Comparative effects of the 5'-triphosphates of 9- β -(2'-azido-2'-deoxy-D-arabinofuranosyl)adenine and 9- β -D-arabinofuranosyladenine on DNA polymerases from L1210 leukemia cells

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Summary

9- β -(2'-Azido-2'-deoxy-D-arabinofuranosyl)adenine (arazide) causes greater and significantly more persistent inhibition of [3 H]-thymidine incorporation into the DNA of neoplastic cells than the related agent 9- β -D-arabinofuranosyladenine (araA). To elucidate the mechanism(s) responsible, we compared the effects of arazide and araA 5'-triphosphates on DNA polymerases α and β of L1210 leukemia cells. Both nucleoside triphosphate analogs inhibited DNA polymerase α activity by competing with dATP; only araATP was inhibitory to DNA polymerase β . Arazide triphosphate was at least four times more active than araATP as an inhibitor of DNA polymerase α . Preincubation of DNA polymerase α with either agent did not result in enzyme inactivation. The results suggest that interference with DNA polymerase α activity by arazide triphosphate may be in part responsible for the inhibition of DNA synthesis produced by arazide in neoplastic cells.

Introduction

9- β -(2'-Azido-2'-deoxy-D-arabinofuranosyl)adenine (arazide) is a purine nucleoside antimetabolite developed to circumvent factors that limit the antineoplastic and antiviral activities of 9- β -D-arabinofuranosyladenine (araA). Thus, arazide is less susceptible to inactivation by adenosine deaminase (EC 3.5.4.4) and is considerably more soluble in aqueous media than araA.¹⁻⁴ Both arazide and araA are substrates for adenosine deaminase; combination of these agents with inhibitors of this enzyme such as 2'-deoxycofomycin (dCF) results in a significant enhancement of anticancer activity in experimental tumor systems.^{2,5-8} This laboratory has shown that daily coadministration of arazide and dCF to animals bearing the P388 leukemia produces markedly greater antineoplastic activity than mixtures of araA and dCF used in the same schedule.² These findings correlated with both greater and more persistent inhibition of [3 H]-thymidine incorporation into the DNA of these neoplastic cells exposed to the combination of arazide and dCF in vivo than to the mixture of araA and dCF, implying that

differences exist in the pharmacological properties of and/or the biochemical lesions created by these two purine nucleoside antimetabolites.

AraA has been shown to be converted to the 5'-triphosphate and in this form to inhibit DNA polymerase⁹⁻¹¹ and ribonucleoside diphosphate reductase^{12,13} activities. Furthermore, a direct relationship has been established between the cancer chemotherapeutic activity of araA and its capacity to interfere with DNA biosynthesis.^{9,10} Our findings with arazide are consistent with the concept that the mechanism of action of this agent is similar to that of araA; however, the molecular basis for the greater potency of arazide as an inhibitor of DNA biosynthesis and as an antineoplastic agent is undefined.

As an approach to the elucidation of the mechanism responsible for the greater activity of arazide relative to that of araA, we have compared the inhibitory activities of the triphosphates of arazide and araA against DNA polymerases α and β isolated from the L1210 leukemia. The findings demonstrate that DNA polymerase α is significantly more sensitive to both of these agents than is DNA polymerase β , and that arazide triphosphate is a more potent competitive inhibitor of DNA polymerase α than is araATP.

Materials and Methods

Tritiated deoxyribonucleoside triphosphates were obtained from New England Nuclear Corp., Boston, MA. Nucleoside triphosphates were purchased from Sigma Chemical Corp., St. Louis, Missouri. Calf thymus DNA was converted to the activated form by partial digestion with DNase I. 9- β -D-Arabinofuranosyladenosine 5'-triphosphate (araATP) was purchased from Calbiochem, San Diego, CA.

Arazide was generously provided by Dr. Frank M. Unger of Sandoz Forschungsinstitut, Vienna, Austria. The 5'-monophosphate was prepared by reaction of arazide with $\text{POCl}_3/\text{PO}(\text{OEt})_3$,¹⁴ followed by purification by chromatography on a column of DEAE-Sephadex (A-25) using a linear gradient of triethylammonium bicarbonate (0 to 0.4 M). Activation of the arazide 5'-monophosphate with 1,1'-carbonyldiimidazole afforded the corresponding phosphorimidazolidate which was condensed with di(tri-*n*-butylammonium)pyrophosphate.¹⁵ The resulting arazide triphosphate was purified using DEAE-Sephadex column chromatography with a linear gradient of triethylammonium bicarbonate (0 to 0.8 M). The purity of arazide triphosphate was established to be >96% by phosphate analysis¹⁶ and high pressure liquid chromatography.

The murine L1210 leukemia, a cell line sensitive to the cytotoxic action of arazide and araA,¹ was employed as the enzyme source. Cells were seeded in Fischer's medium supplemented with 10% horse serum at a concentration of 10^4 cells/ml. Exponentially growing cultures were used for the isolation of cellular DNA polymerases. Procedures for purifying these DNA polymerases from this source have been described previously.¹⁷ In brief, cells were mixed with a homogenization buffer consisting of 0.8 M KCl and 0.5% Triton X-100 and disrupted using a Dounce homogenizer. Nucleic acids were removed from the cell extract by passage through a DEAE-cellulose column which was pre-equilibrated with a buffer consisting of 20 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and 20% glycerol. DNA polymerases were separated by chromatography on a phosphocellulose column using a linear KCl gradient. DNA polymerase β , which eluted at 0.4 M KCl, was free of other cellular DNA polymerases; however, DNA polymerase α , which eluted at 0.18 M KCl, contained some reverse transcriptase activity.¹⁸ DNA polymerase α was separated from the reverse transcriptase activity by further chromatography on a DEAE-cellulose column using a linear KCl gradient.

DNA polymerase α activity was assayed in a 50 μ l reaction mixture which contained 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 8 mM $MgCl_2$, 100 μ M each of dTTP, dCTP and dGTP, and 15 to 20 μ M of [3H]-dATP (2000 cpm/pmole), 10 μ g of activated calf thymus DNA, 10 to 50 μ g of bovine serum albumin, 5 to 10% glycerol, and enzyme. Incubations were conducted at 37° for 30 min unless otherwise indicated. Acid-insoluble radioactivity was collected on a nitrocellulose filter (Millipore: 0.45 μ m), washed several times with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate, once with 70% ethanol, dried, and measured in a liquid scintillation spectrometer. In assays with three natural triphosphate substrates only, 20 μ M [3H]-dGTP (1800 cpm/pmole) was used. Other conditions were identical.

DNA polymerase β activity was assayed using similar conditions except for the following changes: Tris-HCl (pH 9.0), 50 μ M [3H]-dATP and 40 mM KCl were used.

Results

The relative sensitivities of DNA polymerases α and β to araATP and arazide triphosphate were determined and the results are shown in Figure 1. DNA polymerase α was more sensitive than DNA polymerase β to both of the nucleoside antimetabolite triphosphates. Arazide triphosphate was more

Figure 1 Relative sensitivities of DNA polymerase α and DNA polymerase β of the L1210 leukemia to the inhibitory activities of araATP and arazide triphosphate. Enzyme activities were measured by [^3H]-dAMP incorporation into an activated DNA template. Complete activity (100%) of DNA polymerase α and DNA polymerase β were 10.3 and 25.9 pmoles, respectively, of [^3H]-dAMP incorporated in 30 minutes. Other conditions are described in the Methods section. Effect of arazide triphosphate on the activity of DNA polymerase α ($\square$ — $\square$) and DNA polymerase β ($\square$ --- $\square$); effect of araATP on the activities of DNA polymerase α (O—O) and DNA polymerase β (O---O).

inhibitory than araATP to DNA polymerase α ; however, although araATP interfered with the activity of DNA polymerase β , arazide triphosphate did not significantly inhibit the activity of this enzyme. To characterize the nature of the inhibition of the most sensitive enzyme, DNA polymerase α , the effects of increasing concentrations of dATP on the degree of inhibition produced by each of these agents was determined. In assays with activated DNA template, [^3H]-dATP was the rate-limiting substrate and the other three nucleoside triphosphates were in excess. Lineweaver-Burk¹⁹ plots resulted in straight lines that intersected on the ordinate, indicating that inhibition was competitive with respect to dATP (Figures 2A and 2B). The apparent K_m value for dATP with DNA polymerase α was 6.6 μM ; the K_i values for arazide triphosphate and araATP were 1.1 μM and 4.6 μM , respectively. Thus, the mode of inhibition by arazide triphosphate and araATP appeared to be the same, although arazide triphosphate was approximately four times more inhibitory than araATP to DNA polymerase α .

The extent of inhibition with increasing time of incubation was

Figure 2 The effects of varying concentrations of $[^3\text{H}]\text{-dATP}$ on the inhibition of the activity of DNA polymerase α by arazide triphosphate and araATP. Panel A. No inhibitor (O), 2 μM arazide triphosphate (Δ) and 4 μM arazide triphosphate ($\square$). Panel B. No inhibitor (O), 8 μM araATP (Δ) and 16 μM araATP ($\square$).

measured by mixing DNA polymerase α with either 5 μM arazide triphosphate or 20 μM araATP, followed by addition of the other components of the reaction mixture, incubation at 37° and termination of the reaction at selected time intervals thereafter (Figure 3). The results demonstrate that the reaction rate was linear with time for at least up to 120 min in the absence of inhibitor. In the presence of both inhibitors a linear increase in the rate of incorporation of dAMP into DNA occurred, although to a considerably lesser extent than that of the control. The lack of progressive inhibition with time implied that the enzyme was not irreversibly

Figure 3 Time course of the inhibition of DNA polymerase α activity by arazide triphosphate and araATP. The enzyme and inhibitor were mixed at 4° and other components of the reaction mixture were added subsequently and incubated at 37°; the reaction was terminated at the indicated time intervals. Twenty μM [^3H]-dATP was used in the reaction mixture with an activated DNA template. Other conditions were described in the text. Control (O), 5 μM arazide triphosphate (Δ), and 20 μM araATP ($\square$).

inactivated by either arazide triphosphate or araATP. The results shown in Figure 4 lend further support to this conclusion. In these experiments, dATP was omitted from the reaction mixture and [^3H]-dGTP was employed as the radioactive substrate; as expected, a considerable reduction in the rate of [^3H]-dGMP incorporation into DNA occurred. When an appropriate amount of dATP was added, the rate of DNA synthesis increased significantly. However, when dATP was added to the reaction system containing arazide triphosphate, only a slight increase in DNA synthesis was observed. Essentially identical results were obtained in experiments with araATP (data not shown). These results suggest that the purine nucleotide anti-metabolites do not function as effective chain terminators.

Discussion

Arazide appears to have significant advantages over araA as an antineoplastic agent. It is less susceptible to inactivation by adenosine deaminase, seems to be more potent as an anticancer agent, and is more

Figure 4 The effects of arazide triphosphate on the incorporation of [^3H]-dGMP into DNA catalyzed by DNA polymerase α . Twenty μM [^3H]-dGTP was employed in assays with activated DNA template; other conditions were as described in the Methods section. All four nucleotide substrates in the reaction mixture (0—0); three nucleotide substrates only, with dATP omitted (Δ — Δ); at the end of 90 minutes of incubation at 37° , 100 μM dATP was added and the reaction was terminated at 120 minutes (Δ --- Δ); three nucleotide substrates and 20 μM arazide triphosphate ($\square$ — $\square$); at the end of 90 minutes of incubation with three nucleotide substrates and 20 μM arazide triphosphate, 100 μM dATP was added and the reaction was terminated at 120 minutes ($\square$ --- $\square$).

soluble in aqueous media than araA.¹⁻⁴ Exposure of P388 leukemia-bearing mice to arazide results in greater and significantly more persistent inhibition of DNA synthesis in the neoplastic cells than that produced by araA.¹ To characterize the biochemical mechanism responsible for the greater inhibitory effects of arazide on the DNA biosynthetic pathways, we have compared the action of the 5'-triphosphates of araA and arazide on DNA polymerases purified from a susceptible leukemic cell line, the L1210. It had been shown earlier that araATP inhibits DNA polymerase activity by competing with the natural triphosphate substrate, dATP.^{11,20} Our results show that the DNA polymerase α of L1210 cells is inhibited by araATP by a similar mechanism. In addition, we have demonstrated that the mode of inhibition of DNA polymerase α by arazide triphosphate is similar to that of araATP. However, arazide triphosphate was more inhibitory than araATP to DNA polymerase α , the K_i value for arazide triphosphate being at least four times less than that for araATP. Preincubation of DNA polymerase α with either arazide triphosphate or araATP in the presence or absence of

other components of the reaction mixture did not increase inhibition of enzyme activity. Moreover, a lack of progressive inhibition with reaction time indicated that the enzyme was not irreversibly inactivated by either arazide triphosphate or araATP.

DNA polymerase α is considered to be the enzyme responsible for replicative DNA synthesis.²¹ Therefore, the greater inhibitory activity of arazide triphosphate than of araATP for this enzyme may be responsible for the observation that arazide caused a greater and more persistent inhibition of [³H]-thymidine incorporation into the DNA of intact neoplastic cells than did araA. It is not clear, however, how a competitive inhibitor, such as arazide triphosphate can cause exceedingly prolonged inhibition of DNA synthesis in neoplastic cells *in vivo*. Thus, other factors may be responsible for this phenomenon. One of the proposed mechanisms for the cytotoxic action of araA involves incorporation of the arabinosyl nucleotide into replicating DNA, such that further DNA synthesis is inhibited. Recently, Pelling *et al.*²² demonstrated that araA is not an absolute chain terminator of HSV-1 DNA synthesis in virus infected cells, and that the amount of araA incorporated internally into viral DNA is less than the amount incorporated into cellular DNA. Our results with DNA polymerase α also imply that neither arazide triphosphate nor araATP act as effective chain terminators.

The findings suggest that arazide and araA inhibit DNA polymerase α by similar mechanisms, and that the greater potency of arazide as an antineoplastic agent and an inhibitor of DNA biosynthesis may be due at least in part to greater inhibitory activity towards DNA polymerase α .

This research was supported by U.S. Public Health Service grants CA-02817 and CA-28852 and American Cancer Society grant CH 47-D.

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