Biochemical Pharmacology, Vol. 45, No. 8, pp. 1571–1576, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

INHIBITION OF MAMMALIAN DNA POLYMERASE-ASSOCIATED 3' TO 5' EXONUCLEASE ACTIVITY BY 5'-MONOPHOSPHATES OF 3'-AZIDO-3'-DEOXYTHYMIDINE AND 3'-AMINO-3'-DEOXYTHYMIDINE*

Edward G. Bridges, Abdesslem Faraj and Jean-Pierre Sommadossi†
Department of Pharmacology and Division of Clinical Pharmacology, Center for AIDS Research and the Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

(Accepted 5 March 1993)

ABSTRACT-3'-Azido-3'-deoxythymidine 5'-monophosphate (AZT-MP) has been hypothesized by us to possibly affect 3'-azido-3'-deoxythymidine (AZT) excision from host cell DNA. In the present study, AZT-MP inhibited 3' to 5' exonuclease activity of calf thymus DNA polymerase δ at pharmacological relevant intracellular concentrations. Other 2',3'-dideoxynucleoside-5'-monophosphate (ddN-MP) analogs, including 3'-amino-3'-deoxythymidine-5'-monophosphate (AMT-MP), were also assayed as potential inhibitors of 3' to 5' exonuclease activity. The monophosphate derivative of 3'-amino-3'-deoxythymidine (AMT), an *in vivo* toxic catabolite of AZT, was the most potent of the ddN-MP analogs tested, inhibiting this activity by more than 50% at 100 μM. These results suggest that inhibition of 3' to 5' exonuclease activities by AZT-MP and AMT-MP may increase steady-state levels of AZT in host DNA, accounting in part for the cell toxicity associated with this drug. The present study also raises the question of whether AZT-MP inhibition of this activity may lead to potential mutagenic effects due to inhibition of 3' to 5' exonuclease-mediated proofreading functions involved in DNA replication.

3'-Azido-3'-deoxythymidine (AZT), a thymidine analog with potent inhibitory activity against human immunodeficiency virus (HIV) replication *in vitro*, represents a first-line chemotherapeutic agent for the treatment of Acquired Immunodeficiency Syndrome (AIDS). Unfortunately, adverse side effects, manifested as anemia and neutropenia, have been associated with AZT therapy [1]. These hematological side effects correlate to some degree with steady-state levels of AZT incorporated into host cell DNA [2]. Cheng and co-workers recently reported that an exonucleolytic enzyme may remove 3'-terminal AZT or 2',3'-dideoxycytidine (ddC) residues from DNA [3]. An enzymatic excision of 2',3'-didehydro-2',3'-dideoxythymidine (D4T) incorporated into host DNA was also observed by our group [4]. In that study, the presence of nonradiolabeled drug during the chase period did not affect D4T excision but prevented the removal of AZT from DNA. When analogs were crossed during that chase period, cold AZT inhibited radiolabeled D4T excision while D4T had no effect on [^{3}H]AZT removal from DNA (unpublished data), suggesting that AZT or one of its 5'-nucleotide derivatives inhibits the excision process. Previous studies have shown that nucleoside analogs whose 5'-monophosphate derivative remains predominant intracellularly may potentially inhibit the 3' to 5' exonuclease activity associated with calf thymus DNA polymerase δ [5]. While AZT is efficiently converted to 3'-azido-3'-deoxythymidine-5'-monophosphate (AZT-MP) by thymidine kinase, subsequent phosphorylation by thymidylate kinase has been shown to be a rate-limiting step leading to high intracellular AZT-MP concentrations [2,6,7].

The present study was undertaken to determine the effects of AZT-MP, 3'-amino-3'-deoxythymidine-5'-monophosphate (AMT-MP) and various 2',3'-dideoxynucleoside-5'-monophosphate (ddN-MP) analogs on the 3' to 5' exonuclease function of calf thymus DNA polymerase δ (pol δ) [8].

---

* Presented in part at the Fifth International Conference on Antiviral Research, Vancouver, B.C. (Canada), Antivir Res **17**: 87 (Abstr. 83), 1992.

† Corresponding author. Tel. (205) 934-8226; FAX (205) 934-8240.

MATERIALS AND METHODS

Materials. Triethylammonium bicarbonate (TEAB) buffer, thymidine-5'-monophosphate, ammonium formate, TLC cellulose, and AZT were purchased from the Sigma Chemical Co. (St. Louis, MO). Triphenylphosphine was purchased from Aldrich (Milwaukee, WI). DEAE-cellulose and Dowex 50W-X2 were obtained from Whatman (Hillsboro, OR) and Bio-Rad (Richmond, CA), respectively. Poly(dT)$_{10}$ was purchased from Pharmacia (Piscataway, NJ). [^{3}H]dTTP (15 Ci/mmol, 1 mCi/mL) was obtained from ICN (Costa Mesa, CA). Terminal deoxynucleotidyltransferase and T4 polynucleotide kinase were purchased from US Biochemical (Cleveland, OH). Proliferating cell nuclear antigen-dependent pol δ purified from fetal bovine thymus [8] was provided by Drs. C.K. Tan and A. So (University of Miami, Miami, FL). AZT-MP was provided by Dr. A.M. Mian (University of Miami, Miami, FL). 3'-Fluoro-3'-deoxythymidine (FdT), AZT-MP and 3'-azido-2',3'-dideoxyuridine-5'-monophosphate (AZdU-MP) were provided by Dr. R.F. Schinazi (Emory University and Veterans Administration, Atlanta, GA). 2',3'-Didehydro-2',3'-dideoxythymidine-5'-monophosphate (D4T-MP) was a gift of Dr. M.J.M. Hitchcock (Bristol-Myers Squibb, Inc., Wallingford, CT).

Chemical synthesis of 2',3'-dideoxynucleoside-5'-monophosphates. 3'-Fluoro-3'-deoxythymidine-5'-monophosphate (FdT-MP) was prepared by chemical phosphorylation of FdT [9]. The identity and purity (99%) of FdT-MP were confirmed by TLC (*Rf* of FdT 0.85 and of FdT-MP 0.35) and FAB-MS analysis. AMT-MP was prepared by chemical reduction of AZT-MP. Briefly, AZT-MP (20 mg) and triphenylphosphine (32 mg) were dissolved in methanol and the reaction mixture was stirred at room temperature for 3 hr. A solution of TEAB (1.0 M, pH 8.5) was added and the mixture stirred for an additional 2 hr. Methanol was removed under reduced pressure and then triphenylphosphine and triphenylphosphine oxide were removed by filtration. The filtrate was lyophilized and the residue dissolved in water. The resulting solution was applied to a DEAE-cellulose column (bicarbonate form) and washed with water, and the product eluted with a gradient of 0.05 to 0.3 M TEAB. Product-containing fractions were pooled and lyophilized. For complete purification and removal of excess bicarbonate salt, AMT-MP was converted to the chloride form with 1.0 N HCl, applied to a Dowex 50W-X2 column (hydrogen form), and the product eluted with TEAB (0.5 M). Product-containing fractions were collected and lyophilized to obtain 11 mg as pure AMT-MP. The identity and purity of the product were confirmed by TLC (*Rf* of AZT-MP 0.30 and of AMT-MP 0.15), FAB-MS analysis and a ninhydrin assay [10].

3' to 5' Exonuclease assays. Single-stranded DNA was synthesized by incubating poly(dT)$_{10}$ (4 μmol) with 40 μCi of [^{3}H]dTTP in a 400-μL reaction containing 100 mM sodium cacodylate (pH 7.2), 0.2 mM β-mercaptoethanol, 2 mM $CoCl_2$, 10 μg/mL bovine serum albumin (BSA), and 130 units of terminal deoxynucleotidyltransferase for 24 hr at 37°. Unincorporated dTTP was removed by centrifugation through two 3.5-mL Sephadex G-25 spin columns equilibrated with 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA. The average chain length was determined by electrophoresis of product 5'-end labeled with [^{32}P] using T4 polynucleotide kinase on a 10% polyacrylamide-urea sequencing gel.

The 3' to 5' exonuclease activity of pol δ was determined by counting radioactivity associated with single-stranded DNA. Each reaction mixture contained in a final volume of 50 μL, 20 pmol [^{3}H]-3'-labeled poly(dT)$_{16}$ (1000 cpm/pmol), 40 mM HEPES buffer (pH 8.2), 60 μg/mL BSA, 10 mM $MgCl_2$, 5% glycerol, and 0.5 unit of pol δ. One unit of pol δ activity is defined as the removal of 1 nmol dTMP/hr at 37°. Under these reaction conditions, the excision of dTMP from DNA was linear for at least 90 min. After incubation at 37° for 60 min, reaction mixtures were assayed for [^{3}H]dTMP from poly(dT) by applying 45 μL of the reaction to a 2.5-cm diameter DEAE-cellulose filter (DE81, Whatman). The filters were then rinsed three times with 150 mL of 0.3 M ammonium formate (pH 7.9), followed by ethanol, and dried; radioactivity remaining on the filters was determined by scintillation counting.

Nuclease activity using a double-stranded DNA template was performed as previously described [11] with some modifications. A 5'-end-labeled [^{32}P]-oligo $(dT)_{16}$ was annealed to a poly(dA) strand (1:2) and this substrate was incubated in a 10-μL volume reaction as described above for single-stranded DNA. The products of the reaction were separated on a 16% sequencing gel and quantitation was assessed by densitometric scan analysis.

RESULTS AND DISCUSSION

The effects of various concentrations of ddN-MP analogs on the 3' to 5' exonuclease activity of pol δ are shown in Table 1.

Table 1. Effects of 2',3'-dideoxynucleoside-5'-monophosphates on DNA polymerase δ–associated 3' to 5' exonuclease activity*

Nucleotide	% Exonuclease inhibition of pol δ at : 10 μM	50 μM	100 μM	250 μM	500 μM
Thymidine 5'-monophosphate (dTMP)	ND†	ND	0‡	0	56.5 ± 2.5
3'-Amino-3'-deoxythymidine-5'-monophosphate (AMT-MP)	13.0 ± 3.5	37.2 ± 13.4	65.5 ± 7.5	96.5 ± 11.0	ND
3'-Azido-3'-deoxythymidine-5'-monophosphate (AZT-MP)	0	0	38.5 ± 7.5	80.0 ± 2.3	ND
3'-Azido-2',3'-dideoxyuridine-5'-monophosphate (AZdU-MP)	ND	ND	0	12.0 ± 2.3	49.0 ± 10.4
3'-Fluoro-3'-deoxythymidine-5'-monophosphate (FdT-MP)	ND	ND	ND	0	27.0 ± 9.2
2',3'-Didehydro-2',3'-dideoxythymidine-5'-monophosphate (D4T-MP)	ND	ND	ND	0	35.0 ± 5.8

*Assays of 3' to 5' exonuclease activity were performed as described in Materials and Methods except for the addition of nucleotides as indicated. Each set of data represents the arithmetic mean value and the standard deviation of at least three independent experiments.

† Not determined.

‡ No inhibition observed.

Calf thymus pol δ exonuclease activity was inhibited by approximately 50% in the presence of 500 μM dTMP. FdT-MP and D4T-MP at a concentration of 250 μM had no effect and AZdU-MP slightly inhibited exonuclease activity at that concentration. FdT-MP and D4T-MP (500 μM) inhibited pol δ exonuclease activity by approximately 30% and AZdU-MP (500 μM) had a slightly greater effect with an inhibition of approximately 50%. The major inhibitory activity was associated with substitution of the 3'-hydroxyl of dTMP with either an azido or amino group. The calf thymus enzyme was most sensitive to AMT-MP, being inhibited by 50% at a concentration of less than 100 μM; of note, a similar degree of inhibition required a concentration of AZT-MP approximately 3-fold higher than that of AMT-MP.

The severe hematological toxicity associated with AZT may be due to interactions of the triphosphate metabolite with cellular DNA polymerases involved in DNA replication. Studies using purified forms of DNA polymerase α, δ and ε would suggest that the intracellular concentrations of AZT-TP be at least two orders of magnitude greater than those detected in cells to exert an effect on DNA synthesis [11,12]. However, incorporation of AZT-MP into cellular DNA has been observed *in vivo* [2]. Consistent with these results incorporation of AZT-MP into newly synthesized DNA was observed *in vitro* using purified DNA polymerase α and β [13]. Also, the 3' to 5' exonuclease activities of DNA polymerases δ and ε were inhibited by DNA strands with AZT-MP incorporated at the 3'-terminus [12], suggesting that excision of incorporated AZT-MP from DNA may be a critical event in DNA replication. Recently, Cheng and co-workers observed a lack of correlation between incorporation of AZT into DNA and cytotoxicity of K-562 cells [3]. In these studies, AZT incorporation was transient due to excision of AZT-MP from DNA. Exposure of human bone marrow cells to toxic concentrations of AZT, which have been shown to correlate with cellular toxicity [2], showed a lack of removal of AZT from DNA when high intracellular levels of AZT-MP were present [4]. The data of these three studies suggest that toxicity is dependent in part on achieved steady-state levels of AZT in DNA which reflect an equilibrium between incorporation and removal of AZT from host DNA. The direct demonstration herein of an inhibition of calf thymus DNA polymerase δ -associated 3' to 5' exonuclease activity by AZT-MP supports that hypothesis.

Several mammalian 3' to 5' exonuclease activities have been reported [3,8,14]. These enzymes are usually distinguished by a preference for single-stranded or double-stranded DNA and by production of mono- or oligonucleotides [15]. While single-stranded DNA is a preferred substrate for DNA polymerase δ, double-stranded DNA is also hydrolyzed. Therefore, we have also examined whether the 5'-monophosphates of AZT and AMT affect degradation of double-stranded DNA by the same enzyme. Using [^{32}P]-5'-$(dT)_{16}$ annealed to poly(dA) as a template, excision of 3'-terminal nucleotides by calf thymus pol δ was inhibited by 90 and 75% in the presence of a 100 μM concentration of AZT-MP and AMT-MP, respectively. Under these conditions, using the double-stranded DNA template, a similar concentration of 500 μM TMP was required to detect an inhibition of 60-70%. These data suggest that the degree of inhibition of 3' to 5' exonuclease activity by AZT-MP varies as a function of the template and warrant additional studies with other mammalian exonucleases.

The 3' to 5' exonuclease activity associated with calf thymus DNA polymerase δ has been shown to be selectively inhibited *in vitro* by nucleoside 5'-monophosphates [5], and the present study now demonstrates an inhibition by ddN-MP analogs. Previous studies have demonstrated that AZdU, D4T and FdT have intracellular phosphorylation patterns resulting in 5'-monophosphate levels below 10 μM [4,16-18]. The fact that the concentration was much lower than that observed to inhibit exonuclease activity would suggest that these compounds are unlikely to have an influence on 3' to 5' exonuclease activity *in vivo*.

AMT is a highly toxic catabolite of AZT [10] and substantial plasma levels of AMT have been detected in patients treated with AZT [19]. Intracellular phosphorylation studies of AMT are in progress in our laboratory and will permit us to elucidate whether *in vitro* inhibition of exonuclease activity by AMT-MP may be another factor involved in AZT cytotoxicity. Of importance, the data reported herein demonstrate the capability of AZT-MP to

affect a mammalian enzyme at a concentration attainable intracellularly. The high degree of homology in amino acid sequence between the bovine and human pol δ [20] strongly suggests that human pol δ is very likely affected by AZT-MP and AMT-MP. Other exonuclease activities may also be affected by AZT-MP, and the present experiments with double-stranded DNA suggest that a stronger inhibition may even be encountered. The observed inhibition may affect the fidelity of human cellular DNA synthesis since a perturbation of the exonuclease-associated proofreading activity of pol δ would lead to higher rates of misincorporations. Inhibition of DNA polymerase ε by nucleoside 5'-monophosphates was shown to increase the rate of mutation associated with DNA synthesis by the polymerase [21]. Inhibition of exonuclease activity has been suggested in the carcinogenic effects of 6-mercaptopurine, which similar to AZT mostly accumulates within cells as the 5'-monophosphate derivative [5]. This study also raises the question of whether AZT may have potential mutagenic or carcinogenic effects due to the selective inhibition of the 3' to 5' exonuclease proofreading activity associated with DNA polymerases.

ACKNOWLEDGEMENTS. This work was supported by United States Public Health Service Grant HL-42125 (J.-P.S.). J.-P.S. is the recipient of a Faculty Research Award from the American Cancer Society.

REFERENCES

1. Yarchoan R, Mitsuya H, Myers CE and Broder S, Clinical pharmacology of 3'-azido-2',3'-deoxythymidine (Zidovudine) and related dideoxynucleosides. *N Engl J Med* **321**: 726-738, 1989.
2. Sommadossi J-P, Carlisle R and Zhou Z, Cellular pharmacology of 3'-azido-3'-deoxythymidine with evidence of incorporation into DNA of human bone marrow cells. *Mol Pharmacol* **36**: 9-14, 1989.
3. Vazquez-Padua MA, Starnes MC and Cheng Y-C, Incorporation of 3'-azido-3'-deoxythymidine into cellular DNA and its removal in a human leukemic cell line. *Cancer Commun* **2**: 55-62, 1990.
4. Zhu Z, Hitchcock MJ and Sommadossi J-P, Metabolism and DNA interaction of 2',3'-didehydro-2',3'-dideoxythymidine (D4T) in human bone marrow cells. *Mol Pharmacol* **40**: 838-845, 1991.
5. Byrnes JJ, Downey KM, Que BG, Lee MYW, Black VL and So AG, Selective inhibition of the 3' to 5' exonuclease activity associated with DNA polymerases: A mechanism of mutagenesis. *Biochemistry* **16**: 3740-3746, 1977.
6. Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff-Lehrman S, Bolognesi DP, Broder S, Mitsuya H and Barry DW, 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.
7. Fridland A, Connelly MC and Ashmun R, Relationship of deoxynucleotide changes to inhibition of DNA synthesis induced by the antiretroviral agent 3'-azido-3'-deoxythymidine and release of its monophosphate by human lymphoid cells (CCRF-CEM). *Mol Pharmacol* **37**: 665-670, 1990.
8. Ng L, Tan CK, Downey KM and Fisher PA, Enzymologic mechanism of calf thymus DNA polymerase. *J Biol Chem* **266**: 11699-11704, 1991.
9. Yoshikawa M, Kata T and Takenishi T, Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull Chem Soc Jpn* **42**: 3505-3508, 1969.
10. Cretton EM, Xie MY, Bevan RJ, Goudgaon NM, Schinazi RF and Sommadossi J-P, Catabolism of 3'-azido-3'-deoxythymidine in hepatocytes and liver microsomes, with evidence of formation of 3'-amino-3'-deoxythymidine, a highly toxic catabolite for human bone marrow cells. *Mol Pharmacol* **39**: 258-266, 1991.
11. Huang P, Farquhar D and Plunkett W, Selective action of 3'-azido-3'-deoxythymidine 5'-triphosphate on viral reverse transcriptases and human DNA polymerases. *J Biol Chem* **265**: 11914-11918, 1990.

12. Nickle W, Austermann S, Bialek G and Grosse F, Interactions of azidothymidine triphosphate with the cellular DNA polymerases α, δ and ε and with DNA primase. *J Biol Chem* **267:** 848-854, 1992.
13. Copeland WC, Chen MS and Wang TSF, Human DNA polymerases α and β are able to incorporate anti-HIV deoxynucleotides into DNA. *J Biol Chem* **267**: 21459-21464, 1992.
14. Fry M and Loeb LA, *Animal cell DNA polymerases.* CRC Press, Boca Raton, FL, 1986.
15. Kornberg A and Baker TA, *DNA replication.* W.H. Freeman, New York, NY,1992.
16. Zhu Z, Ho H-T, Hitchcock MJ and Sommadossi J-P, Cellular pharmacology of 2',3'-didehydro-2',3'-dideoxythymidine (D4T) in human peripheral blood mononuclear cells. *Biochem Pharmacol* **39:** R15-R19, 1990.
17. Matthes E, Lehman C, Scholaz C, Rosenthal HA and Langen P, Phosphorylation, anti-HIV activity and cytotoxicity of 3'-fluorothymidine. *Biochem Biophys Res Commun* **153:** 825-831, 1988.
18. Zhu Z, Schinazi RF, Chu CK, Williams GJ, Colby CB and Sommadossi J-P, Cellular metabolism of 3'-azido-2',3'-dideoxyuridine with formation of 5'-0-diphosphohexose derivatives by previously unrecognized metabolic pathways for 2'-deoxyuridine analogs. *Mol Pharmacol* **38**: 929-938, 1990.
19. Stagg MP, Cretton EM, Kidd L, Diasio RB and Sommadossi J-P, Clinical pharmacokinetics of 3'-azido-3'-deoxythymidine (zidovudine) and catabolites with formation of a toxic catabolite, 3'-amino-3'-deoxythymidine. *Clin Pharmacol Ther* **51:** 668-676, 1992.
20. Zhang J, Chung DW, Tan CK, Downey KM, Davie EW and So AG, Primary structure of the catalytic subunit of calf thymus DNA polymerase δ: Sequence similarities with other DNA polymerases. *Biochemistry* **30:** 11742-11750, 1991.
21. Thomas DC, Roberts JD, Sabatino RD, Myers TW, Tan CK, Downey KM, So AG, Bambara RA and Kunkel TA, Fidelity of mammalian DNA replication and replicative DNA polymerases. *Biochemistry* **30**: 11751-11759, 1991.