *lane 5*, 96 hr. Densitometric scan analysis showed inhibition of globin levels of 14, 32, and 35% at 48, 72, and 96-hr time points.

ratios, which was quantitatively confirmed by densitometric scan analysis (Table 3).

Nuclear run-on transcription. The decrease in the steady state level of globin mRNA in K-562 cells caused by AZT, demonstrated above by both Northern analysis and *in vitro* translation analysis, may result from a direct inhibition of the rate of transcription or decreased stability of globin transcripts. Transcriptional regulation of globin expression was, therefore, studied with *in vitro* nuclear run-on assays. Because globin mRNA had reached its maximum level by 72 hr (Fig. 3), nuclei from butyric acid-induced K-562 cells exposed to 0, 25, or 100 μM AZT were isolated at this time. Nuclei isolated from 10^7 cells were incubated in the presence of 70 μCi of [α^{32}P]GTP, and the resultant radiolabeled RNA was hybridized to γ-globin and β-actin DNAs, immobilized on nitrocellulose filters, by a dot-blot method. The vector pUC19 was used as a control. This assay showed that K-562 cells exposed to 25 or 100 μM AZT have a substantially decreased rate of globin transcription (Fig. 5). Scan analysis of these transcripts revealed that approximately 20 and 50% inhibition of *in vitro* synthesized globin transcripts occurred in nuclei isolated from cells exposed to 25 and 100 μM AZT, respectively. The low level (approximately 5% compared with control) of background hybridization to the pUC19 vector demonstrated that hybridization was specific for globin sequences and not due to nonspecific hybridization. Globin transcription was sensitive to α-amanitin at a concentration of 1 μg/ml, which is known to selectively inhibit RNA polymerase II transcription (data not shown). The absolute content of globin hybrids ranged from 8 ppm in uninduced nuclei to 45 ppm in induced control nuclei, as determined by scintillation counting. In addition, mean incorporation of [α-^{32}P] GTP per cell into *in vitro* synthesized RNA was the same in AZT-treated cells and in untreated controls (data not shown). In this assay, *in vitro* synthesized actin transcripts above the background hybridization level were not detected.

TABLE 3
In vitro translation of RNA and gel analysis

	0 μM AZT	25 μM AZT	100 μM AZT	250 μM AZT	No RNA input
Total [^{3}H]leucine incorporation (dpm × 10^5)	5.35	5.03	5.60	4.57	0.10
Densitometric areas of specific translation products (arbitrary units)					
Approximate size[a]					
80,000	54.0	46.9	54.4	57.6	
60,000	201	203	216	203	
32,000	343	355	363	347	
22,000	154	174	135	136	
18,000	388	331	309	248	
Globin 15,000	2055	1881	1655	1154	
Globin chain(s)[b]					
ε and ζ	71	58	56	35	
Aγ	137	113	101	46	
Gγ	174	142	125	78	
α	84	77	66	31	

[a] Data were obtained from the autoradiogram illustrated in Fig. 4A.
[b] Data were obtained from the autoradiogram illustrated in Fig. 4B.

Similar data on low actin transcription in K-562 cell nuclei has also been previously reported (26).

Discussion

Previous studies from this laboratory have demonstrated that AZT directly suppresses human bone marrow CFU-GM and BFU-E colony growth in a dose-dependent manner (5), findings that were consistent with the clinical observation that anemia and neutropenia were the major adverse effects of AZT administration to patients with AIDS (4). In a more recent study, we reported that AZT was incorporated into nuclear DNA of human bone marrow cells and that this incorporation correlated to some degree with inhibition of CFU-GM colony formation (9). In the present study, effects of AZT on Hb synthesis machinery through potential genetic mechanism(s) were examined in butyric acid-induced K-562 cells. In particular, the effects of AZT on cell growth, percentage of cells that synthesize Hb, and globin mRNA expression were assessed.

In the present study, AZT inhibited both cell growth and Hb production in K-562 cells at concentrations of ≥100 μM. To determine whether this event was associated with an effect on globin mRNA synthesis, Northern blot analysis was performed. This study demonstrates a substantial dose-dependent decrease in the steady state levels of globin mRNA after exposure of cells to AZT concentrations as low as 25 μM. In contrast, actin steady state mRNA levels were not affected by AZT, suggesting that a general inhibition of mRNA synthesis did not occur. Furthermore, inhibition of globin mRNA steady state levels by AZT was present at each time point during a time course experiment, suggesting that the inhibition occurs throughout the course of induction and is not transient in nature.

Because the Northern blot analysis evaluated AZT effects on only two types of mRNA (globin and actin), effects on other mRNAs were assessed using an *in vitro* translation system. In this system, levels of [^{3}H]leucine-labeled translation products reflect the relative amounts of specific mRNAs present in the sample. As demonstrated by SDS-polyacrylamide gel analysis, essentially all of the mRNAs were equally translated, regardless of the level of AZT exposure, whereas the level of globin mRNA translation products was inhibited by AZT in a dose-dependent manner, in a fashion similar to that demonstrated by Northern analysis. Using a different gel system, which resolves the various globin polypeptide chains, we additionally demonstrated that AZT exposure inhibits all translatable globin mRNAs present in these cells (ζ, ε, Aγ, Gγ, and α) at approximately equal ratios. Therefore, the observed AZT inhibition of globin gene expression is not a result of a general inhibition of mRNA synthesis but appears to be specific for globin genes.

The inhibition of globin mRNA levels may result from several mechanisms, including a decreased rate of transcription, altered processing of heterogeneous nuclear RNA into mature mRNA, a change in globin mRNA transport to the cytoplasm, or decreased stability of globin mRNA. Nuclear run-on transcription assays were, therefore, performed to assess whether AZT inhibition of globin mRNA levels resulted from a decreased rate of globin transcription. These assays, which directly measure the rate of synthesis of specific RNA transcripts, demonstrated that inhibition of globin mRNA synthesis correlates with a decreased rate of globin gene transcription.

These data suggest that intracellular AZT (and/or its phosphorylated metabolites) is modifying the globin gene transcription unit so that fewer RNA polymerase II molecules are actively engaged in transcription of the gene. This may represent a direct, or *cis*, effect of AZT on globin genes or an indirect, or *trans*, effect on a factor(s) that regulates globin gene expression. *Cis* effects may include incorporation of AZT into nuclear DNA in globin gene regulation regions, possibly in specific sequences, thereby influencing the transcription of these genes. Several *trans* effects of AZT on globin gene expression are possible. AZT may modify the activity and/or amount of a specific regulatory factor involved in globin gene transcription. Alternatively, AZT may affect the multistep "program" of differentiation involved in the induction of Hb synthesis in K-562 cells. Several studies have explored whether drugs or physical agents have differential effects on "inducible" versus "constitutive-noninducible" genes. Hamilton and Wetterhahn (27) demonstrated that chromium(VI) inhibited transcription of two glutethimide-inducible genes, 5-aminolevulinate synthetase and cytochrome PB_1 P450, in chicken embryo hepatocytes. Under similar conditions, no effects on transcription of constitutively expressed genes for albumin, conalbumin, or β-actin were observed (27). In that context, it would be important to

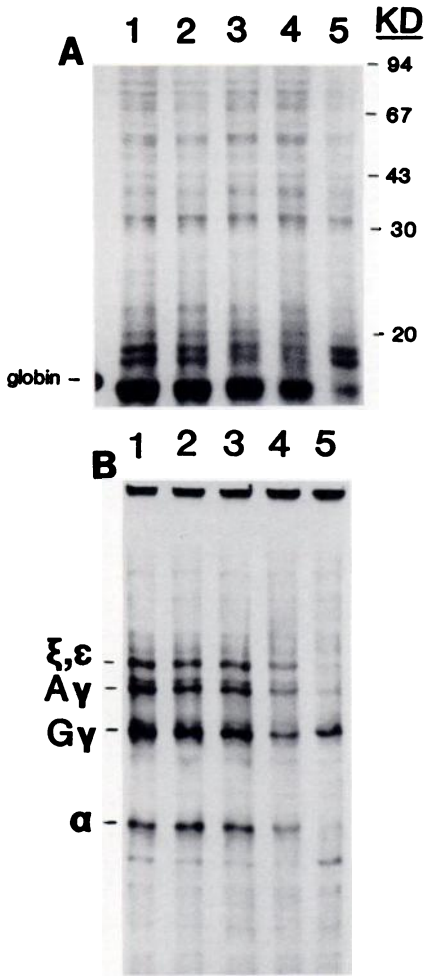

Fig. 4. *In vitro* translation of K-562 cell RNA. Total RNA from cells exposed to various concentrations of AZT was translated *in vitro* with a wheat germ translation system in the presence of [^{3}H]leucine, as described in Experimental Procedures. The translation products were analyzed in two separate gel systems. A, 10% polyacrylamide, 0.1% SDS gel; B, 2% Triton X-100, 6 M urea, 5% acetic acid, 12% polyacrylamide gel. For gel A, protein molecular weight markers were identified by Coomassie blue stain and the globin marker was ^{3}H-labeled translation product of *Xenopus* β-globin mRNA. For gel B, globin chain markers were identified by Coomassie blue staining. For both gels, *lanes 1–4*, translation products of K-562 cell RNA from butyric acid-induced cells exposed to AZT concentrations of 0, 25, 100, and 250 μM, respectively. *Lane 5*, translation products from uninduced cells.

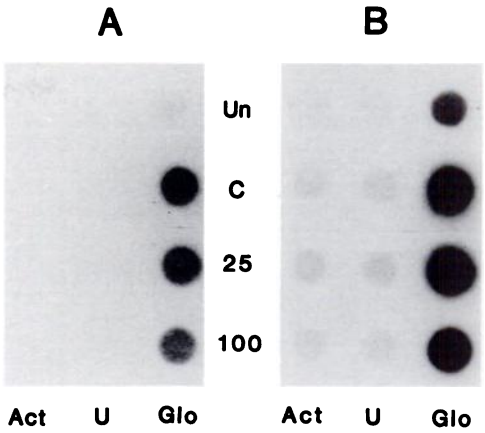

Fig. 5. Effect of AZT on rate of globin transcription. Nuclei were isolated from K-562 cells (exposed to various concentrations of AZT) 72 hr after initiation of the experiment, and run-on transcription assays were performed as described in Experimental Procedures. [α-^{32}P]GTP-labeled RNA was hybridized to linearized plasmid DNA immobilized on nitrocellulose filters. The DNA plasmids were the following: *Act*, plasmid pRβA-1 with a rat β-actin cDNA insert; *U*, plasmid pUC19 used as vector control; *Glo*, plasmid −200 Aγ carrying the human Aγ-globin gene. The same amount of radioactivity (6.3 × 10^6 dpm) from each RNA sample was used in the hybridization. The filter strips were exposed for autoradiography for 7 hr (A) or 40 hr (B). *Un*, nuclei from uninduced cells (negative control); *C*, nuclei from butyric acid-induced cells (positive control); *25*, nuclei from butyric acid-induced cells exposed to 25 μM AZT; *100*, nuclei from butyric acid-induced cells exposed to 100 μM AZT. Densitometric scan analysis showed inhibition of globin hybrids of 20 and 50% at 25 and 100 μM AZT, respectively, after correction for hybridization to pUC19 control DNA.

determine whether AZT specifically inhibits induction of embryonic and fetal globin genes or generally affects inducible gene expression. Other genes reported to be induced by butyric acid in K-562 cells include acetylcholinesterase (28), C-*sis* (29), and 5-aminolevulinate dehydratase, an important enzyme in heme biosynthesis (30). 5′-Aminolevulinate synthetase, the rate-limiting heme synthetic enzyme, is also inducible in K-562 cells (31).

Although the effect of AZT on heme synthesis was not evaluated in our studies, a recent study by Abraham *et al.* (8) demonstrated that hemin can partially overcome the toxicity of AZT to both human and mice bone marrow CFU-E and BFU-E colonies. Preliminary data of these authors suggest that these effects might be due to inhibition of aminolevulinate synthetase by AZT. Therefore, AZT may have multiple effects on Hb biosynthesis machinery, possibly through inhibition of inducible gene expression.

Parallel experiments with DDC, another dideoxynucleoside currently being evaluated in clinical trials, demonstrated that, although DDC markedly inhibited K-562 cell growth (suggesting phosphorylation of DDC to its triphosphate derivative in these cells), no direct effects on Hb production were observed. These results are consistent with the absence of DDC-induced anemia in patients (32), further demonstrating that AZT effects on globin gene expression are specific, as compared with effects of other 2′,3′-dideoxynucleosides, and not related to a general inhibition of cell proliferation.

In summary, the present study provides the first detailed analysis of the effects of AZT on cellular gene expression and their relevance to host cell toxicity. These data demonstrate that AZT-induced anemia may be associated with inhibition of Hb synthesis in differentiating erythroid cells through inhibition of globin gene expression. Elucidation of the molecular mechanisms involved in AZT-induced toxicity will, hopefully, aid in the design of better treatment regimens to overcome these toxic side effects.

Acknowledgments

We are grateful to Ricarda Carlisle, Meng-yu Xie, Mona McPherson, Hiren Patel, Betty Hatcher, and Edward Bridges for technical support and to Bevery Caver and Janna Stockinger for assistance in preparation of this manuscript. We also wish to thank Robert Leboeuf and Martin Johnson for helpful suggestions and Gail Johnson for help with the densitometer.

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