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Antibacterial Activity and Mechanism of Action of 3′-Azido-3′-Deoxythymidine (BW A509U)

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The thymidine analog 3′-azido-3′-deoxythymidine (BW A509U; azidothymidine [AZT]) had potent bactericidal activity against many members of the family *Enterobacteriaceae*, including strains of *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Shigella flexneri*, and *Enterobacter aerogenes*. AZT also had bactericidal activity against *Vibrio cholerae* and the fish pathogen *Vibrio anguillarum*. AZT had no activity against *Pseudomonas aeruginosa*, gram-positive bacteria, anaerobic bacteria, *Mycobacterium tuberculosis*, nontuberculosis mycobacteria, or most fungal pathogens. Several lines of evidence indicated that AZT must be activated to the nucleotide level to inhibit cellular metabolism: (i) AZT was a substrate for *E. coli* thymidine kinase; (ii) spontaneously arising AZT-resistant mutants of *E. coli* ML-30 and *S. typhimurium* were deficient in thymidine kinase; and (iii) intact *E. coli* ML-30 cells converted [^{3}H]AZT to its mono-, di-, and triphosphate metabolites. Of the phosphorylated metabolites, AZT-5′-triphosphate was the most potent inhibitor of replicative DNA synthesis in toluene-permeabilized *E. coli pol A* mutant cells. AZT-treated *E. coli* cultures grown in minimal medium contained highly elongated cells consistent with the inhibition of DNA synthesis. AZT-triphosphate was a specific DNA chain terminator in the in vitro DNA polymerization reaction catalyzed by the Klenow fragment of *E. coli* DNA polymerase I. Thus, DNA chain termination may explain the lethal properties of this compound against susceptible microorganisms.

Nucleoside antibiotics have been under investigation for many years (27). Some of the most clinically effective antiviral agents currently in use are purine or pyrimidine nucleoside analogs (24). For example, ribavirin, a synthetic nucleoside similar in structure to guanosine and inosine, has potent in vitro activity against a broad spectrum of viruses, including the epidemic respiratory viruses (3, 25). Two effective inhibitors of bacteria are 9-β-D-arabinofuranosyladenine and 2′,3′-dideoxyadenosine. Hubert-Habart and Cohen (14) reported the lethality of the former to a purine-requiring strain of *Escherichia coli* B. In this organism, 9-β-D-arabinofuranosyladenine markedly inhibited DNA synthesis and had virtually no effect upon RNA synthesis. In addition, 2′,3′-dideoxyadenosine was shown to be lethal to selected strains of *E. coli* by irreversibly inhibiting DNA synthesis in susceptible microorganisms (5, 28).

As a result of screening synthetic compounds for potential antimicrobial activity, we have observed that compound BW A509U (3′-azido-3′-deoxythymidine, referred to as AZT in this paper; Fig. 1) has potent, bactericidal in vitro activity against various members of the family *Enterobacteriaceae*. This report describes the extent of the in vitro growth-inhibiting activity of AZT and proposes a mechanism to explain its lethal properties. In addition, the antibacterial activity of AZT is discussed in light of the recent finding that this compound inhibits human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) replication and blocks the cytopathic effects of HTLV-III/LAV in vitro (17).

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MATERIALS AND METHODS

Nucleoside analogs. The nucleoside analog AZT (BW A509U) (Fig. 1) was synthesized as previously described (15). AZT-5′-mono-, -di-, and -triphosphates were synthesized from AZT by W. H. Miller, G. A. Freeman, and L. M. Beacham at Wellcome Research Laboratories.

Preparation of [5′-^{3}H]-AZT. (i) Preparation of the 5′-aldehyde of AZT (13). Pyridinium dichromate (1.4 g, 3.7 mmol) was added to a mixture of powdered 0.3-nm molecular sieves (6 g) in dichloromethane (75 ml). AZT (0.5 g, 1.9 mmol) was added last. Thin-layer chromatography, in which silica gel plates (5 by 20 cm) eluted 15 cm with chloroform-methanol (9:1, vol/vol), was used to follow the reaction. The R_f of AZT was 0.33. The product had an R_f of 0.36. The mixture was stirred at ambient temperature for 2.5 h. Ether (75 ml) was added, and the mixture was filtered. The solvents were removed in vacuo, and the residue was chromatographed on silica gel eluted with chloroform-methanol (95:5, vol/vol). The fractions containing the product were combined, and the solvents were removed, giving 0.08 g (16% yield) of clear oil.

(ii) Reduction of the 5′-aldehyde with NaB[^{3}H]$_4$. Sodium boro [^{3}H]hydride (1.72 mg, 500 mCi; New England Nuclear Corp., Boston, Mass.) in 2-propanol–H_2O (3/1[vol/vol], 2.0 ml) was added to a stirred solution of the 5–aldehyde (26.5 mg) in 2-propanol (0.5 ml) at 25°C under argon. After 60 min, thin-layer chromatography showed zero residual aldehyde. Acetone (0.7 ml) was added, and after 5 min, 1 N HCl was added to yield a pH of ~6.

After evaporation to dryness, the residue was dissolved in methanol and applied to a 2-mm Silica Gel 60 preparative

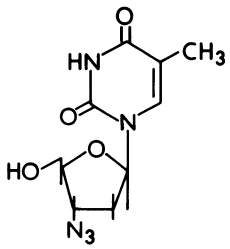

FIG. 1. Structure of AZT (BW A509U).

plate (20 by 20 cm) (Merck & Co., Inc., Rahway, N.J.), and the plate was developed in $CHCl_3/CH_3OH$ (9/1, vol/vol). Removal of the band at an R_f of 0.15 to 0.25 (corresponding to AZT) followed by extraction with acetone (50 ml), filtration, evaporation to dryness, dissolution in ethyl acetate (10 ml), filtration, and evaporation to dryness afforded 15.0 mg of [5'-^{3}H]AZT (BW A509U) as a colorless glass with a specific activity of 2.4 Ci/mmol.

Thin-layer chromatography on Silica Gel 60 (Merck) in $CHCl_3/CH_3OH$ (9/1, vol/vol) showed single-spot material with an R_f (0.37) identical to that of authentic AZT. The radiochemical purity, determined by thin-layer chromatography with a System 200 imaging scanner (Bioscan, Inc., Washington, D.C.), was greater than 96.0%.

(iii) Bacterial strains. Strains of *Vibrio anguillarum* were obtained from the National Collection of Marine Bacteria, Aberdeen, Scotland; the American Type Culture Collection, Rockville, Md.; and the National Fisheries Health Research Laboratory, Kearneysville, W.Va. The remaining organisms used in this study were clinical isolates from the Wellcome Bacterial Collection.

(iv) Susceptibility testing. MICs and MBCs were determined by a microtiter broth dilution technique similar to that described by Gavan and Barry (11). Serial twofold dilutions of AZT were performed with a semiautomated microtiter system (Dynatech Laboratories, Inc., Alexandria, Va.) with Wellcotest broth, a thymidine (dThd)-free, all-purpose bacteriological growth medium prepared by Burroughs Wellcome Co., Research Triangle Park, N.C. NaCl (5%) was added to the test medium when *V. anguillarum* was used. Overnight growth of each strain was diluted in the broth and then added to serial dilutions of each compound to achieve an initial inoculum of approximately 10^6 organisms per ml. The wells were sealed with adhesive cellophane and incubated at 24°C (*V. anguillarum*) or 37°C (the remaining organisms) for 18 to 24 h. The MIC was defined as the lowest concentration of compound that prevented visible growth. MBC determinations were performed by subculturing 0.010-ml samples onto Wellcotest agar plates from wells showing no visible growth and incubating them for 18 to 24 h at 37°C. The MBC was defined as the lowest compound concentration at which ≥99.9% of the original bacteria were killed (10 bacteria or fewer remaining in the 0.010-ml sample).

(v) AZT inhibition of *E. coli* growth and selection of AZT-resistant mutants. Cultures of *E. coli* ML-30 were grown at 37°C with shaking in prewarmed glucose minimal medium (12) in the presence or absence of 2.0 μg of AZT per ml. Cultures were inoculated with exponential-phase cells, and A_{550} of the cultures was monitored. To quantitate the viable bacteria in each culture, samples were removed at various time intervals, washed twice, diluted in minimal salts, and plated onto Luria agar (16). Colonies were counted after overnight incubation at 37°C.

AZT-resistant mutants of *E. coli* ML-30, *Salmonella typhimurium* LT-2, or other gram-negative organisms were obtained after overnight growth in glucose minimal medium or medium A (4), which contained either 10 or 25 μg of AZT per ml. Potential AZT-resistant mutants were streaked onto eosin-methylene blue plates containing 25 μg of AZT per ml; colonies resistant to AZT were selected and stored in 20% glycerol at −20°C until used.

(vi) AZT metabolism in intact *E. coli* ML30 cells. An exponentially growing culture of *E. coli* ML30 cells (0.1 A_{550} unit per ml in minimal salts glucose medium) was supplemented with [5'-^{3}H]AZT (2.79 μg/ml; specific activity of 148 mCi/mmol), and at various times (32, 91, and 166 min) thereafter, 50-ml portions of this culture were passed rapidly through a 47-mm-diameter AP15 glass fiber filter (Millipore Corp., Bedford, Mass.) and the cells were immediately extracted by the method of Payne and Ames (20). Extracts were evaporated to dryness under reduced pressure in an Evapo-Mix apparatus (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N.J.), reconstituted with 500 μl of deionized water, and stored at −20°C until they were analyzed for nucleotides by anion exchange high-performance liquid chromatography (HPLC) (29).

To identify the nucleoside moiety present in the anionic metabolites of [^{3}H]AZT, peak fractions of each radioactive metabolite were collected from the anion exchange HPLC column, desalted by adsorption on charcoal, washed four times with H_2O, and eluted from the charcoal with 50% ethanol–5% ammonium hydroxide–45% H_2O (vol/vol/vol). The desalted metabolites were each concentrated and incubated overnight in 100 μl of 50 mM Tris hydrochloride (pH 8.0) containing 86 U of alkaline phosphatase from calf intestine (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) plus 2.5 U of alkaline phosphatase from *E. coli* (Sigma Chemical Co., St. Louis, Mo.). Samples were heated for 3 min in a boiling-water bath and were then analyzed by reversed-phase HPLC (30).

(vii) Measurement of dThd-phosphorylating activities. Cells in the exponential phase were harvested, washed, and extracted by sonication in 10 mM Tris hydrochloride (pH 7.5) with 1 mM $MgCl_2$. The extract was centrifuged at 100,000 × *g*, and the supernatant was saved. The extracts were desalted through a column of Sephadex G-25 equilibrated with 20 mM Tris hydrochloride (pH 7.5)–2 mM dithiothreitol–10% glycerol and then assayed for dThd-phosphorylating activity. The standard reaction mixtures (60 μl) contained 0.1 mM [^{14}C]dThd (60 cpm/pmol), 2 mM ATP-Mg, 200 mM Tris hydrochloride (pH 7.5), 0.09 mM dCTP as an activator for the enzyme (19), and 10 to 30 μg of appropriate bacterial extract. Reaction mixtures were incubated at 37°C, and 10-μl samples were spotted onto DEAE paper to assay for the extent of dThd phosphorylation (9). Initial velocities were determined from the linear portion of the reaction time courses. Reaction rates were directly proportional to enzyme concentration. Protein concentrations were determined by the Coomassie blue method (2).

(viii) AZT as a substrate for purified *E. coli* dThd kinase. dThd kinase was purified from an extract of ML-30 cells by affinity chromatography with modifications of a method involving a column of dThd-agarose (9). After removal of the precipitate from streptomycin treatment, ammonium sulfate was added to the supernatant to 44% (270 g/liter) saturation and the precipitated protein was dissolved in 20 mM Tris hydrochloride (pH 7.5)–10% glycerol–2 mM dithiothreitol (buffer A). The sample was dialyzed in buffer A and added to the dThd-agarose column. Several column volumes of buffer

A with 0.1 mM dCTP, buffer A with 0.1 mM dCTP–0.5 M NaCl, and buffer A with 0.1 mM dCTP–0.5 M NaCl–2 mM ATP were used to remove unwanted protein. The dThd kinase was eluted with 2 mM dThd in buffer A with 0.1 mM dCTP–1 M NaCl–2 mM ATP–0.5 mg of bovine serum albumin per ml. The enzyme was stored at −70°C and desalted before use. No dTMP and only a trace (5% of the rate of dThd phosphorylation) of ATP-phosphohydrolyzing activity was detected in the enzyme preparation.

The purified enzyme catalyzed the phosphorylation of AZT, and the product was identified by comparison of its retention time to that of AZT-monophosphate by anion exchange HPLC. The rate of phosphorylation was compared to that of dThd by an indirect method measuring the conversion of [α-^{32}P]ATP to [α-^{32}P]ADP (10). The kinetic constants for dThd and AZT were determined as described previously (10) from double-reciprocal plots with computer-assisted least-squares fits to hyperbolae.

(ix) Measurement of replicative DNA synthesis in *E. coli*. Toluene-permeabilized *E. coli* cells were prepared by methods previously described (18). DNA synthesis was measured in reaction mixtures containing 2×10^8 to 4×10^8 toluenized cells, 13.3 mM $MgCl_2$, 1.33 mM ATP, 33 μM concentrations of each deoxynucleotide (dTTP, dATP, dCTP, and dGTP), 1 μC of [^{3}H]dTTP, and 50 mM K_2PO_4 (pH 7.4) in a total volume of 0.3 ml. Reactions were initiated by the addition of toluenized cells. The resultant mixture was incubated 30 to 60 min at 37°C, and the reaction was stopped by adding 3 ml of cold 10% trichloroacetic acid in 0.1 M sodium PP_i. DNA precipitates were collected on glass filters and washed twice with 3-ml portions of 10% trichloroacetic acid in 0.1 M sodium PP_i, and twice with 5-ml portions of 0.01 M HCl. The radioactivity of filters was quantitated by scintillation counting with PCS solubilizer (Fisher Scientific Co., Pittsburgh, Pa.).

(x) AZT-triphosphate as a DNA chain terminator. The dideoxy-DNA chain termination sequencing procedure of Sanger et al. (22) was used to assess DNA chain termination. [^{35}S]dATP was obtained from New England Nuclear. Other than AZT-triphosphate, all reagents were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Sequencing procedures and acrylamide gel electrophoresis were performed as recommended by the manufacturer (Bethesda Research Laboratories). Specifically, the AZT reaction contained 300 μM AZT-triphosphate, 1.6 μM dTTP, 33 μM dCTP, 33 μM dGTP, 1.3 μM [^{35}S]dATP (500 Ci/mmol), 0.2 μg of single-stranded M13mp8 template, 1.0 ng of 15-base primer, and 0.34 U of the Klenow fragment of *E. coli* DNA polymerase I. The ddTTP reaction was the same as the AZT reaction except that 200 μM 2′,3′-ddTTP replaced AZT-triphosphate.

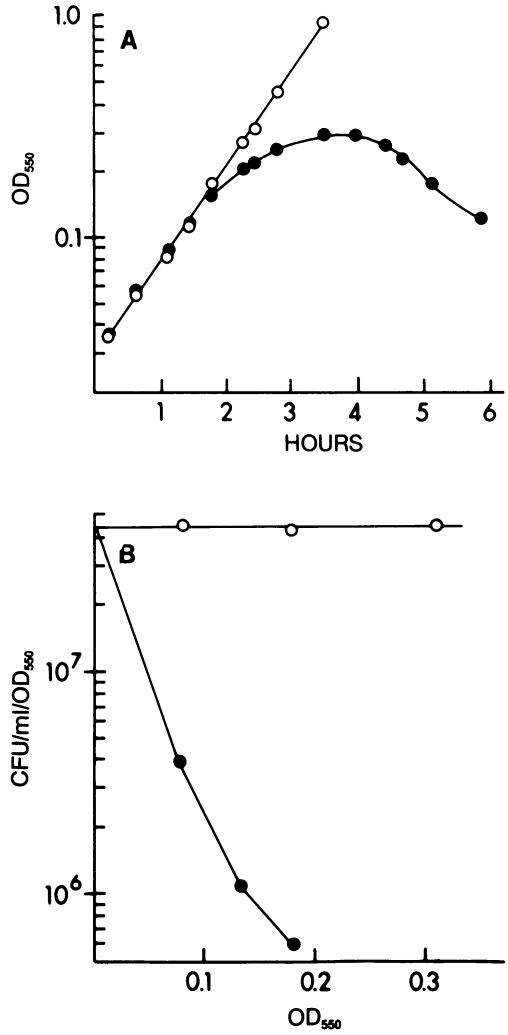

FIG. 2. Effect of AZT on growing cultures of *E. coli* ML-30. *E. coli* ML30 was grown at 37°C with shaking in glucose minimal medium in the presence (●) and absence (○) of 2.0 μg of AZT per ml. OD_{550}s were monitered and plotted (A). Samples of each culture were removed at various time intervals, washed twice, diluted in minimal salts, and plated onto Luria agar. CFU were counted after overnight incubation at 37°C (B).

TABLE 1. MICs of AZT against clinical isolates

Organism (no. of isolates)	MIC (μg/ml)[a]	
	Range	50%
Escherichia coli (9)	0.0025–1.0	0.5
Salmonella typhimurium (6)	0.03–0.4	0.03
Shigella flexneri (6)	0.025–0.2	0.1
Klebsiella pneumoniae (6)	0.1–3.1	3.1
Enterobacter aerogenes (6)	0.3–2.0	0.8
Proteus mirabilis (8)	6.2–25.0	12.5
Vibrio anguillarum (6)	0.2–4.0	1.0
Vibrio cholerae (12)	1.0–31.0	8.0
Yersinia enterocolitica (11)	0.4–12.5	6.2
Haemophilus influenzae (8)	6.2–25.0	12.5

[a] 50% MIC for 50% of isolates.

RESULTS

In vitro antibacterial activity. AZT was tested against a variety of microorganisms for antibacterial activity with a broth dilution microtiter system as previously described (11). Potent bactericidal activity was observed against a variety of potentially pathogenic gram-negative bacteria, including *E. coli, S. typhimurium, Shigella flexneri, Klebsiella pneumoniae,* and *Enterobacter aerogenes* (Table 1). AZT was also active against several isolates of *V. anguillarum*, the etiological agent of vibriosis, a serious infectious disease that affects marine fish (6). MBCs were generally within a twofold dilution of the MICs (data not shown). This compound was moderately active in vitro against isolates of *Vibrio cholerae* and *Yersinia enterocolitica* (Table 1). In contrast, AZT showed no activity against selected gram-

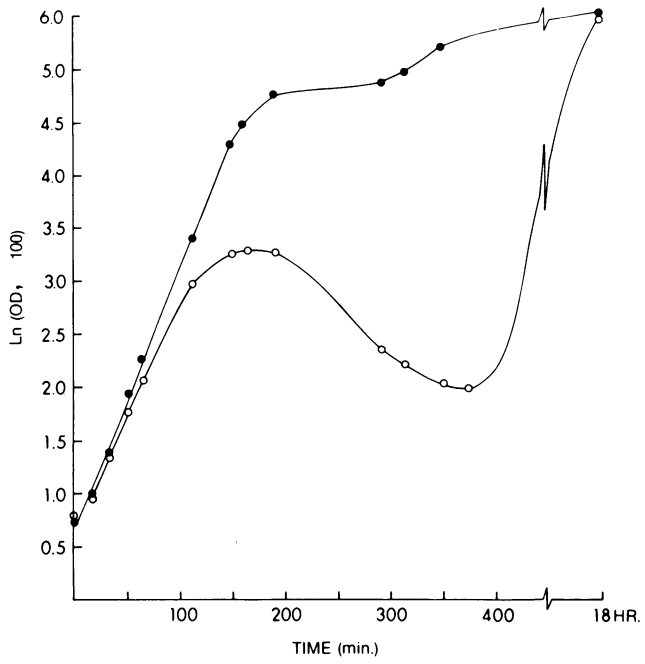

FIG. 3. Emergence of AZT-resistant mutants. *E. coli* ML-30 was grown overnight in rich medium in the absence (●) or presence (○) of 25 μg of AZT per ml. The *A* 600 was measured as a function of time.

positive bacteria (including *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*) or against *Mycobacterium avium*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bordetella bronchiseptica*, and *Campylobacter jejuni*.

Kinetics of AZT inhibition of bacterial growth. The effect of 2.0 μg of AZT per ml on the growth rate of *E. coli* ML30 is shown in Fig. 2A. The optical density at 550 nm (OD_{550}) increased exponentially for both control and AZT-treated cultures for about 2 doublings, after which the drug-treated culture became inhibited and eventually decreased in OD, an indication of culture death due to cell lysis. The numbers of viable bacteria contained in these cultures were determined at several time intervals; the control culture showed a constant number of CFU per milliliter per OD unit, whereas a time-dependent decrease occurred in the drug-treated culture (Fig. 2B). This indicated that fewer viable bacteria accounted for the observed increase in the OD_{550}, suggesting that the drug-inhibited cells were larger than those in the uninhibited control. Microscopic examination showed that AZT-treated cultures contained highly elongated bacteria compared to the control cells (data not shown). Bacterial elongation could be detected as early as 30 min after drug addition, and a reduction in CFUs was observed within 10 min after AZT addition, suggesting that cell division was immediately inhibited even though the OD continued to increase.

Detection of AZT-resistant mutants. The results of an experiment in which *E. coli* ML30 cells were exposed to 25 μg of AZT per ml are shown in Fig. 3. Consistent with previous results at lower AZT concentrations, cell lysis began to occur about 200 min after drug addition. However, the ODs of both the AZT-treated and control cultures were the same after overnight incubation (Fig. 3), indicating the emergence of AZT-resistant bacteria. Resistance might evolve by the induction of AZT-degradative or -modifying enzymes, by a mutational event affecting AZT uptake, or by the metabolic inactivation of AZT. To distinguish between the two suggested modes of resistance, AZT-resistant colonies were isolated and grown for 20 to 30 generations in the absence of any drug and then reexposed to 25 μg of AZT per ml. The growth rates of these cultures were unaffected by high levels of AZT (data not shown), suggesting that resistance was due to a stable mutational event rather than the induction of an enzyme(s).

In addition to the resistance of *E. coli* ML30 and *S. typhimurium* LT-2, rapid in vitro emergence of AZT resisance has been demonstrated in selected strains of *Shigella flexneri*, *K. pneumoniae*, *Pasteurella multocida*, and *Proteus vulgaris* (data not shown).

AZT metabolism in *E. coli* ML30 cells. The nucleoside structure of AZT suggested the strong likelihood that a phosphorylated metabolite of this compound might be implicated in its lethal properties. To investigate this possibility, exponential-phase *E. coli* ML30 cells were grown in the presence of radiolabeled AZT (2.8 μg/ml). The bacteria were then harvested and lysed with 1 M cold formic acid, and the resultant concentrated extracts were analyzed by anion exchange HPLC. Column eluates were monitored both for UV A_{254} and for radioactivity, and the results of this analysis are presented in Fig. 4. It was clear from the profiles that extracts from these cells contained radioactivity peaks that were coeluted with authentic samples of AZT-diphosphate and -triphosphate. The identities of these two radioactive metabolites were further verified by their conversion via alkaline phosphatase to a radioactive substance having the same retention time as AZT by reversed-phase HPLC. These results indicate that [^{3}H]AZT was metabolized se-

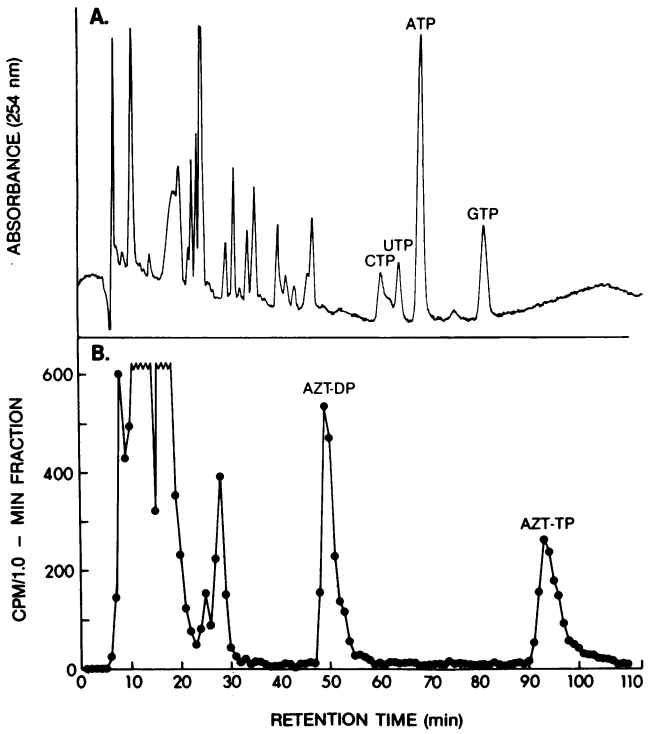

FIG. 4. HPLC elution profile of an extract from [^{3}H]AZT-treated *E. coli* ML30 cells. Exponentially growing cells were extracted after a 91-min incubation with [^{3}H]AZT (2.8 μg/ml). (A) UV (254 nm) elution profile; (B) radioactivity elution profile of the bacterial cell extract.

quentially to its 5'-mono-, -di-, and -triphosphate derivatives in intact *E. coli* ML30 cells. Multiple peaks of [^{3}H]AZT metabolites were evident in the monophosphate region of the elution profile (Fig. 4B). After purification and subsequent treatment with alkaline phosphatase, these radioactive metabolites yielded either [^{3}H]AZT or an unidentified compound that was eluted ahead of AZT from the reversed-phase HPLC column.

Role of dThd kinase for AZT activity. If the enzyme dThd kinase is critical to the antibacterial action of AZT, two predictions can be made. First, AZT should serve as a substrate for *E. coli* dThd kinase, and second, this enzyme might be significantly altered or missing in AZT-resistant mutants. To test the first prediction, AZT was compared to dThd as a substrate for purified *E. coli* dThd kinase. The results showed that AZT was a substrate for this enzyme (Table 2). A comparison of K_m and relative V_{max} values shows that AZT was about one-fifth as efficient as dThd as a substrate for dThd kinase but that it was a reasonably good substrate nonetheless. To examine the second prediction, cell extracts of AZT-susceptible and -resistant strains of *E. coli* ML30 and *S. typhimurium* were analyzed for dThd kinase activity. These results (Table 3) show that AZT-resistant mutant strains of *E. coli* ML30 and *S. typhimurium* contained 70 to 300 times less dThd kinase activity than did the AZT-susceptible parental strains.

If a phosphorylated metabolite of AZT is responsible for the growth inhibition of susceptible cells, one would further predict that various phosphorylated species of AZT would differ in their effects on bacterial cells. To examine this hypothesis, AZT and chemically synthesized 5'-mono-, -di-, and -triphosphate nucleoside analogs of AZT were tested for their ability to inhibit DNA synthesis in toluene-permeabilized *E. coli* cells. These experiments were done with a DNA polymerase I-deficient strain, *E. coli* D110. Cells with a *polA* mutation grow quite normally and make DNA normally; however, they are defective in their ability to repair DNA damage. Therefore, this experiment primarily measured the effect that AZT and AZT-phosphates had upon replicative DNA synthesis in these cells.

The results of these experiments are shown in Table 4. The data clearly show the ranking of inhibitory potency to be AZT-triphosphate > AZT-diphosphate > AZT-monophosphate > AZT, supporting the idea that the 5'-triphosphate species is the active inhibitor. The other compounds are presumably sequentially metabolized to AZT-triphosphate inside AZT-susceptible microorganisms.

AZT-triphosphate as a DNA chain terminator. If AZT is incorporated into DNA, the absence of the 3'-hydroxyl group would prevent further elongation of the DNA chain. To experimentally test this possibility, we used the dideoxy-DNA chain termination sequencing procedure of Sanger et al. (22) to monitor for DNA chain termination. Briefly, the approach was as follows. A single-stranded DNA template was annealed to a complementary primer which had a 3'-hydroxyl group and which was elongated by the Klenow fragment of *E. coli* DNA polymerase I in the presence of dCTP–dGTP–dTTP–deoxyadenosine 5'-[α-^{35}S] thiotriphosphate. Because the Klenow fragment lacks the 5' → 3' exonuclease activity of the holoenzyme, all synthesized products have a common 5'-end. These products can be seen on autoradiographs of sequencing gels which can resolve DNA chains differing in length by only 1 nucleotide. In the absence of DNA chain terminators, the synthesized products are hundreds of nucleotides long. Shorter chains are synthesized if a known DNA chain terminator (for example, 2',3'-ddTTP) is added to the reaction. The resultant DNA chains, which incorporate dideoxy-TTP, terminate specifically at residues where dTTP would normally be incorporated. The ratio of deoxynucleotide to chain terminator is adjusted so that only an occasional terminator is incorporated.

The results of such an experiment, in which AZT-triphosphate was added to one of the reactions, are shown in Fig. 5. It is quite clear that AZT-triphosphate (AZT lane) elicited the same base-specific stops as ddTTP (ddT lane). AZT-triphosphate was thus specifically incorporated into elongating DNA chains by the Klenow fragment of DNA polymerase I leading to dThd base-specific chain termination.

TABLE 2. Kinetic constants of purified *E. coli* dThd kinase with AZT

Substrate	Relative v (%)[a]	K'_m (μM)[b]	v/K'_m[c]
dThd	100	14	7.1
AZT	32	21	1.5

[a] Rate with 1 mM phosphate acceptor–1 mM[^{14}C]ATP-Mg–100 mM Tris hydrochloride (pH 7.5)–enzyme–0.5 M dCDP (as the enzyme activator in place of dCTP). The assay measured the phosphate acceptor-dependent conversion of ATP to ADP (see Materials and Methods). The velocity (v) with dThd was arbitrarily set at 100%.

[b] Apparent K_m values (K'_m) determined under the standard assay conditions with [^{14}C]dThd from 3 to 40 μM (see Materials and Methods). The value for AZT was determined from its K_is (K_i value calculated from slopes of the double-reciprocal plots) as a competitive inhibitor of dThd phosphorylation. Because AZT is a substrate, K_is = K'_m (23).

[c] Relative efficiency.

TABLE 3. dThd-phosphorylating activity of AZT-susceptible and -resistant bacterial extracts[a]

Bacterial strain	Phenotype for AZT	dThd-phosphorylating activity[b]
E. coli ML-30	Susceptible	2,800
E. coli ML-30	Resistant	40
S. typhimurium	Susceptible	8,400
S. typhimurium	Resistant	20

[a] Activity was determined with [^{14}C]dThd as the phosphate acceptor under standard conditions as described in Materials and Methods.

[b] Expressed as picomoles per minute per milligram of protein.

DISCUSSION

The dThd analog AZT has potent in vitro bactericidal activity against certain members of the family *Enterobacteriaceae*. For example, in vitro MICs of AZT against selected strains of *E. coli*, *S. typhimurium*, and *Shigella flexneri* are as low as 2.5 to 30 ng/ml. Spontaneously occurring AZT-resistant mutants lack significant levels of dThd kinase, and

TABLE 4. Inhibition of replicative DNA synthesis in *E. coli* D110

Compound	% Inhibition at a compound concn (M) of:			
	10^{-7}	10^{-6}	10^{-5}	10^{-4}
AZT	NT[a]	NT	NT	3
AZT-monophosphate	NT	NT	0, 3.5[b]	23, 14.5
AZT-diphosphate	0	21, 30.4	52, 40.5	NT
AZT-triphosphate	14, 10.7	49, 44.6	70	NT

[a] NT, Not tested.

[b] Double values represent the results of duplicate experiments.

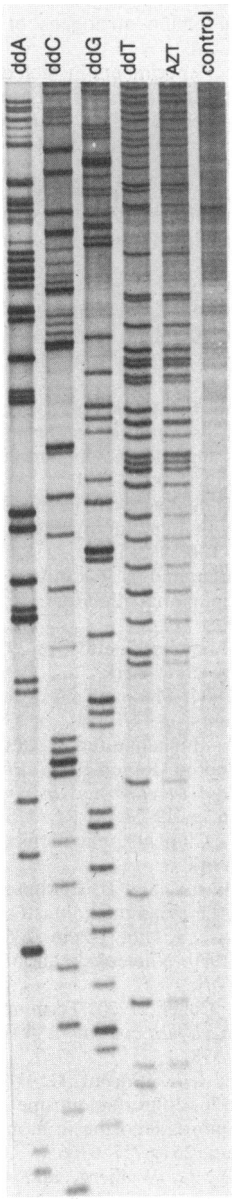

FIG. 5. AZT-triphosphate as a DNA chain terminator. Autoradiograph of an 8% acrylamide, 7 M urea gel. ddATP(ddA), ddCTP (ddC), ddGTP (ddG), and ddTTP (ddT) lanes represent typical Sanger dideoxy-sequencing reactions with the Klenow fragment of *E. coli polI*. The control lane shows a reaction performed in the absence of any chain terminators and was included to detect any artifactual terminations. The AZT lane represents a reaction identical to the dd-TTP reaction except that 300 μM AZT-triphosphate replaced 200 μM dd-TTP. The dTTP concentration was 1.6 μM in both the dd-TTP and AZT-triphosphate reactions.

it is clear from data presented in this study that AZT must be metabolically activated to the nucleotide level to inhibit cellular metabolism. Some of the clinically important microorganisms not inhibited by AZT include *P. aeruginosa*, *Mycobacterium tuberculosis*, *Mycobacterium intracellulare*, and *Candida* species. Saito and Tomioka (21) have reported that these particular organisms lack dThd kinase activity, which might explain their insusceptibility to AZT. In contrast, *S. typhimurium*, an organism exquisitely sensitive to AZT, contains approximately 11-fold more dThd kinase activity than *E. coli* does (21). Although cell-associated dThd kinase levels appear to correlate with AZT susceptibility in some organisms, cell permeability undoubtedly plays a role as well. For example, *Proteus mirabilis* contains approximately one-third of the dThd kinase level of *E. coli* and yet is only marginally susceptible to AZT, with MICs ranging from 6.2 to 25 μg/ml (Table 1).

Several lines of evidence indicate that AZT-triphosphate is the active metabolite of AZT in drug-susceptible bacterial cells. AZT is a relatively good substrate for *E. coli* dThd kinase, and intact cells convert the compound to its mono-, di-, and triphosphate metabolites. As mentioned above, spontaneously arising mutants of *E. coli* and *S. typhimurium* were found to be deficient in dThd kinase. In addition, of the three phosphorylated derivatives, AZT-triphosphate was found to be the most potent inhibitor of replicative DNA synthesis in *E. coli* D110, with an I_{50} of approximately 1 μM under these experimental conditions.

The mechanism of action of AZT was relatively easy to predict because AZT lacks the 3'-hydroxyl required for DNA polymerase-mediated chain elongation. Incorporation of AZT-triphosphate into DNA could be expected to result in DNA chain termination, and we experimentally verified this prediction. The mechanism of action of AZT is not novel. For example, Atkinson et al. (1) showed that the nucleotide analog ddTTP inhibited DNA synthesis and related reactions catalyzed by *E. coli* DNA polymerase I. Furthermore, they demonstrated that the inhibitory effect of ddTTP on DNA polymerase I was due to its incorporation into the growing DNA chain in the place of dTMP (1). In addition to the inhibition of DNA polymerase I, ddTTP also inhibits the DNA synthesis by RNA-directed DNA polymerases of Moloney murine leukemia virus (26) and Rous sarcoma virus (7).

Related to these observations, it has recently been shown (17) that AZT inhibits the reverse transcriptase of HTLV-III/LAV and blocks the expression of the p24 *gag* protein of HTLV-III/LAV in H9 cells after exposure to virus. In addition, the triphosphate derivative of AZT selectively binds to the HTLV-III/LAV reverse transcriptase, and incorporation of this nucleoside moiety into a growing DNA strand terminates DNA elongation and consequently inhibits DNA synthesis (8). Thus, the mechanism of AZT antibacterial activity appears to be basically similar to that of the anit-HTLV-III activity of this compound. If AZT becomes a primary or adjunct therapy in the treatment of acquired immune deficiency syndrome, its potent, albeit selective antibacterial activity should be taken into consideration. Recently it has been shown that AZT acts synergistically with trimethoprim in vitro against some strains of gram-negative bacteria, including species of *Salmonella*, *Shigella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus*, *Providencia*, *Morganella*, and *Escherichia* (M. Bushby, personal communication). It is hoped that in certain patients this additional activity will contribute to the therapeutic effectiveness of AZT against this important disease.

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