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BIOLOGICAL ACTIVITIES AND MODES OF ACTION OF 9-α-D-ARABINOFURANOSYLADENINE AND 9-α-D-ARABINOFURANOSYL-8-AZAADENINE

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Abstract—From earlier studies it is known that 9-α-D-arabinofuranosyladenine (α-araA) and 9-α-D-arabinofuranosyl-8-azaadenine (α-ara-8-azaA) have *in vitro* antiviral activity, are cytotoxic, and are metabolized in mammalian cells to the triphosphates. This study was designed to compare the *in vivo* antiviral activities of these compounds and their loci of action with those of 9-β-D-arabinofuranosyladenine (β-araA). The latter compound selectively inhibits DNA synthesis in intact cells, and its triphosphate is a known inhibitor of DNA polymerases and ribonucleotide reductase. Whereas β-araA was significantly effective in the treatment of systemic herpes simplex virus type 1 (HSV-1) infections in mice, α-araA and α-ara-8-azaA were therapeutically ineffective. α-AraATP at a concentration of ~1 mM did not inhibit (1) DNA polymerases present in crude extracts of cultured H.Ep.-2 cells; (2) DNA polymerases present in extracts of KB cells; (3) partially purified DNA polymerase-α from mouse embryo cells; or (4) DNA polymerases induced by HSV-1 and HSV-2. DNA polymerase-β from mouse embryo cells was inhibited to a small extent by 10^{-4}M α-araATP. In contrast, all of these enzymes were inhibited by β-araATP at a concentration of 10^{-5}M (as shown in these or in earlier studies). The reductions of CDP and UDP by ribonucleotide reductase from L1210 cells were not inhibited by α-araATP (~10^{-3}M), whereas β-araATP produced 70–80 per cent inhibition at this concentration. In cultured H.Ep.-2 cells, α-ara-8-azaA inhibited the incorporation of thymidine, uridine, and formate into macromolecules, but it was without effect on the incorporation of adenine and hypoxanthine, and produced marginal inhibition of the incorporation of leucine. α-Ara-8-azaA produced a dose-dependent inhibition of the accumulation of [^{14}C] formyl-glycinamide ribonucleotide in H.Ep.-2 cells treated with azaserine and [^{14}C] formate. These results indicate that the α-nucleosides inhibit nucleic acid synthesis by mechanisms different from those of β-araA.

We have reported earlier that 9-α-D-arabinofuranosyladenine (α-araA)§ and 9-α-D-arabinofuranosyl-8-azaadenine (α-ara-8-azaA) exert significant antiviral effects against herpes simplex virus type 1 (HSV-1) *in vitro* [1] and that these compounds are cytotoxic to cultured mammalian cells [2]. α-Ara-8-azaA is a substrate for adenosine kinase and its principal metabolite in cell culture is α-ara-8-azaATP [2]. α-AraA is also anabolized in mammalian cells to the triphosphate [3]. 9-β-D-Arabinofuranosyladenine (β-AraA) is important both as an antiviral agent and as an antitumor agent, but its antitumor activity is limited by its ready deamination so that it is active only in the presence of inhibitors of adenosine deaminase (ADA) [4, 5]. α-AraA and α-ara-8-azaA are resistant to attack by ADA [2]; hence, if α-araA should have sites of action similar to those of β-araA, it would be the preferred agent because it would not be necessary to use it in combination with an ADA inhibitor. It was therefore desirable to compare the biological activities and the biochemical properties of the α-arabinosyl compounds with those of β-araA. We report here three such studies. The first is a quantitative evaluation of α-araA and α-ara-8-azaA for *in vivo* therapeutic efficacy against lethal HSV-1 infections in mice. The second is a comparison of the effects of α-araATP and β-araATP on ribonucleotide reductase and on cellular and HSV-induced DNA polymerases. These enzymes were chosen because each was known to be inhibited by β-araATP [6–13]. The third was a study in cultured cells of the metabolic effects of α-ara-8-azaA, which was selected for study over α-araA because it is more toxic and because it was the compound with which we conducted earlier metabolic studies [2].

While this work was in progress, Müller *et al.* [3, 11, 12, 14], using a sample of α-araATP from the same batch utilized herein, reported it to be an effective inhibitor of DNA polymerase-α and therefore similar in mode of action to β-araATP. Our results, some of which have been reported in preliminary form [15], differ from those of Müller and his associates and reopen the question as to the

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§ Abbreviations: α-AraA, 9-α-D-arabinofuranosyladenine; α-ara-8-azaA, 9-α-D-arabinofuranosyl-8-azaadenine; β-araA, 9-β-D-arabinofuranosyladenine; HSV-1, herpes simplex virus, type 1; HSV-2, herpes simplex virus, type 2; and ADA, adenosine deaminase.

mechanisms of action of the α-arabinosyl compounds.

MATERIALS AND METHODS

Compounds

α-AraA and α-ara-8-azaA were synthesized in our laboratory by procedures described earlier [16]. α-AraA was converted to α-araATP by Terra Marine Bioresearch, La Jolla, CA. The product was analyzed in our laboratories by high pressure liquid chromatography (h.p.l.c.) with a Waters Associates (Milford, MA) model 202 apparatus. Chromatography was performed with a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ), 4.6 mm i.d. × 25 cm. Elution was achieved with a linear gradient of 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, at a flow rate of 2 ml/min, and detection of eluted materials was accomplished by measurement of u.v. absorption at 254 nm. Integrations of peak areas were made with a Hewlett-Packard model 3380-A digital electronic integrator, and quantitations were achieved using peak area–nmole relationships derived from determinations of known nucleotides. Three peaks were obtained. The principal peak, which eluted with a retention time indicative of a triphosphate and about the same as ATP (34 min), amounted to 64–67 per cent of the sample. There were two smaller peaks with retention times close to those of ADP (15–17 min); these were presumed to be isomeric diphosphates (α-araADP, α-araA-3′,5′-diphosphate, or α-araA-2′,5′-diphosphate). To confirm that all of the phosphates present were derivatives of α-araA, a sample of α-araATP was treated overnight with crude snake venom (Ross Allen's Reptile Institute, Silver Springs, FL) and then chromatographed on a μBondapak C_{18} column (Waters Associates), 3.9 mm i.d. × 30 cm. The solvent consisted of H_2O–acetonitrile (95:5, v/v) and the flow rate was 0.8 ml/min. Detection of peaks was accomplished as described above for ion exchange chromatography. A single peak was obtained with a retention time (14 min) the same as that of α-araA. We have shown earlier that this procedure gives a good separation of the α- and β-anomers of ara-8-azaA [2].

β-AraA and azaserine were obtained from the Warner–Lambert/Parke–Davis Pharmaceutical Research Laboratories, Detroit, MI. The following radioactive compounds were obtained from the New England Nuclear Corp., Boston, MA: sodium [^{14}C]formate (6.54 mCi/mmole) [1-^{14}C]-L-leucine (16.4 mCi/mmole) [8-^{14}C]hypoxanthine (48.6 mCi/mmole) [8-^{14}C]adenine (15.7 mCi/mmole) [2-^{14}C]uridine (55.5 mCi/mmole) [2-^{14}C]thymidine (57.6 mCi/mmole) and [^{3}H-methyl]thymidine 5′-triphosphate (18 Ci/mmole).

Evaluation of α-araA and α-ara-8-azaA for antiviral efficacy in the treatment of systemic HSV infections in mice.

Random-bred, female ICR Swiss mice (18–22 g) were obtained from the Charles River Breeding Laboratories (Wilmington, MA) and given food and water *ab lib*. Mice were inoculated i.p. with 2 LD_{50} of HSV-1 (strain HS-123). Stock virus was prepared as a 10% mouse brain suspension and was diluted in phosphate-buffered saline to yield the desired challenge virus concentration. Compounds were administered to mice i.p., once daily for 7 days, beginning 24 hr after virus inoculation. The compounds were suspended in 0.9% NaCl solution containing 0.3% hydroxypropyl cellulose at three different concentrations so that a single dose volume of 0.01 ml/g of body weight represented the approximate LD_{10}, $LD_{10}/2$, and $LD_{10}/4$ for each compound.

Twenty untreated virus-infected mice served as the virus control group. Ten uninfected animals were sham-injected i.p. with 0.2 ml of the virus diluent alone (no virus), and ten uninfected animals were sham-injected i.p. on the q.d. 1–7 day schedule with the drug diluent alone (no drug). In addition, a group of ten uninfected, untreated mice was held as normal controls. β-AraA was employed as a positive control drug and was concomitantly administered i.p. to groups of ten virus-infected and ten uninfected animals on the same schedule (q.d. 1–7 days) as the α-nucleoside analog-treated groups. The animals were observed daily for deaths and weight loss over a 21-day observation period, and results were expressed in terms of the percentage of survivors in the virus-infected, drug-treated groups compared to that observed in the virus-infected control (untreated) group, or in terms of the mean survival time of animals dying on or before day 21 post-infection in the drug-treated groups compared to that observed in the control group.

All data were subjected to statistical evaluation to determine the significance of differences observed. The Chi-square test was used to compare the mortality of control and drug-treated mice, while Student's *t*-test was employed to compare the differences in mean survival time of virus-infected control and drug-treated animals. A P value of < 0.05 was considered indicative of a statistically significant difference.

Assays of DNA polymerases

Effects of α- and β-araATP were determined on mixed DNA polymerases present in crude supernatant fractions from cultured H.Ep.-2 cells, on partially purified DNA polymerase-α and DNA polymerase-β from mouse embryo cells, and on DNA polymerases present in extracts from whole HSV-infected and uninfected KB cells.

DNA polymerases from mouse embryo cells and H.Ep.-2 cells. Partially purified DNA polymerases-α and -β from mouse embryo cells [17] were obtained from Dr. V. Sagar Sethi, Anthem Research, Inc., Rockville, MD.

For preparation of the crude polymerases from H.Ep.-2 cells, the cells were homogenized in a Potter-Elvehjem glass homogenizer with a Teflon pestle and the homogenate was centrifuged at 100,000 *g*; the supernatant fraction was used as the enzyme fraction. Polymerase activity was determined by incubating the enzyme with [^{14}C]dTTP, unlabeled dCTP, dATP, and dGTP, and primer-template DNA, as described in Fig. 1. Polymerase activity was measured by the incorporation of label into acid-insoluble material determined by the

method of Bollum [18]. Studies with the purified polymerase-α and β from mouse embryo cells were performed in a manner similar to that described in Table 2.

Preparation of uninfected and HSV-infected KB cells. Uninfected KB cells were grown in suspension culture as described previously [19]. Cells were harvested at a density of approximately 500,000 cells/ml by low-speed centrifugation, washed twice by suspension and centrifugation in cold 20 mM Tris–HCl (pH 7.5) containing 150 mM KCl, frozen rapidly by immersion in liquid nitrogen, and stored at −76°.

To obtain cells infected with HSV, nearly confluent roller cultures of KB cells were exposed to either the HF strain of HSV-1 or the X-79 strain of HSV-2 at an input multiplicity of 5 plaque-forming units/cell. After a 1-hr period of adsorption, virus growth medium (Eagle's minimum essential medium supplemented with Hanks' salts, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 1.2 mM arginine, 100 units/ml penicillin G, 50 μg/ml streptomycin sulfate, and 10% unheated calf serum) was added. Incubation at 37° was allowed to proceed until 18 hr post-infection, when HSV-induced DNA polymerase activity had attained a maximum level. Infected cells were detached by agitation (HSV-1) or scraping (HSV-2), collected, washed, and stored in the same manner as described for uninfected cells.

Preparation of whole KB cell extracts. Extracts were prepared from whole uninfected and HSV-infected KB cells using the method of Powell and Purifoy [20]. All steps were conducted at 0–4°. In brief, cells were thawed, suspended in hypotonic buffer, and disrupted ultrasonically. The sonically treated suspension was made 1.7 M in KCl, left on ice for 60 min, clarified by ultracentrifugation, and dialyzed extensively against 50 mM Tris–HCl (pH 7.5) containing 0.5 mM dithiothreitol, 0.2% Nonidet P-40, and 20% glycerol. The precipitate that formed during dialysis was removed by ultracentrifugation, and the supernatant liquid, which contained most of the DNA polymerase activities detectable in the unclarified sonically treated suspension, was divided into small aliquots and stored at −76°.

KB cell and HSV-specific DNA polymerase assays. Polymerase-specific reaction mixtures were modifications of those developed by Weissbach and associates [21, 22]. The basic reaction mixture for assay of both α-polymerase and β-polymerase activities in extracts from uninfected KB cells consisted of: 50 mM Tris–HCl (pH 8.5), 7.5 mM $MgCl_2$, 500 μg/ml bovine serum albumin, 750 μg/ml activated calf thymus DNA, 25 μM [^{3}H]dATP (sp. act. 50–200 cpm/pmole) and dCTP, dGTP, and dTTP at 100 μM each. Dithiothreitol at a final concentration of 0.5 mM was included in the α-polymerase assay. To distinguish α-polymerase from β-polymerase, dithiothreitol was omitted and *N*-ethylmaleimide and KCl were added to final concentrations of 5 mM and 100 mM respectively. Separation of α-polymerase and β-polymerase activities into characteristic peaks on sucrose density gradients [23] established that KB α-polymerase did not react in the β-polymerase assay. KB β-polymerase reacted to a greatly diminished extent in the α-polymerase assay. When extracts from uninfected cells were assayed using the α-polymerase assay, however, the contribution of β-polymerase to total activity detected was negligible.

HSV-specific DNA polymerase activity was assayed in extracts from HSV-infected cells, using a reaction mixture that contained 100 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 0.5 mM dithiothreitol, and 120 mM K_2SO_4. Bovine serum albumin, activated calf thymus DNA, [^{3}H]dATP, dCTP, dGTP and dTTP were all present at the same final concentrations employed for α-polymerase and β-polymerase assays. The reaction mixture was specific for the viral DNA polymerase: optimal activity was obtained in assays of extracts from HSV-infected cells, but under identical assay conditions only an extremely low level of activity was detected in an extract from uninfected cells. The residual activity may have been due to cellular γ-polymerase [24]. All assays were incubated at 37° for 15 min. Acid-precipitable radioactivity was quantitated as described previously [13].

Studies with ribonucleotide reductase

Ribonucleotide reductase was prepared from cultured H.Ep.-2 cells essentially as described in published work [25]. The fraction used was that precipitated by 25–40% ammonium sulfate. Conditions of the assay are given in Table 3.

Nucleic acid synthesis in H.Ep.-2 cells,

H.Ep.-2 cells were grown in suspension culture in SRI-14 medium [26]. To logarithmically growing cell cultures (~2 × 10^5 cells/ml) was added α-ara-8-azaA at the desired concentration, followed 1 hr later by the labeled precursors at the concentrations shown in the legend to Table 4. The cells were harvested 4 hr after addition of the labeled precursors and were washed free of medium with 0.85% NaCl solution. Soluble materials were removed by extraction with boiling 80% ethanol. The residue was extracted with hot 10% NaCl solution; crude polynucleotides (DNA + RNA) were precipitated from the extract by the addition of 4 vol. of ethanol. The polynucleotides were redissolved in water and the concentration was determined by measurement of u.v. absorbance at 254 nm. Portions of this solution were pipetted into vials for determination of radioactivity in a Packard TriCarb liquid scintillation spectrometer. These procedures have been described in detail elsewhere [27].

De novo *synthesis of purine nucleotides*

The effects of α-ara-8-azaA on synthesis of purine nucleotides *de novo* were determined by a previously described modification [28] of the method of LePage and Jones [29], which involves measurement of the inhibition of synthesis of [^{14}C]formylglycinamide ribonucleotide in cells grown in the presence of azaserine and [^{14}C]formate. Additional details are given in Table 5.

RESULTS

Lack of in vivo *antiviral activity of α-araA and α-ara-8-azaA against HSV-1*

Although β-araA produced a highly significant antiviral effect against HSV-1 *in vivo*, treatment with

Table 1. Effect of treatment with 9-β-D-arabinofuranosyladenine, 9-α-D-arabinofuranosyladenine, or 9-α-D-arabinofuranosyl-8-azaadenine on mortality of random-bred Swiss mice inoculated i.p. with 2 LD_{50} of herpes simplex virus type 1

Drug	Drug dosage* ($mg \cdot kg^{-1} \cdot day^{-1}$)	Virus-infected animals: Mortality (No. dead/total)	Virus-infected animals: Mortality (%)	Virus-infected animals: Mean survival time (days)†	Uninfected animals: Mortality (No. dead/total)	Uninfected animals: Mortality (%)
None	0	18/20	90	7.9	0/30	0
β-AraA	500	1/10	10‡	15.0§	0/10	0
β-AraA	250	1/10	10‡	7.0	0/10	0
β-AraA	125	0/10	0‡		0/10	0
α-AraA	420	10/10	100	7.6	0/10	0
α-AraA	210	8/10	80	8.1	0/10	0
α-AraA	105	10/10	100	7.5	0/10	0
α-Ara-8-azaA	80	10/10	100	7.4	1/10	10
α-Ara-8-azaA	40	9/10	90	7.2	0/10	0
α-Ara-8-azaA	20	7/10	70	7.7	0/10	0

* Drugs were administered i.p. once daily for 7 days beginning 24 hr after virus inoculation.
† Only animals dying on or before day 21 after virus inoculation were considered.
‡ Probability (P) that the observed increase in the number of survivors was due to chance < 0.0005 (χ^2-test).
§ Probability (P) that the observed increase in the mean survival time was due to chance < 0.005 (Student's t-test).

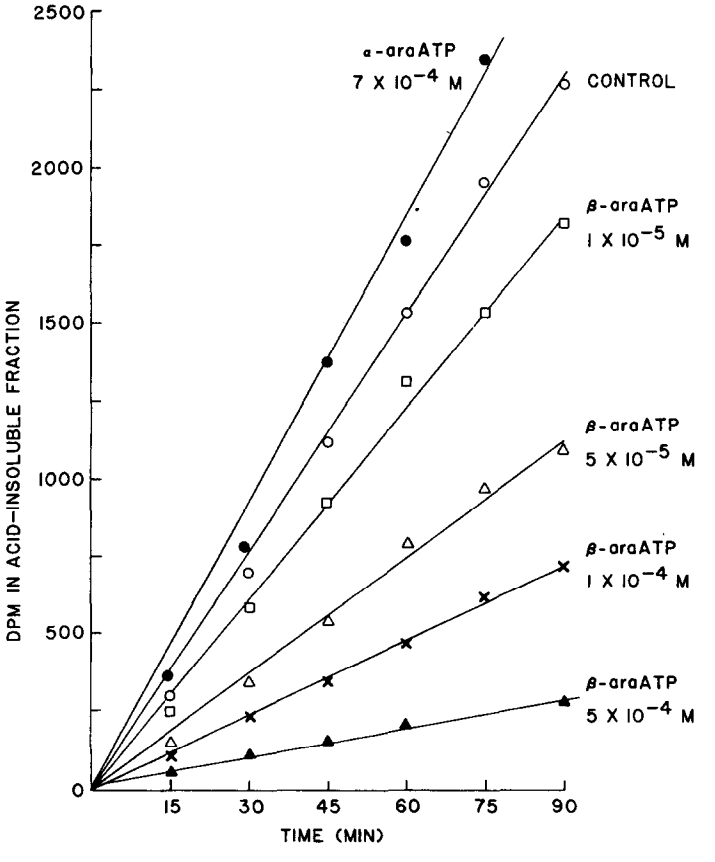

Fig. 1. Effects of β-araATP and α-araATP on the DNA polymerase activity of cell-free preparations from H.Ep.-2 cells. The reaction mixture (final volume 0.5 ml) contained [2-^{14}C]dTTP (50 nmoles, 0.1 μCi); 50 nmoles each of dATP, dGTP, and dCTP; $MgCl_2$ (4 μmoles); primer-template DNA (denatured calf thymus DNA) (200 μg); mercaptoethanol (0.5 μmole); and the enzyme preparation in phosphate buffer, pH 7.0. Incubation was at 37°. At 15-min intervals portions of the mixture were removed and assayed for incorporation of carbon-14 into acid-insoluble material (see text). The figure shows results for only the highest concentration (7×10^{-4} M) of α-araATP. Each point is the average of duplicate assays. Also assayed were four other concentrations of α-araATP (ranging from 7×10^{-6} to 4×10^{-4} M), which also showed no inhibition of polymerase activity.

either α-araA or α-ara-8-azaA was without therapeutic benefit. β-AraA reduced the mortality from 90 per cent in the untreated, virus-infected control group to 0–10 per cent in the virus-infected, drug-treated groups (Table 1). In addition, β-araA at 500 $mg \cdot kg^{-1} \cdot day^{-1}$ significantly increased the mean survival time of dying animals from 7.9 days in the control (untreated) group to 15.0 days. No such increases were observed in the numbers of survivors or in the mean survival times of HSV-infected mice treated with α-araA or α-ara-8-azaA.

Effects of nucleotide analogs on DNA polymerases

The effects of α- and β-araATP on mixed DNA polymerases present in crude supernatant fractions of H.Ep.-2 cells are shown in Fig. 1. α-AraATP was non-inhibitory at concentrations up through 0.7×10^{-3}M, the highest concentration assayed. β-AraATP was inhibitory at a concentration of 1×10^{-5}M, and increased concentrations gave proportionately increased inhibitions. Table 2 shows the effects of the anomeric triphosphates on the polymerases from mouse embryo cells. β-AraATP inhibited both polymerases to about the same degree, and significant inhibition was produced by a concentration of 1×10^{-5}M. α-AraATP did not inhibit DNA polymerase-α and produced a small inhibition of DNA polymerase-β at a concentration of 0.7×10^{-4}M.

Figure 2 illustrates the effects of a α-araATP on DNA polymerases in whole cell extracts prepared from uninfected or HSV-infected KB cells. The concentrations of α-araATP employed ranged from 4×10^{-6} to 1.8×10^{-4}M. The ratio of α-araATP concentration to dATP concentration (inhibitor: substrate or I/S ratio) thus varied from 0.16:1 to 7.2:1 (approximately 50-fold). In an extract from uninfected cells, neither α-polymerase nor β-polymerase was affected significantly by α-araATP at the tested concentrations. Activity in the presence of α-araATP was never less than 93 per cent nor more than 103 per cent of control activity. When extracts from HSV-1- or HSV-2-infected cells were assayed under conditions specific for HSV-induced DNA polymerase, α-araATP appeared to be slightly inhibitory. At the highest α-araATP concentration, activity was reduced to about 74 per cent of control. Under identical assay conditions, however, α-araATP failed to inhibit activity in a preparation of HSV-1 DNA polymerase which had been partially purified by chromatography on DEAE-cellulose (data not shown).

In contrast to α-araATP, β-araATP is a potent inhibitor of KB cell α-polymerase and the HSV-1 DNA polymerase; KB cell β-polymerase is considerably less sensitive [13]. In the current study, β-araATP at an I/S ratio of 7.2:1 (corresponding to the highest I/S ratio of α-araATP tested) reduced KB α-polymerase activity to approximately 7 per cent of conrol and HSV-1 DNA polymerase activity to approximately 2 per cent of control (data not shown). As indicated in Fig. 2, β-araATP was also a strong inhibitor of virus-specific DNA polymerase activity in an extract from HSV-2-infected KB cells; at an I/S ratio of 8:1, approximately 2 per cent of control activity remained.

Effects of nucleotide analogs on ribonucleotide reductase

As shown in Table 3, β-araATP at a concentration of 5×10^{-5}M inhibited the reduction of CDP and at a concentration of 5×10^{-4}M inhibited the reduc-

Table 2. Inhibition of partially purified DNA polymerase-α and -β from mouse embryo cells by α-araATP and β-araATP*

Inhibitor	Concn (M)	Activity of DNA polymerase (% of control)	
		α-Polymerase	β-Polymerase
α-AraATP	0.7×10^{-5}	100	89
	0.7×10^{-4}	87	69
β-AraATP	1×10^{-5}	66	55
	1×10^{-4}	31	24

* Effects of α- and β-araATP on the activities of partially purified mouse embryo cell DNA polymerase-α and -β preparations were determined by incubating each of the enzymes with the nucleotide analogs for 30 min at 37° in a reaction mixture (100 μl) containing 20 μM [^{3}H]dTTP (2.5 μCi/10 μl, ~2000 dpm/pmole) as substrate; 20 μg activated (nicked) calf thymus DNA as primer-template; 20 μM dATP; 1.0 mM dCTP; 1.0 mM dGTP; 15 μM NaF; 5 mM dithiothreitol; 50 mM KCl; 0.8 mM $MgCl_2$; and 50 mM Tris–HCl buffer, pH 8.0. All tubes were held in an ice bath at 4° while additions were made. The reaction was initiated by the addition of the [^{3}H]dTTP with immediate transfer to a 37° water bath. To terminate the reaction, 0.5 ml of a 0.1 M sodium pyrophosphate solution and 50 μl of carrier RNA (yeast tRNA, stripped; 4 mg/ml) was added to each tube. After thorough mixing, 0.5 ml of a cold 25% TCA solution was added to each tube and the contents were mixed again. Precipitates were allowed to form for at least 15 min at 4° before collection of samples on Millipore Celotate filters (25 mm), premoistened with cold 5% TCA, using a Millipore model 3025 Sampling Manifold and vacuum pump. Samples were washed six times with ice-cold 5% TCA and dried under an infrared heat lamp. The dried filters were then transferred to counting vials containing 0.2 ml distilled water and solubilized with 1.0 ml NCS Solubilizer (Amersham/Searle, Arlington Heights, IL) by heating in an oven at 40–50° for 30 min. A toluene-base scintillation mixture was added to each vial and the radioactivity was measured in a Packard liquid scintillation counter.

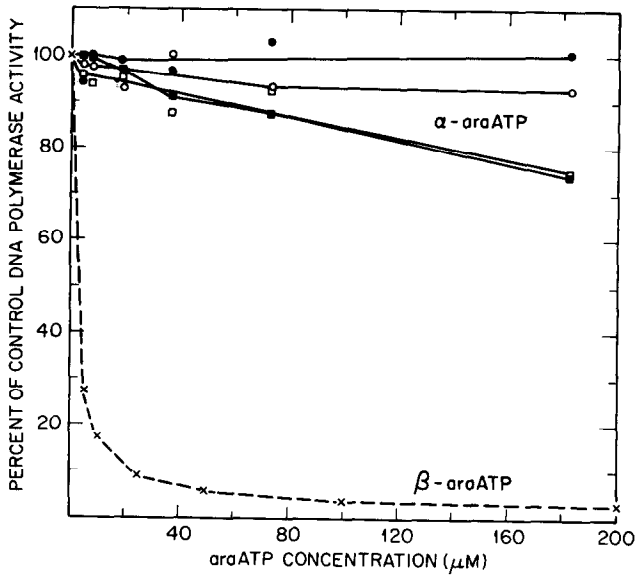

Fig. 2. Response of DNA polymerase activities in extracts from whole uninfected and HSV-infected KB cells to α-araATP or β-araATP. DNA polymerase-specific assays were conducted as described in Materials and Methods. Each value is the mean of duplicate assays, corrected for assay background. Key: extract from uninfected KB cells with α-araATP in the α-polymerase assay (●); extract from uninfected KB cells with α-araATP in the β-polymerase assay (○); extract from HSV-1-infected KB cells with α-araATP in the HSV polymerase assay (□); extract from HSV-2-infected KB cells with α-araATP in the HSV-specific polymerase assay (■); and extract from HSV-2-infected KB cells with β-araATP in the HSV-specific polymerase assay, (X).

Table 3. Effects of α-AraATP and β-AraATP on the reduction of CDP and UDP by ribonucleotide reductase from H.Ep.-2 cells*

Substrate	Inhibitor concn (M)	Deoxynucleotide formation (% Inhibition)
CDP	α-AraATP (4×10^{-5})	0
	α-AraATP (0.7×10^{-4})	0
	α-AraATP (4×10^{-4})	0
	α-AraATP (0.7×10^{-3})	0
	β-AraATP (5×10^{-5})	34
	β-AraATP (1×10^{-4})	41
	β-AraATP (5×10^{-4})	72
	β-AraATP (1×10^{-3})	77
UDP	α-AraATP (0.7×10^{-4})	0
	α-AraATP (4×10^{-4})	0
	α-AraATP (0.7×10^{-3})	0
	α-AraATP (2×10^{-3})	0
	β-AraATP (1×10^{-4})	0
	β-AraATP (5×10^{-4})	48
	β-AraATP (1×10^{-3})	71
	β-AraATP (3×10^{-3})	94

* The incubation mixture contained, in a final volume of 0.2 ml: 8.3 μM potassium phosphate buffer (pH7); 4.4 μM ATP; 2.7 μM magnesium acetate; 8.3 μM sodium fluoride; 0.06 μM ferrous chloride; 6.2 μM dithioerythritol; 0.4 μM (0.1 μCi) [2-^{14}C]CDP or [2-^{14}C]UDP; and the enzyme. After incubation for 30 min at 37°, the reaction was stopped by rapid heating to boiling for 2 min. Protein was removed by centrifugation; to the supernatant fraction was added crude snake venom (1 mg), alkaline phosphatase (0.2 unit), and deoxycytidine or deoxyuridine (50 μg). After incubation at 37° for 90 min, the reaction mixture was subjected to chromatography for separation of deoxycytidine or deoxyuridine. Areas containing these nucleosides were cut out and assayed for radioactivity. For CDP, values are the average of two experiments; for UDP, the results are for a single experiment. These procedures are described in detail elsewhere [25].

Table 4. Effects of α-arabinosyl-8-azaadenine on the incorporation of precursors into polynucleotides of cultured H.Ep.-2 cells*

Precursor	Specific activity of polynucleotides: % of control at α-ara-8-azaA concn (μM) of			
	11	19	38	94
Sodium [^{14}C]formate	110	84	81	39
[8-^{14}C]Hypoxanthine	103	100	97	90
[8-^{14}C]Adenine	94	99	117	94
[2-^{14}C]Thymidine	87	91	81	53
[2-^{14}C]Uridine	90	84	90	64
[^{14}C]Leucine	102	107	79	78

* α-Ara-8-azaA was added to proliferating suspension cultures 1 hr before the addition of the labeled precursors, each of which was present at a concentration of 0.0385 μCi/ml. The micromolar concentrations of the individual precursors were: formate, 38; hypoxanthine, 0.79; adenine, 2.4; thymidine, 0.67; uridine, 0.69; and leucine, 2.3. The cells were harvested 4 hr after addition of the labeled compounds, and the polynucleotides were isolated and assayed as described in the text. Results are for duplicate assays in a single experiment.

tion of UDP. α-AraATP at a concentration of 0.7×10^{-3} M was without effect on the reduction of CDP and was also without effect on the reduction of UDP at concentrations through 2×10^{-3} M.

Metabolic effects of α-ara-8-azaA

When α-ara-8-azaA was assayed for inhibition of incorporation of a number of precursors into polynucleotides, significant inhibitions were observed with formate, uridine, and thymidine, but not with adenine or hypoxanthine (Table 4). There was perhaps a small inhibition of the incorporation of leucine. A further study of the effects on utilization of [^{14}C]formate is shown in Table 5. α-Ara-8-azaA strongly inhibited the incorporation of formate into formylglycinamide ribonucleotide in cells that had been pretreated with azaserine.

DISCUSSION

The cytotoxicity and the antiviral and antitumor activities of β-araA are associated with a selective inhibition of DNA synthesis, which is generally ascribed to inhibition of DNA polymerases [4, 5, 11]. Ribonucleotide reductase from some sources is also highly sensitive to β-araATP [6, 9, 30], and its inhibition may contribute to the effects of araA on DNA synthesis. The results of the present study show that α-araATP at concentrations in the mM range did not inhibit ribonucleotide reductase from H.Ep.-2 cells; DNA polymerases from H.Ep.-2 cells, KB cells, or mouse embryo cells; or DNA polymerases from HSV-1 or HSV-2-infected cells. All of these enzymes were inhibited by β-araATP at a concentration of 5×10^{-5} M or lower. Therefore, if DNA polymerases and ribonucleotide reductase are the primary sites of action of β-araA, it would appear that α-araA and β-araA do not inhibit cell proliferation and virus replication by the same mechanism. In addition to the results reported here, Derse and Cheng [31], using a sample of α-araATP from the same batch used in this study, found no inhibition of highly purified DNA polymerases-α and β from HeLa cells. In contrast, Müller and his associates [3, 11, 12, 14], also using a sample of α-araATP obtained from us, reported that it was as effective

Table 5. Inhibition by α-ara-8-azaA of the synthesis of formylglycinamide ribonucleotide in azaserine-treated H.Ep.-2 cells*

Concn of α-ara-8-azaA (M)	^{14}C Present in formylglycinamide derivatives (% of control)
3.7×10^{-6}	127
7.4×10^{-6}	119
22×10^{-6}	68
37×10^{-6}	66
74×10^{-6}	37
220×10^{-6}	34

* To suspension cultures of H.Ep.-2 cells (~4×10^7 cells in 100 ml of medium) asaserine was added to a concentration of 58 μM, followed 30 min later by α-ara-8-azaA. Thirty minutes after addition of nucleoside, 25 μCi (3.8 μmoles) of sodium [14C]formate was added. Two hours after addition of labeled formate, the cells were harvested and assayed by paper chromatography and autoradiography (see text). The formylglycinamide derivatives include the ribonucleotide, the ribonucleoside, and ribonucleoside polyphosphates. Results are for duplicate assays of a single experiment.

as β-araATP, or more so, as an inhibitor of DNA polymerase-α from rabbit kidney cells. We cannot explain this conflicting result from Müller's laboratory other than to speculate that it may be due to a peculiarity of the rabbit kidney enzyme or may reflect some differences in conditions of assay. The bulk of the evidence, consisting of observations in three different laboratories with polymerases from four different types of cells, would appear to be against inhibition of DNA polymerases being a site of action of α-araA. It is to be noted that Müller *et al.* found with HSV-2, as did we with both HSV-1 and HSV-2, that α-araATP did not inhibit the virus-specific polymerase [14].

Study of the effects of α-ara-8-azaA on the incorporation of precursors into macromolecules (Table 4) showed inhibition of utilization of formate, thymidine, and uridine, with formate representing the strongest inhibition. Further study with formate (Table 5) showed that α-ara-8-azaA inhibited an early site on the pathway of synthesis of purine nucleotides, an action produced by many other purine and nucleoside analogs [28, 29]. It may be, then, that inhibition of DNA and RNA synthesis, as measured by utilization of thymidine and uridine, simply reflects inhibition of synthesis of purine nucleotides required for RNA and DNA synthesis. These results with α-ara-8-azaA differ from those of Müller *et al.* [3] with α-araA. These investigators found α-araA to inhibit thymidine incorporation selectively and to be incorporated in small amounts into DNA of L5178Y cells. In contrast, we failed to detect incorporation of [8-^{14}C]-α-ara-8-azaA into polynucleotides [2]. Thus, although it would be expected that such closely related compounds as α-araA and its 8-aza analog would have the same mechanism of action, our results with α-ara-8-azaA, unlike those of Müller *et al.* with α-araA, provide no evidence for selective effects on DNA synthesis.

These studies have also demonstrated the failure of these α-arabinosyl compounds to inhibit lethal HSV-1 infections in mice at nontoxic doses, despite the earlier finding of antiviral activity *in vitro* [1, 32].

The fact that this study has failed to reveal common sites of action for β-araA and α-araA (or α-ara-8-azaA) raises anew the question as to the biochemical bases for the cytotoxicity of these α-nucleosides. Since nucleosides in the "unnatural" α-configuration are generally considered to be biologically inert, the mechanisms of action of the α-arabinosyl derivatives are of some importance for an understanding of the relationship of structure to the biological activities of nucleoside analogs. Although the results reported herein and in our earlier study [2] do not define the mode of action of α-ara-8-azaA, they are consistent, as far as they go, with this agent functioning as a ribonucleoside analog, rather than as a deoxyribonucleoside analog; thus, it is a good substrate for adenosine kinase [2] and a moderately good inhibitor of purine synthesis *de novo* (Table 5).

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