0026-895X/89/010009-06$02.00/0

MOLECULAR PHARMACOLOGY, 36:009-014

ACCELERATED COMMUNICATION

Cellular Pharmacology of 3′-Azido-3′-Deoxythymidine with Evidence of Incorporation into DNA of Human Bone Marrow Cells

JEAN-PIERRE SOMMADOSSI, RICARDA CARLISLE, and ZHU ZHOU

Department of Pharmacology, Center for AIDS Research, and Comprehensive Cancer Center, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received December 9, 1988; Accepted April 12, 1989

SUMMARY

We previously demonstrated that 3′-azido-3′-deoxythymidine (AZT) inhibits growth proliferation of human bone marrow progenitor cells *in vitro* [*Antimicrob. Agents Chemother.* 31:452–454 (1987)]. The present study evaluates the effect of toxic concentrations of AZT on possible sites of toxicity in human bone marrow cells. Exposure of cells over a 6-hr period to AZT concentrations between 0.5 and 50 μM resulted in a decreased incorporation of tritiated deoxyguanosine into DNA. Unchanged AZT and its phosphorylated metabolites accumulated within cells after exposure to 10 μM [^{3}H]AZT. 3′-Azido-3′-deoxythymidine-5′-monophosphate was the predominant metabolite, reaching a concentration of 49.2 ± 14.1 pmol/10^6 cells after 48 hr, and a continuous increase was observed in all phosphorylated derivative levels between 2 and 48 hr of incubation. Using a highly sensitive and specific DNA polymerase assay, endogenous deoxyribonucleotide pool size(s) were analyzed for 48 hr after incubation of cells with a pharmacologically relevant concentration of 10 μM AZT. After a 6-hr exposure, 2′-deoxycytidine-5′-triphosphate and 2′-deoxythymidine-5′-triphosphate pools represented approximately 86 and 70% of the control values; levels returned to normal after 24 hr and remained subsequently unchanged. Nucleic acids of human bone marrow cells exposed for 24 hr to 10 μM [^{3}H]AZT were purified and analyzed by cesium sulfate density gradient. No radioactivity was detected in the RNA region, whereas a significant amount was associated with the DNA region. Hydrolysis of radiolabeled DNA and subsequent analysis by high performance liquid chromatography demonstrated specific incorporation of AZT into DNA. In additional studies, the amount of AZT incorporated into DNA was correlated with the initial extracellular AZT concentration. In particular, a significant relationship ($p < 0.0001$) between the level of AZT incorporated into DNA and the inhibition of clonal growth was observed at concentrations of AZT between 1 and 25 μM (IC_{50} and IC_{85} for human bone marrow cells). In summary, these studies demonstrate that AZT is incorporated into DNA of human bone marrow cells and suggest that incorporation of AZT into DNA may be one mechanism responsible for AZT-induced bone marrow toxicity. In contrast, imbalance of deoxyribonucleotide pools by AZT appears unlikely to be associated with inhibition of DNA synthesis and toxicity in human bone marrow cells.

AZT is the first clinically approved drug for the treatment of AIDS. The antiretroviral effects of AZT are thought to be due to its conversion through cellular kinases, to AZT-TP, which competitively inhibits the human immunodeficiency virus reverse transcriptase, terminates the newly synthesized viral DNA chain, or both (1–4). The major limitation to AZT therapy is bone marrow toxicity, manifested as anemia and neutropenia, which requires a dosage reduction or discontinuation of treatment in approximately 40% of patients (5). Consistent with these toxic manifestations, we previously demonstrated that AZT produced a dose-dependent inhibition of human CFU-GM and erythroid burst-forming unit colonies at clinically achievable AZT concentrations (6).

Several hypotheses have been suggested for the biochemical mechanism(s) responsible for the cytotoxic effects of AZT for human host cells. One hypothesis is that thymidylate kinase is inhibited by AZT-MP, resulting in decreased formation of dTTP needed for DNA synthesis (7). Significant variation in the degree of AZT-induced perturbation of deoxyribonucleotide pools has been reported (1, 8–10), which may reflect differences

This work was supported by Public Health Service Grants HL 42125, AI 25784, and NO1 RR 00032. J.-P.S. is a recipient of a Junior Faculty Research Award from the American Cancer Society.

ABBREVIATIONS: AZT, 3′-azido-3′-deoxythymidine; AIDS, acquired immunodeficiency syndrome; AZT-TP, 3′-azido-3′-deoxythymidine-5′-triphosphate; AZT-MP, 3′-azido-3′-deoxythymidine-5′-monophosphate, AZT-DP, 3′-azido-3′-deoxythymidine-5′-diphosphate; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography; CFU-GM, colony forming unit granulocyte-macrophage; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

among cell populations. These differences in cell sensitivity indicate that confirmation of that mechanism will require studies using cells representative of the toxicity site, such as human bone marrow cells. St. Clair *et al.* (2) have suggested that incorporation of AZT into newly synthesized DNA, with inhibition of DNA elongation by chain termination, may be another mechanism of AZT toxicity; however, these studies were performed with purified DNA templates and the incorporation of AZT into host cellular DNA using intact cells has not been examined.

The relative contribution of hypothesized mechanisms to AZT bone marrow toxicity is unclear from these previous studies, which usually have focused on one mechanism without considering the others and which have not been conducted in human bone marrow cells. Furthermore, the correlation of a biochemical event with toxicity has never been demonstrated. Therefore, in the present study, following analysis of AZT metabolism, we evaluated the effects of specific toxic concentrations of AZT on endogenous deoxyribonucleotide pools and AZT incorporation into nucleic acids of human bone marrow cells. A correlation between incorporation of AZT into cellular DNA and toxicity, as assessed by a colony-forming assay (6), is reported. These studies have been presented in part previously (11).

Materials and Methods

Chemicals. [*methyl*-^{3}H]AZT (3 Ci/mmol) and radiolabeled nucleosides, [8-^{3}H]2′-deoxyguanosine (12 Ci/mmol), [2-^{3}H]adenine (15 Ci/mmol), and [6-^{3}H]uridine (20 Ci/mmol), were purchased from Moravek Biochemicals (Brea, CA). The purity of radiolabeled AZT was ≥99%, as ascertained by the HPLC technique described below. [*methyl*-^{3}H]dTTP (21 Ci/mmol), [8-^{3}H]dATP (22 Ci/mmol), [8-^{3}H]dGTP (10 Ci/mmol), and [5-^{3}H]dCTP (18 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). Nonlabeled standards, AZT, AZT-MP, AZT-DP, and AZT-TP were generous gifts of Dr. Raymond F. Schinazi (Veterans Administration Medical Center and Emory University, Atlanta, GA). Nucleosides and nucleotides, RNAase A and T_1, pronase B, and alkaline phosphatase were obtained from Sigma Chemical Co. (St. Louis, MO). Micrococcal nuclease, snake venom, and spleen phosphodiesterase were purchased from Worthington Biochemicals (Fairfield, NJ). All other chemicals and reagents were of the highest analytical grade available.

Bone marrow cell isolation. Human rib specimens, obtained during thoracic surgery according to a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham, were the source for bone marrow cells. Cells were flushed from the ribs, under sterile conditions, with Hanks' balanced salt solution in phenol red with 10% fetal calf serum and 1% penicillin-streptomycin. The cell mixture was centrifuged at 1200 rpm in a Beckman GPR centrifuge for 10 min and the supernatant was discarded. After resuspension in Hanks' balanced salt solution with 10% fetal calf serum and 1% penicillin-streptomycin, cells were gently layered onto 10 ml of Ficoll-Hypaque (Sigma) and were centrifuged at 1500 rpm in a Beckman GPR centrifuge for 35 min. The mononuclear cell layer was removed and the cell suspension was washed twice at 1200 rpm for 10 min. Cells were counted with a hemacytometer and viability was >98%, as assessed by trypan blue exclusion. Under these conditions, approximately 400×10^6 cells/rib were collected.

Effect of AZT on nucleic acid synthesis of human bone marrow cells. Human bone marrow cells (2×10^6 cells/ml) were suspended in McCoy's 5A nutrient medium that contained 15% dialyzed fetal bovine serum. After the addition of 0.5, 5, or 50 μM AZT, cells were maintained at 37° under an atmosphere of 5% CO_2. At specified times, aliquots of cells were exposed for 60 min to [^{3}H]2′-deoxyguanosine (3 μCi/ml), to measure DNA synthesis, or [^{3}H]adenine (3 μCi/ml), to measure RNA synthesis. At the end of the pulse, the medium was removed and cells were washed three times with cold phosphate-buffered saline. TCA-soluble activities were determined by extracting the cell monolayer twice with 2 ml of cold 5% TCA. Incorporation of [^{3}H]2′-deoxyguanosine into DNA was determined by further washing the pellet with methanol and then ether. The pellet was dried overnight at 70° and then digested with 300 μl of 1 N KOH. Samples were mixed with scintillation fluid and counted for radioactivity in a Beckman LS-5801 liquid scintillation counter. When incorporation of [^{3}H]adenine into RNA was investigated, the acid-insoluble activities were washed with 0.2 N $HC10_4$ and RNA was hydrolyzed at 37° for 1 hr in 0.3 N NaOH. After acidification with 5.2 N $HC10_4$ and centrifugation, the supernatant was removed and counted in a Beckman LS-5801 liquid scintillation counter for determination of radioactivity incorporation into RNA.

Effect of AZT on endogenous deoxyribonucleotide pools. Human bone marrow cells (1×10^5 cells/ml) were exposed to 10 μM AZT (IC_{75}) (6), in McCoy's medium with nutrients, for specific time periods of between 6 and 48 hr. A total of 2×10^7 cells was used for each time point and, at the end of the incubation period, cells were extracted with 60% cold methanol. Cell extracts were stored overnight at −20° and then centrifuged at 15,000 rpm for 5 min in an IEC B20A centrifuge and the pellets were discarded. Supernatants, to which water was added, were lyophilized to dryness. A second extraction was performed with 2 ml of 0.4 N $HC10_4$ for 30 min on ice. After centrifugation, the supernatant was neutralized with 1.5 ml of 1 N KOH and subsequently lyophilized to dryness. The deoxyribonucleoside-5′-triphosphate levels were enzymatically determined as follows. The dATP and dTTP assay mixtures contained a total final volume of 180 μl. This included 0.02 A_{260} units of poly[d(A,T)] (Pharmacia, Piscataway, NJ), 1.8 μmol of $MgCl_2$, 1.8 μmol of dAMP, 18 μmol of HEPES buffer, pH 7.4, and 0.75 Richardson units of DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, IN). The dATP assay included 250 pmol (2 μCi) of [^{3}H]dTTP and 0 to 80 pmol of unlabeled dATP. The dTTP assay contained 1 nmol (8 μCi) of [^{3}H]dATP and 0 to 80 pmol of unlabeled dTTP. The reaction mixtures were identical for the dGTP and dCTP assays, except that 0.02 A_{260} units of poly[d(I,C)] (Pharmacia) and 2 and 3.0 units of DNA polymerase I were used for dGTP and dCTP determinations, respectively. The dGTP assay included 500 pmol (6.6 μCi) of [^{3}H]dCTP and 0 to 40 pmol of dGTP, whereas the dCTP assay included 1 nmol (6 μCi) of [^{3}H]dGTP and 0 to 50 pmol of dCTP. Assays were performed at 37° for 35 min. Samples (50 μl) were spotted on squares of Whatman 3-mm filter paper. Precipitation of nucleotides was performed with a mixture of ice-cold 10% TCA and 1% sodium pyrophosphate. The filters were washed twice with ice-cold 5% TCA followed by 95% ethanol. Filters were then dried and radioactivity was determined in a Beckman LS-5801 liquid scintillation counter. This assay had a sensitivity limit of approximately 1–2 pmol/10^6 cells for each deoxyribonucleotide.

Analysis of [^{3}H]AZT metabolites by HPLC. A high performance liquid chromatograph (Hewlett-Packard 1090) that was equipped with automatic injector, filter spectrophotometric detector, and chromatographic terminal (Hewlett-Packard 3393A) was used for analysis of AZT metabolites. All analyses were performed using a modified HPLC method previously described (1), which can simultaneously resolve the unchanged AZT and its mono-, di-, and triphosphate derivatives. Anion exchange chromatography was performed using a Partisil 10 SAX column (Whatman, Inc., Clifton, NJ). Elution was carried out at 1 ml/min with 15 mM KH_2PO_4 (pH 3.5) and a 55-min linear gradient of 1 M KH_2PO_4 (pH 3.5) from 0 to 100%, starting at the time of injection. Under the conditions defined above, the retention times of the unlabeled markers, AZT, AZT-MP, AZT-DP, and AZT-TP, were 5.8, 11, 29, and 56 min, respectively. The total radioactivity applied to the column was recovered in 65 min.

Incorporation of AZT into nucleic acids of human bone marrow cells. Human bone marrow cells (2×10^6 cells/ml) were suspended

in McCoy's 5A medium that was supplemented with nutrients and 10% dialyzed heat-inactivated fetal bovine serum, at 37° in a 5% CO_2 incubator. After addition of 1, 5, 10, 15, or 25 μM [^{3}H]AZT at a final specific activity of approximately 750 mCi/mmol, cells were incubated for 24 hr. Cells were then washed twice in phosphate-buffered saline and resuspended in 2 ml of 0.015 M sodium citrate/0.15 M sodium chloride solution (pH 7.0). Digestion of cells was accomplished by adding 0.2% sodium dodecyl sulfate (20 μl) and 250 μg/ml proteinase K and incubating the mixture overnight at 37°. Nucleic acids were extracted with a 1:1 mixture of phenol (containing 0.1% hydroxyquinoline and 0.2% β-mercaptoethanol) and a chloroform/isoamyl alcohol solution (24:1, v/v). A second extraction was performed with 2 ml of the chloroform/isoamyl alcohol solution. Nucleic acids were precipitated by adding 0.3 M sodium acetate and 2 volumes of ice-cold absolute ethanol. After 3 hr at −20°, nucleic acids were centrifuged at 7000 rpm from 30 min in an IEC B20A centrifuge and the precipitate was washed with ice-cold 70% ethanol. After resuspension in 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, RNA was hydolyzed for 2 hr at 37° with 50 μl of heat-inactivated RNAse A and 50 μl of heat-inactivated RNAse T_1. Samples (100 μl) were spotted on squares of Whatman 3-mm filter paper and filters were washed twice with ice-cold 5% TCA followed by 95% ethanol. Filters were dried and radioactivity was determined in a Beckman LS-5801 liquid scintillation counter. The amount of DNA in each sample was ascertained by a fluorometry technique, as described previously (12).

Cesium sulfate density gradient centrifugation. After phenol extraction and ethanol precipitation (see above), nucleic acids were analyzed by cesium sulfate gradient centrifugation in a solution (4 ml) containing 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 1.54 g/ml cesium sulfate. Centrifugation was performed at 33,000 rpm for 60 hr at 20°, using a Beckman SW60 Ti rotor. Fractions (0.1 ml) were collected from the top to the bottom of the tube, using a Buchler Auto-Densi Flow II pump driver. The density of each fraction was determined with an ABBE refractometer.

Digestion of [^{3}H]AZT-labeled DNA. After cesium sulfate density gradient centrifugation, samples with a density of 1.41 to 1.43 g/ml were combined and dialyzed overnight against 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, after which DNA was precipitated in 0.3 M sodium acetate and ice-cold absolute ethanol. DNA was then dissolved in 20 μl of Tris·HCl (pH 7.4), 1 mM EDTA, and heat denatured by boiling at 100° for 5 min. The DNA was hydrolyzed at 37° for 30 min with 50 μl of micrococcalnuclease (12 units/ml), in 10 mM Tris·HCl (pH 8.8), 2 mM $CaCl_2$. After addition of a mixture containing 1 μl of 50 mM EDTA, 3 μl of 1 M sodium acetate buffer (pH 6.5), and 3 μl of 0.1 M $MgCl_2$, degradation to nucleosides was carried out at 37° for 24 hr with spleen phosphodiesterase (2 units), snake venom phosphodiesterase (2 units), and alkaline phosphatease (0.3 units). Aliquots were subsequently analyzed by HPLC using the methodology described above.

Clonogenic assay for drug cytotoxicity. The culture assay of CFU-GM was performed using a bilayer soft agar method as we previously described (6), except that human recombinant GM-CSF (50 units/ml) (Genzyme, Boston, MA) was used as a source of colony-stimulating factor. McCoy's 5A nutrient medium that was supplemented with 15% dialyzed fetal bovine serum (heat inactivated at 56° for 30 min) was used in all experiments. Briefly, mononuclear cells (10^5/ml) were exposed for 24 hr at 37°, in 5 ml of McCoy's 5A nutrient medium, to AZT concentrations of between 1 and 25 μM. Cells were then washed twice with fresh cold incubation medium and cells were subsequently cloned in 0.3% agar. After 14 days of incubation at 37° in a humidified atmosphere of 5% CO_2 in air, colonies (≥50 cells) were counted by using an inverted microscope. The clonogenic efficiency was between 0.05% and 0.1% in all experiments.

Results

Intracellular metabolism of AZT in human bone marrow cells. After incubation of human bone marrow cells with 10 μM [^{3}H]AZT, AZT was rapidly phosphorylated within cells, as evidenced by the substantial amount of AZT-5′-phosphate derivatives. The intracellular concentrations of AZT and its metabolites after 2-, 6-, 24-, and 48-hr incubation periods are shown in Table 1. Continuing elevation of AZT-MP, AZT-DP, and AZT-TP intracellular concentrations was observed during the 48-hr incubation. Table 2 suggests that the increase in AZT phosphorylated metabolites over time was not due to an increased content of anabolizing enzymes, because the percentage of cells in S phase did not substantially change over time or in the presence of AZT. AZT-MP accounted for the majority (88 to 89%) of intracellular metabolites at all time points. Concentrations of AZT-DP and AZT-TP were in the same range.

Effect of AZT on nucleic acid synthesis. When human bone marrow cells in suspension were exposed to either 0.5, 5 or 50 μM AZT over a 6-hr period, deoxyguanosine incorporation into DNA was progressively inhibited (Fig. 1). By the 6th hour, DNA synthesis was decreased to approximately 65 and 50% of control in the presence of 5 and 50 μM, respectively. Only a slight decrease in the incorporation of deoxyguanosine (approximately 12%) was observed in the presence of 0.5 μM AZT. In contrast, RNA synthesis, as measured by incorporation of [^{3}H] adenine, was totally unaffected by concentrations of AZT as high as 100 μM (data not shown).

Effect of AZT on endogeneous deoxyribonucleotide pools. Intracellular concentrations of dCTP, dTTP, dATP, and dGTP were determined in human bone marrow cells (10^6 cells/ml) after exposure to a pharmacologically relevant concentration of AZT (10 μM) for varying time periods. After a 6-

TABLE 1

Metabolism of [^{3}H]AZT in human bone marrow cells

Human bone marrow cells (2 × 10^6 cells/ml) were suspended in McCoy's 5A medium that was supplemented with nutrients and 10% dialyzed heat-inactivated fetal bovine serum, at 37° in a 5% CO_2 incubator. The experiment was initiated with the addition of 10 μM [^{3}H]AZT (specific activity, 200 mCi/mmol) and cells were exposed to drug for varying time periods. Cell viability was 95% or greater, as assessed by trypan blue exclusion, and cell number was constant over 48 hr. Medium was then removed and cells were washed three times with cold Hanks' balanced salt solution. Extraction of cell pellets was performed with 0.5 ml of cold 0.5 N perchloric acid. After centrifugation at 1500 rpm for 5 min at 4°, supernatants were neutralized with 100 μl of 5 M potassium bicarbonate. Aliquots were analyzed by HPLC as described in Materials and Methods. Values are mean ± standard deviation from three experiments.

Time of Exposure	Intracellular Concentration		
	AZT-MP	AZT-DP	AZT-TP
hr	*pmol/10^6 cells*		
2	32.2 ± 9.1	0.04 ± 0.02	0.05 ± 0.04
6	40.1 ± 15.5	0.1 ± 0.04	0.06 ± 0.02
24	44.5 ± 19.5	0.12 ± 0.03	0.08 ± 0.04
48	49.2 ± 14.1	0.18 ± 0.08	0.12 ± 0.02

TABLE 2

Constituency of bone marrow cells as determined by microfluorometric analysis

Cell cycle analysis was determined with a Becton-Dickinson FACS Scan by DNA histogram flow cytometry. A total of 1.0 to 1.5 × 10^6 cells were centrifuged at 160 × *g* for 5 min. The medium was discarded and the pellet was stained by a hypotonic solution of propidium iodide.

Cell Cycle	Bone Marrow Cell Constituency		
	Control, 0 hr	Control, 24 hr	10 μM AZT, 24 hr
	%		
G_0–G_1 phase	79	69	69
S phase	19	31	28
G_2-M phase	2	0	4

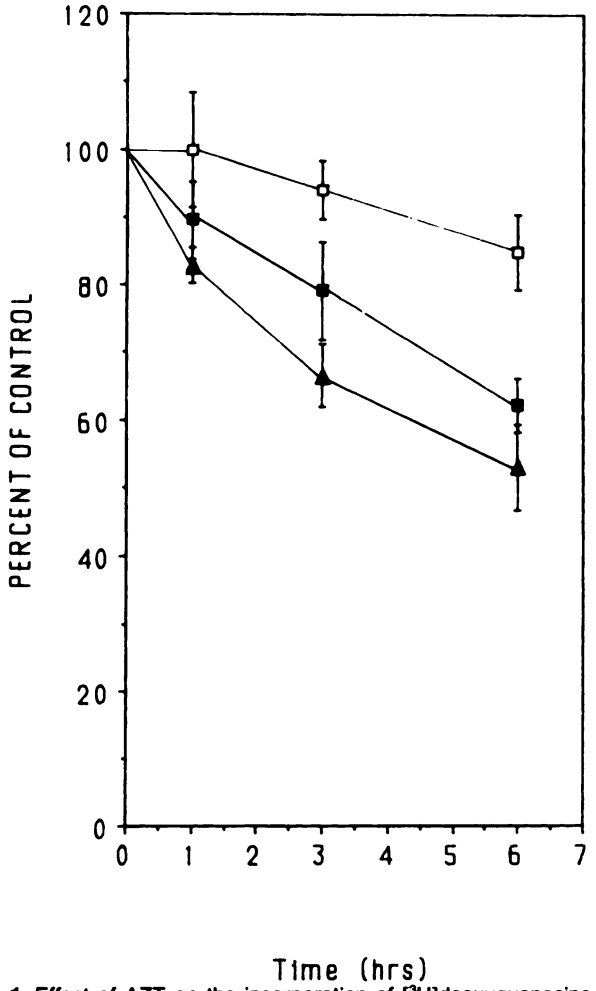

Fig. 1. Effect of AZT on the incorporation of [^{3}H]deoxyguanosine into DNA by human bone marrow cells. Experiments were performed as described in Materials and Methods, with exposure of cells for 1, 3, and 6 hr to 0.5 (□), 5 (■), or 50 μM AZT (▲). Each *point* represents the mean ± standard deviation of at least two experiments with different marrow cells.

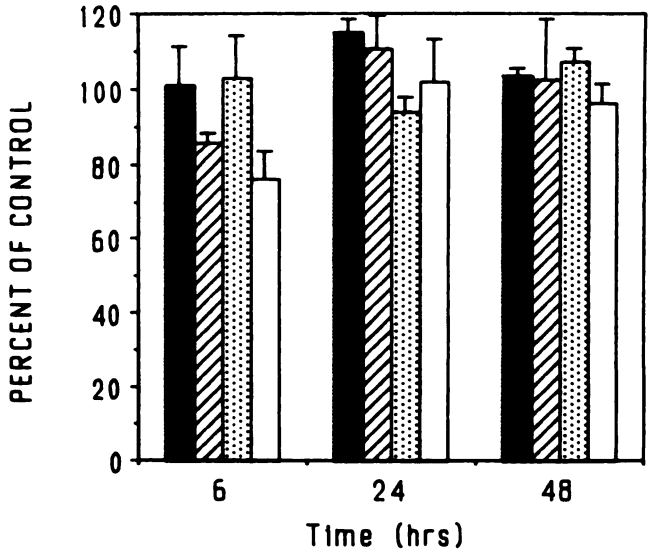

Fig. 2. Effect of AZT on pool size of endogenous deoxyribonucleoside-5′-triphosphate in human bone marrow cells. *Columns*, mean percentage inhibition of dATP (■), dGTP (▨), dCTP (▩), and dTTP (□) as compared with control in at least two experiments; *bars*, standard deviation.

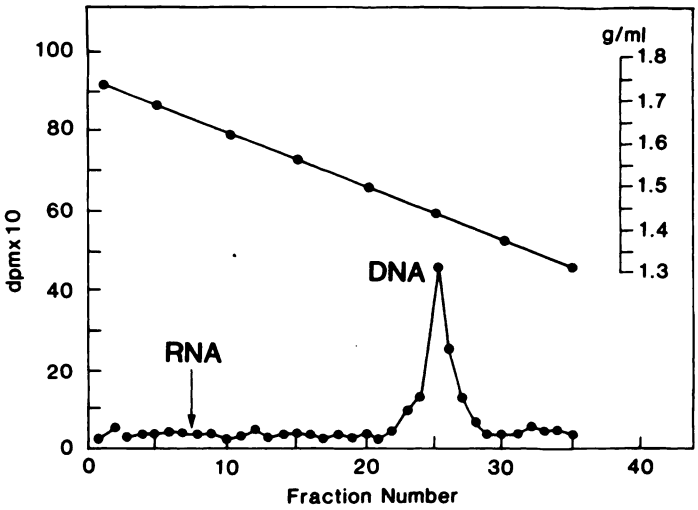

Fig. 3. Cesium sulfate density gradient of [^{3}H]AZT-radiolabeled nucleic acids. Human bone marrow cells at a density of 2×10^6 cells/ml were incubated with 10 μM [^{3}H]AZT (specific activity, 750 mCi/mmol) for 24 hr. Purification of nucleic acids and centrifugation were performed as described in Materials and Methods. Fractions of 100 μl were collected and 90-μl samples were spotted on squares of Whatman 3-mm filter paper. Filters were washed twice with 5% TCA followed by 95% ethanol. Filters were dried and radioactivity was determined.

hr exposure, dCTP and dTTP pools represented approximately 86 and 70% of the control values, respectively, whereas the dGTP and dATP levels were unchanged (Fig. 2). All deoxyribonucleotide pools were unaffected following a 24- or 48-hr exposure to 10 μM AZT. These data demonstrate that depletion of dTTP pools in human bone marrow cells is minimal, transient, and rapidly reversible.

Analysis of AZT incorporation into nucleic acids. Cellular nucleic acids from human bone marrow cells that were exposed for 24 hr to 10 μM [^{3}H]AZT were purified and analyzed by cesium sulfate density gradient. No radioactivity was detected in the RNA region, whereas a significant amount of tritium label was associated with the DNA region (density, 1.42 g/ml) (Fig. 3). Evidence that all the radioactivity detectable in DNA is associated with AZT was obtained by hydrolyzing the DNA to nucleosides and subsequently analyzing the nucleoside mixture by an HPLC methodology described in Materials and Methods. Fig. 4 shows the radiochromatogram of enzymatically digested DNA. All radioactivity coeluted with an authentic AZT standard, demonstrating that the detected radioactivity is specific for the incorporation of [^{3}H]AZT into DNA. To determine to what extent AZT was incorporated into DNA, kinetics were investigated at concentrations of AZT ranging from 1 to 25 μM. The amounts of AZT incorporated into DNA were determined after DNA purification by a disc filter assay, which appeared to be quantitatively more precise than a cesium sulfate gradient analysis. Fig. 5 demonstrates that, after a 24-hr exposure, there is an apparent linear relationship between the initial extracellular concentration of AZT and the amount of AZT incorporated into DNA.

Correlation of toxicity with AZT incorporation into DNA of human bone marrow cells. To evaluate whether the level of AZT incorporation into DNA correlated with the toxic effects of AZT for human bone marrow cells, studies were undertaken to compare the amount of AZT incorporated into

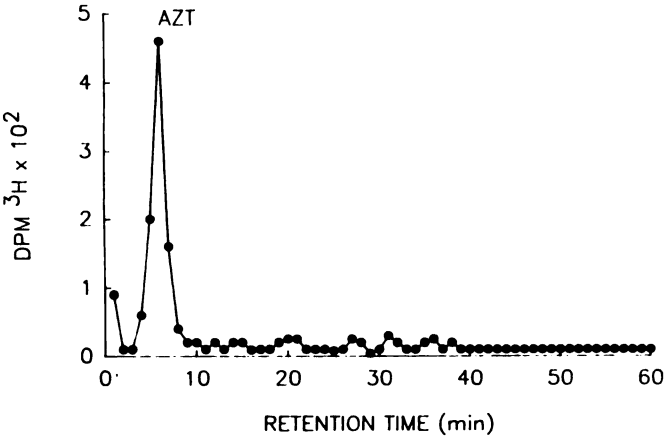

Fig. 4. HPLC analysis of enzymatically digested DNA labeled with 10 μM [^{3}H]AZT. Purified DNA was digested to nucleosides by the sequential action of micrococcal nuclease, snake venom and spleen phosphodiesterase, and alkaline phosphatase, as described in Materials and Methods.

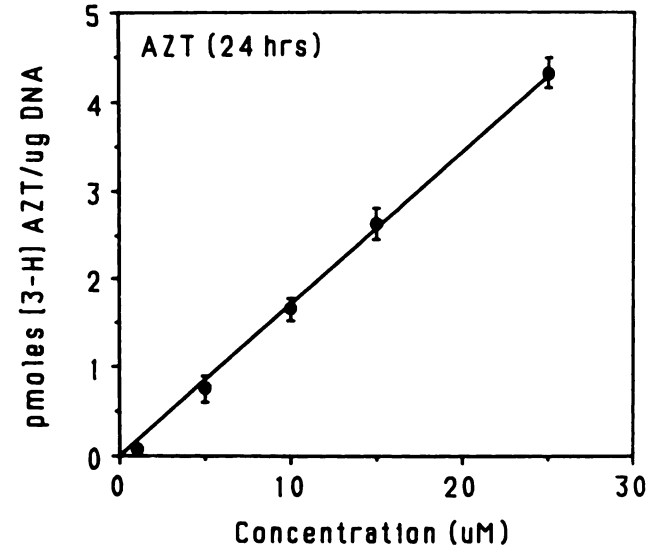

Fig. 5. Relationship of AZT incorporation into DNA after 24 hr versus the initial extracellular concentration of AZT. Each *point* represents the mean ± standard deviation of three separate experiments.

DNA with the clonogenic survival fraction after exposure for 24 hr to 1, 5, 10, 15, or 25 μM AZT. Fig. 6 shows that, by 50% inhibition of CFU-GM colony formation, the decline in the survival fraction was proportionate to the amount of AZT incorporated into DNA. Correlation between incorporation into DNA and inhibition of clonal growth at concentrations of AZT lower than the IC_{50} (1 μM) is uncertain because amounts of radioactivity associated with DNA were less than the limit of sensitivity.

Discussion

In a previous report, we demonstrated that AZT directly suppressed human bone marrow progenitor colony growth in a dose-dependent manner, by direct interaction with CFU-GM and erythroid burst-forming unit precursor cells (6). These findings were consistent with the clinical observation that anemia and neutropenia were the major adverse effects of AZT administration to patients with AIDS (13). In an attempt to correlate AZT toxicity with hypothesized sites of toxicity, the

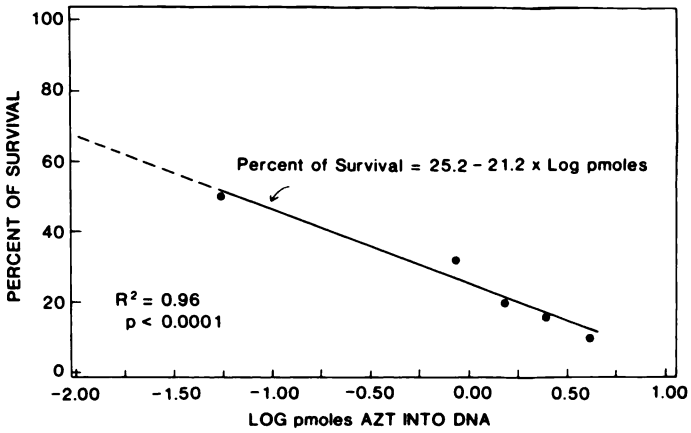

Fig. 6. Relationship between AZT-induced inhibition of human granulocyte-macrophage clonal growth and incorporation of AZT into cellular DNA (pmol/μg of DNA). Human bone marrow cells were exposed for 24 h to AZT concentrations ranging from 1 to 25 μM. The effect of AZT on human bone marrow cells as assessed by a colony-forming assay has been previously described (6). Values shown are the mean of two experiments, with values varying less than 10% at each studied concentration.

present study examined the effect of a range of previously determined toxic concentrations of AZT on (i) formation of intracellular AZT phosphorylated metabolites; (ii) nucleic acid biosynthesis; and (iii) incorporation of AZT into cellular DNA of human bone marrow cells.

Fig. 1 demonstrates that AZT toxicity in human bone marrow cells is probably due to its inhibitory effect on DNA synthesis, as previously speculated by others (7). Intracellular anabolic phosphorylation of AZT and other dideoxynucleosides with formation of 5′-triphosphate derivatives has been demonstrated to be a major factor in exerting their anti-HIV effect (1, 10). In contrast, Balzarini *et al.* (14) have suggested that the absolute intracellular concentration of AZT-TP appears not to be correlated with the cytotoxic effect of the drug. Table 1 demonstrates that AZT is activated to phosphorylated metabolites in human bone marrow cells. As observed in all human cell lines (1, 14), conversion of AZT-MP to AZT-DP was the rate-limiting step, leading to a high intracellular level of AZT-MP. Of particular importance was that all 5′-phosphorylated AZT metabolites continued to accumulate as a function of time, up to 48 hr in human bone marrow cells. This represents a very different pattern than data reported by Balzarini *et al.* (14) in ATH8 and Molt 4 human cell lines, where the intracellular AZT metabolites reached maximum levels at 5 hr and subsequently declined (14). Our data suggest that AZT-TP may have a quite long half-life in human bone marrow cells, which may be relevant to its cytotoxic effect. Deoxyribonucleoside-5′-triphosphate pool imbalance with, essentially, depletion of dTTP pools as a result of inhibition of thymidylate kinase by AZT-MP has been hypothesized as a major mechanism of AZT-induced toxicity (1, 7). In contrast, a recent study by Hao *et al.* (10) demonstrated that AZT, at pharmacologically meaningful concentrations, had no effect on dTTP pools; however, studies were performed in H_9 and Molt 4 cell lines, which are quite insensitive to AZT toxicity, with IC_{50} values of approximately 1 mM (1) and 63 μM (14), respectively. Fig. 2 demonstrates that, at a toxic concentration of 10 μM AZT (an IC_{75} value for human granulocyte-macrophage progenitor cells), dTTP pools were not markedly inhibited over a 6-hr exposure, with approx-

imately 70% of control levels remaining. Furthermore, this slight decrease was only transient and by 24 hr no inhibition was observed. This indicates that AZT-induced toxicity of human bone marrow cells is probably not associated with a decrease of the dTTP pool leading to inhibition of DNA synthesis and further confirms results from our laboratory, which indicated that thymidine was unable to reverse the toxic effects of AZT for human granulocyte-macrophage precursor cells (15).

Recent studies (2, 3) have suggested that AZT may be incorporated into newly synthesized DNA; however, to our knowledge, no study has yet demonstrated that AZT adds to cellular DNA in an intact cell system. Our studies provide the first detailed analysis of the incorporation of AZT into DNA of human bone marrow cells and the potential role of this incorporation in the AZT cytotoxic effects. Using cesium sulfate density gradient centrifugation, all radioactive AZT found in nucleic acids was associated with DNA and the specificity of the incorporation was demonstrated by DNA hydrolysis to nucleosides and HPLC analysis (Fig. 4). Of note, the amount of AZT incorporated into DNA correlated with the initial extracellular concentration of AZT, as shown in Fig. 5. The potential role of AZT incorporation into DNA in mediating toxicity for human bone marrow cells is further suggested by increased concentrations of AZT in DNA correlating with increased toxicity, as assessed by the CFU-GM clonogenic assay (6). The relationship between AZT incorporation into DNA and the clonogenic survival fraction after exposure to varying amounts of AZT is reported in Fig. 6. A highly significant relationship ($p < 0.0001$) between the extent of AZT incorporation into cellular DNA (probably at the chain termini) of human bone marrow progenitor cells and the toxicity (assessed by inhibition of clonal growth) was demonstrated for AZT concentrations higher than the IC_{50} of 1 μM (Fig. 6). This demonstrates that AZT incorporation into DNA is probably one mechanism responsible for cell toxicity. However, the minimal (not detectable) amount of AZT incorporated into DNA at concentrations less than or equal to the IC_{50} suggests that another mechanism of toxicity may be involved. Studies are underway to elucidate the molecular mechanisms and consequences of this AZT incorporation into DNA and how it relates to toxicity. A better understanding of these mechanisms should, hopefully, lead to more selective chemotherapeutic approaches for the treatment of AIDS.

References

1. Furman, P. A., S. Nusinoff-Lehrman, J. A. Fyfe, M. H. St. Clair, K. L. Weinhold, J. L. Rideout, G. A. Freeman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333–8337 (1986).
2. St. Clair, M. H., C. A. Richards, T. Spector, K. J. Weinhold, W. H. Miller, A. J. Langlois, and P. A. Furman. 3′-Azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob. Agents Chemother.* **31**:1972–1977 (1987).
3. Ono, K., M. Ogasawara, Y. Iwata, H. Nakane, T. Fujii, K. Sawai, and M. Saneyoshi. Inhibition of reverse transcriptase activity by 2′,3′-dideoxythymidine 5-triphosphate and its derivatives modified on the 3′-position. *Biochem. Biophys. Res. Commun.* **140**:498–507 (1986).
4. Cheng, Y.-C., G. E. Dutchman, K. F. Bastow, M. G. Sarngadharan, and R. Y. C. Ting. Human immunodeficiency virus reverse transcriptase: general properties and its interaction with nucleoside triphosphate analogs. *J. Biol. Chem.* **262**:2187–2189 (1987).
5. Hirsh, M. S. Azidothymidine. *J. Infect. Dis.* **157**:427–431 (1988).
6. Sommadossi, J.-P., and R. Carlisle. Toxicity of 3′-azido-3′-deoxythymidine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine for normal human hematopoietic progenitor cells *in vitro*. *Antimicrob. Agents Chemother.* **31**:452–454 (1987).
7. Mitsuya, H., and S. Broder. Strategies for antiviral therapy in AIDS. *Nature (Lond.)* **325**:773–778 (1987).
8. Balzarini, J., D. A. Cooney, S. Broder, and D. G. Johns. Biochemical basis for the cytostatic effects of the potent anti-HIV (human immunodeficiency virus) drug 3′-azido-2′,3′-dideoxythymidine (AZT). *Arch. Int. Physiol. Biochim.* **95**:B-52 (1987).
9. Frick, L. W., D. J. Nelson, M. H. St. Clair, P. A. Furman, and T. A. Krenitsky. Effects of 3′-azido-3′-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. *Biochem. Biophys. Res. Commun.* **154**:124–129 (1988).
10. Hao, Z., D. A. Cooney, N. R. Hartman, C. F. Perno, A. Fridland, A. L. DeVico, M. G. Sarngadharan, S. Broder, and D. G. Johns. Factors determining the activity of 2′,3′-dideoxynucleosides in suppressing human immunodeficiency virus *in vitro*. *Mol. Pharmacol.* **34**:431–435 (1988).
11. Zhu, Z., R. Carlisle, and J.-P. Sommadossi. Studies on mechanism of toxicity of 3′-azido-3′-deoxythymidine (AZT) in human bone marrow (HBM) cells. *Intersci. Conf. Antimicrob. Agents Chemother. Proc.* **28**:1466 (1988).
12. Labarca, C., and K. Paigen. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**:344–352 (1980).
13. Richman, D. D., M. A. Fischl, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, M. S. Hirsch, G. G. Jackson, D. T. Durack, S. Nusinoff-Lehrman, and the AZT Collaborative Working Group. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N. Engl. J. Med.* **317**:192–197 (1987).
14. Balzarini, J., R. Pauwells, M. Baba, P. Herdewijn, E. De Clercq, S. Broder, and D. G. Johns. The *in vitro* and *in vivo* anti-retrovirus activity and intracellular metabolism of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine are highly dependent on the cell species. *Biochem. Pharmacol.* **37**:897–903 (1988).
15. Sommadossi, J.-P., R. Carlisle, R. F. Schinazi, and Z. Zhou. Uridine reverses the toxicity of 3′-azido-3′-deoxythymidine in normal human granulocyte-macrophage progenitor cells *in vitro* without impairment of antiretroviral activity. *Antimicrob. Agents Chemother.* **32**:997–1001 (1988).

Send reprint requests to: Dr. Jean-Pierre Sommadossi, Division of Clinical Pharmacology, University of Alabama at Birmingham, UAB Station Box 600, Birmingham, AL 35294.