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3′-Azido-3′-Deoxythymidine (AZT) Monophosphate: an Inhibitor of Exonucleolytic Repair of AZT-Terminated DNA

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A 3′-exonuclease(s) that excised 3′-azido-3′-deoxythymidine (AZT) monophosphate (AZTMP) from the 3′ terminus of DNA was partially purified from two human cell lines. AZTMP inhibited the hydrolysis of AZTMP-terminated single-stranded and double-stranded DNA substrates. Thus, high levels of AZTMP might inhibit the exonuclease and trigger the toxicity of AZT by impairing the repair of AZTMP-terminated DNA.

3′-Azido-3′-deoxythymidine (AZT) is used to treat infections of human immunodeficiency virus, the etiologic agent of AIDS (2, 5, 14, 23). Anemia and neutropenia are the most common serious side effects of AZT treatment (9, 16). The mechanisms of this hematopoietic cytotoxicity may involve impairment of cellular DNA synthesis. Although AZT triphosphate is an inefficient substrate for cellular DNA polymerases, AZT monophosphate (AZTMP) is incorporated into DNA (18–20). The amount of AZTMP incorporated into the DNA of human bone marrow cells increases linearly with the dose of AZT (18). However, AZT does not inhibit cell growth until a threshold level is present, and the amount of AZTMP in the DNA decreases after AZT is removed from the medium (20). Furthermore, an exonuclease that excises AZTMP from DNA may prevent cytotoxicity by AZT (20). However, the DNA repair capacity appears to be inadequate at cytotoxic concentrations of AZT.

Many 3′-exonucleases are inhibited by nucleoside 5′-monophosphates (4, 8, 10). Thus, the AZTMP that accumulates to millimolar concentrations in cells exposed to toxic levels of AZT (1) might inhibit the exonuclease-catalyzed repair of AZTMP-terminated DNA and thereby trigger the hematopoietic cytotoxicity. To test this hypothesis, we partially purified a 3′-exonuclease from both K562 cells and CCRF-CEM cells and studied the ability of AZTMP to inhibit excision of AZTMP from DNA.

A 3′-exonuclease was partially purified by DNA-cellulose chromatography as described by Vazquez-Padua et al. (20). Of the total exonuclease activity, 10 to 15% was associated with DNA polymerase γ. The exonuclease was isolated from all detectable DNA polymerizing activity by fast protein liquid chromatography Mono Q anion-exchange chromatography with a 10-ml linear gradient from 0 to 0.5 M NaCl. Thus, this 3′-exonuclease was not associated with DNA polymerase δ, γ, or ε. Studies described herein were conducted with enzyme from CCRF-CEM cells purified by DNA-cellulose chromatography (20) and with enzyme further purified by Mono Q chromatography. The additional purification step did not affect the results. Furthermore, similar results were obtained with the exonuclease from K562 cells. The exonuclease had an optimal pH of 8.0 and required a divalent cation (Mg^{2+} or Mn^{2+}) for activity. The optimal cation concentration was 2.5 mM Mg^{2+} or 0.5 mM Mn^{2+}.

The 3′-AZTMP-terminated DNA substrate (d44:[5′-^{32}P]21mer-AZTMP) was prepared by incubating 6 μM d44:[5′-^{32}P]21mer (15), 40 μM AZT triphosphate, and 60 U of *Escherichia coli* polymerase I (Klenow) (Boehringer Mannheim) for 90 min at 37°C. The product was purified by electrophoresis through a 15% acrylamide–7 M urea gel. The product band was excised, and the [5′-^{32}P]21mer-AZTMP was recovered by electroelution. The [5′-^{32}P]21mer-AZTMP was used as a substrate either alone or annealed to the d44mer (15) or the pGEM4z plasmid (Promega Biotec).

The 3′-exonuclease hydrolyzed [5′-^{32}P]21mer-AZTMP in a time-dependent manner to give the products n-1 (first hydrolysis product) and n-2, etc. (Fig. 1). The rate of substrate disappearance was equal to the rate of formation of shorter primer products, indicating that no competing enzymatic activity was present. The first hydrolysis product (n-1) comigrated with the authentic [5′-^{32}P]21mer, indicating that the exonuclease sequentially excised single-nucleotide residues (data not shown). Furthermore, the appearance of the shorter products indicated that the 3′-exonuclease was not specific for a 3′-AZTMP-terminated primer. In a separate experiment, the [5′-^{32}P]21mer and the [5′-^{32}P]21mer AZTMP were utilized equally as substrates (data not shown).

Single-stranded DNA was the preferred substrate for the exonuclease (Fig. 2). Either [5′-^{32}P]21mer-AZTMP or d44:[5′-^{32}P]21mer-AZTMP was a substrate in the presence of Mg^{2+} or Mn^{2+}. The template primer formed by annealing [5′-^{32}P]21mer-AZTMP to pGEM4z was a substrate only with Mg^{2+} as the cation. No difference in rate was observed at 50 or 100 nM [5′-^{32}P]21mer-AZTMP. This suggested that the K_m for [5′-^{32}P]21mer-AZTMP was <10 nM.

AZTMP inhibited the hydrolysis of AZTMP-terminated single-stranded and double-stranded DNA substrates. Inhibition by AZTMP required Mn^{2+}, whereas GMP, dGMP, and arabinosyl-GMP inhibited the exonuclease in the presence of either cation (Fig. 3). CMP, dCMP, UMP, dUMP, and TMP also inhibited activity in the presence of either cation (data not shown). GMP was the most potent inhibitor tested. The 50% inhibitory concentrations (IC_{50}s) of AZTMP and GMP were approximately 250 and 50 μM, respectively. These values were obtained by titrating the exonuclease activity with either AZTMP or GMP and per-

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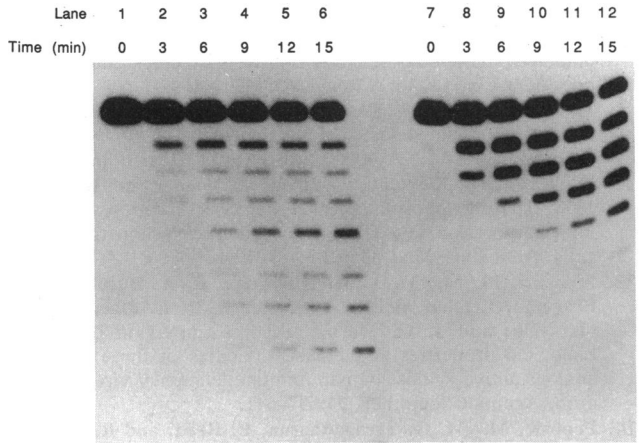

FIG. 1. Time course for exonucleolytic digestion of AZTMP-terminated DNA. Reaction mixtures (40 μl) contained 50 mM Tris-HCl (pH 8.0), 0.5 mg of bovine serum albumin per ml, 0.075 μM [5′-^{32}P]21mer-AZTMP, enzyme (purified through DNA-cellulose), and either 5 mM $MgCl_2$ (lanes 1 to 6) or 0.5 mM $MnCl_2$ (lanes 7 to 12). Reactions were initiated with enzyme, and 5 μl was removed at the indicated times and quenched with gel loading buffer (80% formamide, 50 mM Tris-borate, 1 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). The samples were heated for 3 min at 90°C and cooled rapidly on ice prior to being electrophoresed on a 15% acrylamide–7 M urea gel. After electrophoresis of the gel, Kodak XAR-2 film was exposed to the gel at −70°C with an enhancer screen. This result was reproduced five times with five different enzyme preparations.

forming densitometric analysis of the autoradiograms (data not shown).

When inhibition of the exonuclease by AZTMP was examined in the presence of physiological concentrations of

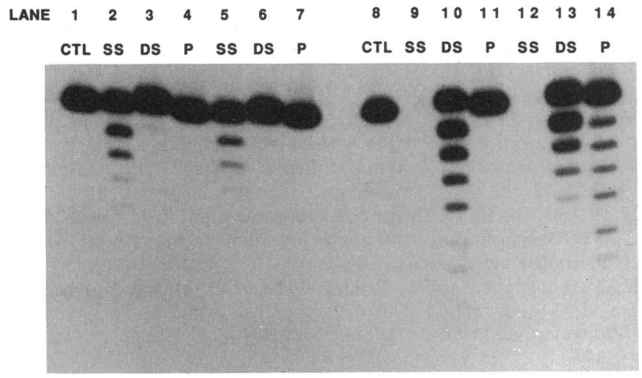

FIG. 2. Substrate specificity. Reaction mixtures (20 μl) contained 50 mM Tris-HCl (pH 8.0), 0.5 mg of bovine serum albumin per ml, enzyme (purified through Mono Q), either 0.5 mM $MnCl_2$ (lanes 1 to 4 and 8 to 11) or 2.5 mM $MgCl_2$ (lanes 5 to 7 and 12 to 14), and template primer (0.075 μM). The DNA substrates used were single-stranded [5′-^{32}P]21mer-AZTMP (SS), double-stranded d44 mer:[5′-^{32}P]21mer-AZTMP (DS), and plasmid pGEM4z:[5′-^{32}P] 21mer-AZTMP (P). The reactions were initiated with 0.48 μg (lanes 2 to 7) or 38 μg (lanes 9 to 14) of enzyme per ml. The control samples (CTL) (lanes 1 and 8) lacked enzyme. After 10 min, 10 μl was removed and quenched with the gel loading buffer. Product formation was analyzed by sequencing gel electrophoresis as described in the legend to Fig. 1. Similar results were observed with a second enzyme preparation purified through DNA-cellulose.

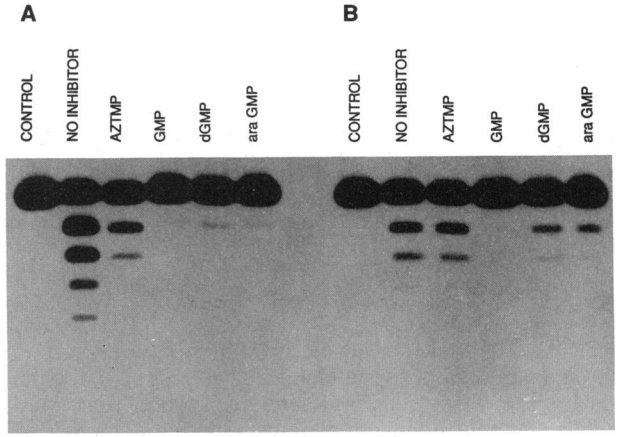

FIG. 3. Inhibition by nucleoside 5′-monophosphates. Reaction mixtures (20 μl) contained 50 mM Tris-HCl (pH 8.0), 0.5 mg of bovine serum albumin per ml, 0.075 μM [5′-^{32}P]21mer-AZTMP, enzyme (purified through Mono Q), and either 0.5 mM $MnCl_2$ (A) or 2.5 mM $MgCl_2$ (B) and 1.0 mM of the indicated nucleoside 5′-monophosphate. The control samples lacked enzyme. After 10 min, 10 μl was removed and quenched with the gel loading buffer. Product formation was analyzed by sequencing gel electrophoresis as described in the legend to Fig. 1. Similar results were observed with a second enzyme preparation purified through DNA-cellulose. ara GMP, arabinosyl-GMP.

Mg^{2+} and Mn^{2+} (2.5 mM and 30 μM, respectively [11, 21]), 1.0 mM AZTMP inhibited the exonuclease approximately 30%. The inhibition was maximal at approximately 100 μM $MgCl_2$ and was unaffected by the presence of 2.5 mM Mg^{2+}. Although these data demonstrate the potential for AZTMP to inhibit excision of AZTMP from DNA, their physiological significance remains uncertain. More information concerning the concentrations of AZTMP and Mn^{2+} and the metal requirements of the exonuclease in the cellular microenvironment is required in order to extrapolate less equivocally.

Because uridine reverses the toxicity of AZT without affecting its antiviral efficacy (17), we examined whether uridine or uridine nucleotides could abrogate AZTMP inhibition of the exonuclease. However, none of the 13 compounds (uracil, uridine, UMP, UDP, UTP, dUMP, dUDP, dUTP, 2′-deoxyuridine, 3′-deoxyuridine, UDP-glucose, UDP-glucuronic acid, and UDP-galactose) at 1.0 mM alleviated the inhibition by AZTMP. As expected, inhibition was increased by UMP or dUMP, which also inhibited the exonuclease (see above). Thus, uridine reverses the toxicity of AZT either via a metabolite not tested here or by a different mechanism.

When cells are incubated with radiolabelled AZT and then chased with nonradiolabelled AZT, no excision of AZTMP from DNA is observed (24). Under these conditions, intracellular AZTMP levels are maintained, and they may inhibit excision of AZTMP from DNA. When AZT is removed after 24 h of exposure of the cells to the drug, a time-dependent loss of AZTMP from the DNA is observed (20). Thus, these results are consistent with the presence of a 3′-exonuclease that can repair AZTMP-terminated DNA but that is sensitive to inhibition by AZTMP.

Comparison of the IC_{50}s of AZT and the intracellular concentrations of AZTMP in various rapidly dividing lymphoblastic cell lines further suggests that the toxicity of AZT may be related to high levels of AZTMP. For example, the IC_{50} of AZT in CEM cells is 50 μM (6). CEM cells exposed

to 25 μM AZT for 24 h accumulate 900 μM AZTMP (6). Similar results have been obtained with Molt4F cells (1). In contrast, the IC_{50} of AZT in L1210 cells is 900 μM, and AZTMP does not accumulate in these cells (1).

Finally, high monophosphate levels of other dideoxynucleotide analogs may contribute to their stem cell toxicity. For example, 3′-fluoro-2′,3′-dideoxythymidine (FLT) is relatively toxic in pluripotent stem cell assays (13) and causes anemia in simian immunodeficiency virus-infected monkeys (3). Interestingly, the anabolic profile of FLT is very similar to that of AZT in that FLT monophosphate accumulates to high levels in cells treated with FLT (12). In contrast, 2′,3′-dideoxycytidine, 2′,3′-dideoxy-2′,3′-didehydrothymidine, and 2′,3′-dideoxyinosine, which do not generate high intracellular monophosphate concentrations (1, 7), are much less toxic to stem cells and seldom produce dose-limiting anemia and neutropenia in patients admitted to a clinic (22). Therefore, the results of the present study and other studies discussed herein suggest that nucleoside analogs such as AZT, which generate high levels of monophosphate, may trigger toxicity by inhibiting exonucleolytic repair of chain-terminated DNA.

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