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In Vitro Biological Activity of 9-β-D-Arabinofuranosyl-2-Fluoroadenine and the Biochemical Actions of Its Triphosphate on DNA Polymerases and Ribonucleotide Reductase from HeLa Cells

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SUMMARY

9-β-D-Arabinofuranosyl-2-fluoroadenine (2-F-araA) inhibited the growth *in vitro* of HeLa cells by 50% at a concentration of 0.25 μM and depressed the replication of herpes simplex virus Types 1 and 2 by 99% at 25 μM. The analogue served as a substrate for cytoplasmic but not mitochondrial deoxycytidine (dCyd) kinase partially purified from human peripheral chronic lymphocytic leukemic blast cells. The K_m values of dCyd and 2-F-araA for the cytoplasmic enzyme were 5 μM and 213 μM, respectively. However, at concentrations of 0.4 mM, the analogue was phosphorylated 2.9 times faster than dCyd. The 5′-triphosphate of 2-F-araA was examined for its biochemical effects on partially purified ribonucleotide reductase and highly purified DNA α- and β-polymerases from HeLa cells. 2-F-araATP was a potent inhibitor of ribonucleotide reductase; the concentration required for 50% inhibition of ADP reduction (0.3 mM ADP; 5 mM GTP or dGTP) was 1 μM and for CDP reduction (0.15 mM CDP; 5 mM ATP) was 8.5 μM. Furthermore, 2-F-araATP was a competitive inhibitor (K_i = 1.2 μM) with respect to dATP (K_m = 3.8 μM) of DNA α-polymerase, whereas DNA β-polymerase was relatively insensitive to the drug. The results suggest that the cytotoxic actions of 2-F-araA may be due, in part, to a "self-potentiating" inhibition of DNA synthesis. That is, by inhibiting the formation of competing dATP, 2-F-araATP may potentiate its inhibition of DNA synthesis.

INTRODUCTION

2F-araA[3] is known to be cytotoxic to cells in culture and to exhibit antitumor activity in mice (1–3). Unlike araA, 2-F-araA was a poor substrate for adenosine deaminase (3). It was phosphorylated to the corresponding 5′-triphosphate in L1210 cells in culture and *in vivo*, and it inhibited DNA synthesis (2–4). Results of previous work demonstrated that the first step in the activation of 2-F-araA in L1210 cells was its phosphorylation to 2-F-araAMP by deoxycytidine kinase (1). Utilizing highly purified enzymes from human leukemic blast cells we have now examined 2-F-araA as a substrate for cytoplasmic dCyd kinase and for mitochondrial pyrimidine deoxynucleoside kinase. In the present study we also report the action of 2-F-araATP on two key enzymes of DNA synthesis, namely ribonucleotide reductase and DNA polymerase. A preliminary report of this work has been presented (5). Antiherpes virus activity of 2-F-araA was assessed in view of the known antiviral activity of araA (6).

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade or better. Nucleosides, nucleoside di- and triphosphates, deoxynucleoside triphosphates, and calf thymus DNA were purchased from Sigma Chemical Company (St. Louis, Mo.). AraATP was purchased from P-L Biochemicals (Milwaukee, Wisc.). We are indebted to Dr. J. A. Montgomery, Southern Research Institute, for 2-F-araA and 2-F-araAMP and to Dr. Robert Naylor, P-L Biochemicals, for phosphorylation of 2-F-araAMP to 2-F-araATP. [^{14}C]CDP, [^{14}C]ADP, and [^{3}H]dATP were purchased from Amersham/Searle Corporation (Arlington Heights, Ill.).

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[3] The abbreviations used are: 2-F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; araA, 9-β-D-arabinofuranosyladenine; 2-F-araATP, the 5′-triphosphate of 9-β-D-arabinofuranosyl-2-fluoroadenine; HSV-1 and HSV-2, herpes simplex virus Types 1 and 2; dCyd, deoxycytidine; dPyd, pyrimidine deoxynucleoside; I_{50}, concentration for 50% inhibition.

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[γ-^{32}P]ATP was obtained from New England Nuclear Corporation (Boston, Mass.).

Cells and Viruses

The antiviral activity of 2-F-araA against HSV-1 (KOS strain) and HSV-2 (333 strain) *in vitro* and its cytotoxicity to HeLa S3 cells in culture were determined by procedures described previously (7).

For ribonucleotide reductase and DNA polymerase preparations, HeLa S3 cells were cultured in spinner flasks with RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (50 units/ml). Cells were harvested in the log phase of growth, collected by centrifugation, and rinsed with phosphate-buffered saline (0.14 M NaCl, 4.0 mM KCl, 0.5 mM $NaHPO_4$, 0.15 mM $KHPO_4$).

Enzyme Purifications

Deoxycytidine kinase. The procedures used for purifying the cytoplasmic and mitochondrial dCyd kinases from peripheral blast cells of patients with chronic lymphocytic leukemia have been described previously (8). The specific activity of the final cytoplasmic enzyme preparation used was 3.4×10^4 units/mg or higher.

Ribonucleotide reductase. The procedure used for partial purification of ribonucleotide reductase from human cell lines has been described previously (9). Purification involved streptomycin sulfate precipitation and ammonium sulfate fractionation. The enzyme activity was found in the 35–50% ammonium sulfate fraction. The specific activity of the final preparation used was 120 pmoles of CDP reduced/min/mg of protein.

DNA polymerases. Purification of HeLa DNA α- and β-polymerases were performed as previously described (10). α-Polymerase was purified to a specific activity of 8×10^3 units/mg; the specific activity of β-polymerase was 1.4×10^4 units/mg.

Enzyme assays. Conditions for the assay of dCyd kinase have been described previously (8). For determination of K_m and V_{max} values the modified procedures of Doberson and Greer (11) were used, which measure the transfer of ^{32}P from [γ-^{32}P]ATP to substrate, dCyd, or 2-F-araA. One unit of kinase activity is defined as the amount of enzyme catalyzing the phosphorylation of 1 nmole of dCyd per minute under the conditions specified.

The conditions for the assay of CDP and ADP reductases have been described earlier (9). CDP reductase activity was determined by the method of Steeper and Steuart (12) with the use of Dowex 1-borate ion-exchange chromatography for separation of deoxy- and ribonucleotides. ADP reductase activity was determined by the method of Cory *et al.* (13). An enzyme sample heated for 2 min in a boiling water bath prior to the addition of the labeled substrate served as the reaction blank. The incubation was carried out at 37° for 60 min, and the reaction was linear with respect to time and enzyme concentration during this incubation period. ATP (5 mM) was included in the assay as an activator of CDP reductase; GTP or dGTP (5 mM) was included as an activator of ADP reductase.

DNA α- and β-polymerase activities were measured as previously described (10). The α-polymerase assay mixture contained in 0.1 ml: 50 mM Tris-HCl (pH 8.5); 7.5 mM $MgCl_2$; heat-inactivated bovine serum albumin (0.5 mg/ml); 0.5 mM dithiothreitol; 100 μM each of dTTP, dCTP, and dGTP; 10 μM [^{3}H]dATP (5 μCi/ml); and activated calf thymus DNA (240 μg/ml). The β-polymerase assay mixture was identical with α-polymerase except that KCl was present at a concentration of 0.1 M. One unit of polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmole of [^{3}H]dAMP per hour into acid-precipitable product under the conditions described.

Protein determination. Protein concentrations were determined by the method of Bradford (14), using bovine serum albumin as the standard.

RESULTS

Cytotoxicity and antiviral activity. 2-F-AraA was inhibitory to the growth of HeLa cells in culture, as shown in Fig. 1A. The ID_{50} was determined to be 0.25 μM under the conditions described. The drug was less effective against the replication of HSV-1 and HSV-2 (Fig. 1B) and was inhibitory only at concentrations that were toxic to host cells (5 μM).

Phosphorylation by dCyd kinase. As compared with dCyd at a concentration of 0.4 mM, 2-F-araA (0.4 mM) was a good substrate for the cytoplasmic dCyd kinase but was a poor substrate for the mitochondrial dPyd kinase (data not shown). Utilizing the method of Doberson and Greer (11), we measured the rates of cytoplasmic dCyd kinase-catalyzed phosphorylation at varied concentrations of dCyd and 2-F-araA. The data are presented in the form of a Lineweaver-Burk plot in Fig. 2;

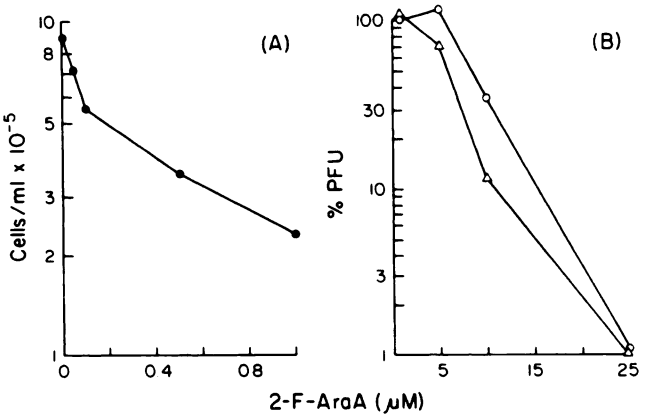

FIG. 1. *Effects of 2-F-araA on HeLa cells and HSV-1 and HSV-2*

A. Inhibition of HeLa cell growth by 2-F-araA. Exponentially growing HeLa cells (2×10^5/ml) were continuously exposed to the indicated concentrations of 2-F-araA. At 47 hr the fraction of surviving cells was determined by counting the number of cells which excluded trypan blue dye.

B. Inhibition of HSV-1 and HSV-2 replication of 2-F-araA. The yields of HSV-1 (O——O) and HSV-2 (Δ——Δ), expressed as percentage of control, are plotted versus the indicated concentrations of 2-F-araA. Virus-infected HeLa BU cells were exposed to the drug from the time of infection to 28 hr after infection, at which time the cultures were placed at −70° until subsequent virus titration. Control plaque-forming units (*PFU*) for HSV-1 and HSV-2 were 2×10^6/ml and 2×10^7/ml, respectively.

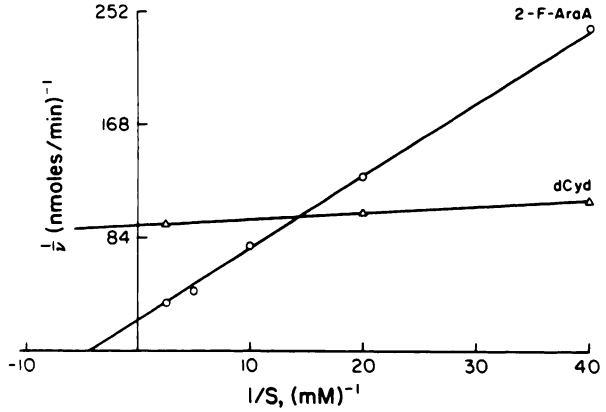

FIG. 2. *Phosphorylation of dCyd and 2-F-araA by cytoplasmic dCyd kinase*

Phosphorylation was measured by the transfer of ^{32}P from [γ-^{32}P] ATP to varied concentrations of dCyd (Δ——Δ) or 2-F-araA (○——○). The data are presented as a Lineweaver-Burk plot.

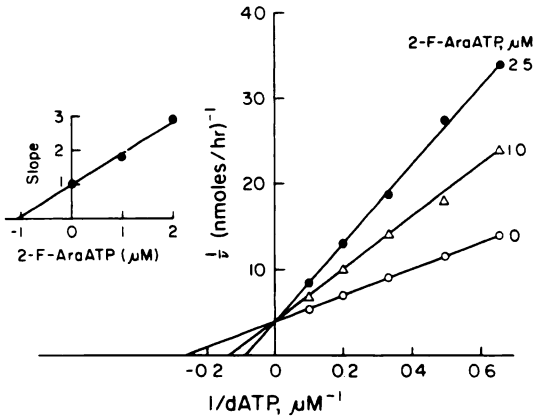

FIG. 4. *Competitive inhibition of HeLa DNA α-polymerase by 2-F-araATP*

HeLa α-polymerase reactions were determined as described under Experimental Procedures at variable concentrations of [^{3}H]dATP and 0 (○——○), 1.0 μM (Δ——Δ), and 2.5 μM (●——●) 2-F-araATP. The data are presented as a Lineweaver-Burk plot. The *inset* shows the slope replot used to determine the K_i value of 2-F-araATP.

only a portion of the data for dCyd are shown for comparative purposes. 2-F-araA had a K_m value of 213 μM, compared with 5 μM for dCyd. However, the maximal velocity of 2-F-araA phosphorylation was approximately 4 times greater than dCyd.

Effect of 2-F-AraATP on HeLa DNA Polymerases. The inhibition of HeLa DNA α- and β-polymerases by 2-F-araATP is shown in Fig. 3. At identical deoxynucleoside triphosphate concentrations, 2-F-araATP was much more inhibitory to DNA α-polymerase than to β-polymerase. Detailed kinetic studies were performed with DNA α-polymerase at fixed concentrations of 2-F-araATP and varied concentrations of dATP. The data are displayed in the Lineweaver-Burk plot shown in Fig. 4. 2-F-araATP competitively inhibited the incorporation of dAMP into DNA. The K_m and K_i values of dATP and 2-F-araATP were 3.8 μM and 1.2 μM, respectively. 2-F-araATP was noncompetitive with respect to dTTP, dCTP, or dGTP (data not shown).

Action of 2-F-AraATP on ribonucleotide reductase. Figure 5A and B demonstrate that 2-F-araATP strongly inhibited ADP and CDP reductase activities with I_{50} values of 1 μM and 8.5 μM, respectively. By comparison, araATP was a weaker inhibitor for both types of ribonucleotide reduction. The I_{50} values of araATP and dATP were 28 μM and 4 μM, respectively, for ADP reduction, with an activator of either GTP or dGTP.

DISCUSSION

2-F-AraA was a potent inhibitor of HeLa cell growth in culture. Unlike araA, which is a selective antiherpes virus agent, 2-F-araA inhibited herpes simplex virus replication only at concentrations which were cytotoxic to HeLa cells. It is therefore unlikely that this compound could be used as an antiviral agent; however, it is conceivable that cancer patients being treated with 2-F-araA may have a lower incidence of herpes simplex virus infections. The differential effects of the drug on cell and virus growth reflect either that the biochemical environment of the cell is grossly altered after virus infection or that the sites of action responsible for its toxicity differ.

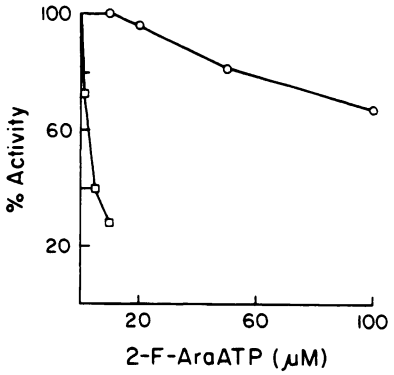

FIG. 3. *Inhibition of HeLa DNA α- and β-polymerase by 2-F-araATP*

HeLa DNA α-polymerase (□——□) and β-polymerase (○——○) reactions were determined as described under Experimental Procedures in the presence of 1 μM [^{3}H]dATP and the indicated concentrations of 2-F-araATP. Activity is expressed as percentage of control reactions performed in the absence of 2-F-araATP.

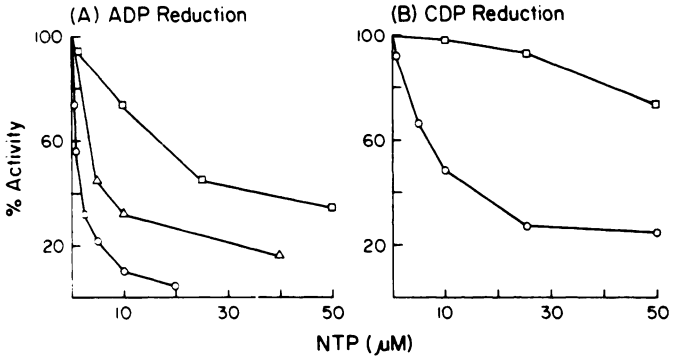

FIG. 5. *Inhibition of HeLa cell ribonucleotide reductase by 2-F-araATP*

ADP (A) and CDP (B) reductase activity was measured as described under Experimental Procedures. The percentage activity remaining is plotted versus the indicated concentrations of 2-F-araATP (○——○), dATP (Δ——Δ), and araATP (□——□).

The behavior of 2-F-araA toward both enzyme species in human cells responsible for the phosphorylation of dCyd revealed that cytoplasmic dCyd kinase, but not mitochondrial dPyd kinase, utilized 2-F-araA as a substrate. The kinetic affinity of the cytoplasmic enzyme for 2-F-araA was lower than for dCyd, but the maximal velocity of phosphorylation was greater for the drug. This could account for the efficiency of the phosphorylation of 2-F-araA in cells. The lack of the phosphorylation by mitochondrial dPyd kinase suggests that this compound may inhibit only nuclear DNA synthesis.

2-F-AraATP was examined for its effects on both major types of nuclear DNA polymerase. It was a potent inhibitor of HeLa DNA α-polymerase, the replicative DNA polymerase, and was competitive with respect to dATP; HeLa β-polymerase was relatively insensitive to the drug. 2-F-araATP also was inhibitory to DNA α-polymerase from L1210 leukemia cells (K_i = 11 μM); DNA β-polymerase was much less sensitive (15).

Since dATP and araATP are known inhibitors of ribonucleotide reductase (16), 2-F-araATP was compared with these compounds as inhibitors of partially purified ribonucleotide reductase from HeLa cells. It was found to be more potent than dATP and araATP as an inhibitor of CDP and ADP reduction. Similar results were obtained in studies of inhibition of ribonucleotide reductase prepared from human epidermoid carcinoma cells and from L1210 leukemia cells (15). Further purification of this enzyme is required before detailed kinetic studies are conducted. The inhibition by 2-F-araATP of DNA α-polymerase and ribonucleotide reductase suggests that 2-F-araATP may exert its action through a "self-potentiation" mechanism by decreasing dATP formation.

REFERENCES

1. Brockman, R. W., Y.-C. Cheng, F. M. Schabel, Jr., and J. A. Montgomery. Metabolism and chemotherapeutic activity of 9-β-D-arabinofuranosyl-2-fluoroadenine against murine leukemia L1210 and evidence for its phosphorylation by deoxycytidine kinase. *Cancer Res.* **40**:3610–3615 (1980).
2. Dow, L. W., D. E. Bell, L. Poulakos, and A. Fridland. Differences in metabolism and cytotoxicity between 9-β-D-arabinofuranosyladenine and 9-β-D-arabinofuranosyl-2-fluoroadenine in human leukemic lymphoblasts. *Cancer Res.* **40**:1405–1410 (1980).
3. Brockman, R. W., F. M. Schabel, Jr., and J. A. Montgomery. Biological activity of 9-β-D-arabinofuranosyl-2-fluoroadenine, a metabolically stable analog of 9-β-D-arabinofuranosyladenine. *Biochem. Pharmacol.* **26**:2193–2196 (1977).
4. Cheng, Y.-C., J. Tsou, and R. W. Brockman. Enzyme activity responsible for the phosphorylation of 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) to F-araAMP in L1210 cells. *Fed. Proc.* **38**:370 (1979).
5. Tseng, W.-C., D. Derse, Y.-C. Cheng, R. W. Brockman, and L. L. Bennett, Jr. Self-potentiation mechanisms of action of 9-β-D-arabinofuranosyl-2-fluoroadenine. *F. A. S. E. B.* **2497**:98 (1981).
6. Keeney, R. Z., and R. A. Buchanan. Clinical application of adenine arabinoside. *Ann. N. Y. Acad. Sci.* **255**:185–189 (1975).
7. Cheng, Y.-C., B. Domin, R. A. Sharma, and M. Bobek. Antiviral action and cellular toxicity of four thymidine analogues: 5-ethyl, 5-vinyl-, 5-propyl-, and 5-allyl-2′-deoxyuridine. *Antimicrob. Agents Chemother.* **10**:119 (1976).
8. Cheng, Y.-C., B. Domin, and L.-S. Lee. Human deoxycytidine kinase purification and characterization of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemic patients. *Biochim. Biophys. Acta* **481**:481–492 (1977).
9. Chang, C.-H., and Y.-C. Cheng. Demonstration of two components and dissociation of adenosine diphosphate-cytidine diphosphate reductase from cultured human lymphoblast cells (Molt-4F). *Cancer Res.* **39**:436–442 (1979).
10. Derse, D., Y.-C. Cheng, P. A. Furman, M. H. St. Clair, and G. B. Elion. Inhibition of purified human and herpes simplex virus-induced DNA polymerases by 9-(2-hydroxyethoxymethyl)guanine triphosphate. *J. Biol. Chem.* **256**:11447–11451 (1981).
11. Doberson, M. J., and S. Greer. An assay for pyrimidine deoxyribonucleoside kinase using γ-^{32}P-labelled ATP. *Anal. Biochem.* **67**:602–610 (1975).
12. Steeper, J. R., and C. D. Steuart. A rapid assay for reductase activity in mammalian cell extracts. *Anal. Biochem.* **34**:123–130 (1970).
13. Cory, J. G., F. A. Russell, and M. M. Mansell. A convenient assay for ADP reductase activity using Dowex-Bovante columns. *Anal. Biochem.* **55**:449–4456 (1973).
14. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
15. White, L., S. C. Shaddix, Y.-C. Cheng, B. W. Brockman, and L. L. Bennett, Jr. Inhibition of ribonucleotide reductase and DNA polymerases of tumor cells by 9-β-D-arabinfuranosy-2-fluoroadenine 5′-triphosphate. *Proc. Am. Assoc. Cancer Res.* **22**:33 (1981).
16. Chang, C.-H., and Y.-C. Cheng. Effects of deoxyadenosine triphosphate and 9-β-D-arabinofuranosyladenine 5′-triphosphate on human ribonucleotide reductase from Molt-6F cells and the concept of "self potentiation." *Cancer Res.* **40**:3555–3558 (1980).

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