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Action of 9-β-D-Arabinofuranosyl-2-fluoroadenine on RNA Metabolism

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SUMMARY

The action of 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) on RNA metabolism was evaluated both in whole cells and in cell-free systems. F-ara-A was converted to its 5′-triphosphate, F-ara-ATP, in cells and then incorporated into RNA as well as DNA. F-ara-A inhibited RNA synthesis in cultured cells in a concentration-dependent manner. This inhibition was mediated mainly by F-ara-ATP. Experiments using isolated nuclei demonstrated that RNA polymerases I, II, and III accounted for 24, 73, and 3% of the total RNA synthesis activity, respectively. About 88% of the total inhibition was attributed to the suppression of RNA polymerase II activity. In cultured cells, F-ara-A was preferentially incorporated into the poly(A)$^+$ RNA fraction. Approximately 78% of the incorporated F-ara-A monophosphate residues were located at the terminal position of the RNA chain. The incorporation of F-ara-A monophosphate into mRNA resulted in premature termination of the RNA transcript and impaired its functioning as a template for protein synthesis. The inhibitory action of F-ara-A on RNA metabolism is a unique property of this compound, differing from the action of arabinosylcytosine and arabinosyladenine.

F-ara-A is a derivative of ara-A. The use of ara-A as an antitumor agent is limited, due to its rapid conversion to arabinosylhypoxanthine by adenosine deaminase (1, 2). In contrast, F-ara-A is resistant to inactivation by adenosine deaminase and thus overcomes this drawback of ara-A as a therapeutic agent (3, 4). Studies with murine models have demonstrated that F-ara-A is effective in therapy of mice bearing L1210 leukemia (3, 5) and P388 lymphocytic leukemia (6). Clinical investigations have demonstrated that this compound is also effective in the treatment of indolent lymphocytic neoplasms (7, 8).

After being transported into cells, F-ara-A is converted to the active 5′-triphosphate, F-ara-ATP, by cellular enzymes. F-ara-ATP is then incorporated into both DNA and RNA (9, 10). We and others have demonstrated that F-ara-ATP competed with dATP for incorporation into the A sites of the elongating DNA strand and terminated further DNA synthesis (11, 12). This activity may be potentiated by the inhibition of ribonucleotide reductase by F-ara-ATP, an action that decreases the value of dATP/F-ara-ATP and may result in greater F-ara-AMP incorporation into DNA (11, 12). Results from studies in CEM and HL-60 cells indicate a correlation between the loss of clonogenicity and the incorporation of F-ara-A into DNA (10, 11).

The ability to incorporate into RNA is one of the properties of F-ara-A that differ from those of ara-A, which is incorporated exclusively into DNA (13–15). The biological consequences of F-ara-A on RNA metabolism, however, remain unknown. Although Brockman *et al.* (3) showed that 4–40 μM F-ara-A did not inhibit the incorporation of [^{3}H]uridine into RNA in L1210 cells, studies in CEM cells showed that RNA synthesis was affected by 100 μM F-ara-A (4). Subsequently, little additional information has become available concerning the possible action of F-ara-ATP on RNA metabolism. The present project was conducted to investigate the action of F-ara-ATP on RNA synthesis and function, both in intact cells and in cell-free systems. Our studies showed that F-ara-A was preferentially incorporated into the poly(A)$^+$ RNA fraction in cultured cells. This action resulted in a premature termination of RNA transcripts and impaired the ability of RNA to function as a template for protein synthesis.

Experimental Procedures

Materials. F-ara-A was supplied by Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. F-ara-ATP was chemically synthesized in our laboratory by Dr. Alina Sen, using a described procedure (16). F-[8-^{3}H]ara-A (specific activity, 11 Ci/mmol) was from Moravek Biochemicals,

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ABBREVIATIONS: F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-ATP, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate; ara-A, 9-β-D-arabinofuranosyladenine; DHFR, dihydrofolate reductase; F-ara-AMP, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate; HPLC, high pressure liquid chromatography.

Inc. (Brea, CA). [5-^{3}H]Uridine and [α-^{32}P]UTP were purchased from ICN Radiochemicals (Irvine, CA). Spleen phosphodiesterase was from Sigma Chemical Co. (St. Louis, MO), and micrococcal nuclease was from Worthington Biochemical Corp. (Freehold, NJ). A reticulocyte lysate translation system employing [^{35}S]methionine was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade or of the greatest purity available.

Cell culture. Human T lymphoblastoid CEM cells were maintained in exponential growth in RPMI 1640 suspension culture medium supplemented with 5% fetal bovine serum. The cell number and mean cell volume were determined by a Coulter counter equipped with a model C-1000 particle size analyzer (17). Mouse sarcoma S180 cells were cultured in minimum essential medium (Eagle) supplemented with 10% calf serum. The methotrexate-resistant S180-500R cells were maintained in the same medium plus 500 μM methotrexate.

HPLC analysis of nucleosides and nucleotides. Cells were incubated with various concentrations of F-ara-A for 5 hr, washed with cold phosphate-buffered saline, and then extracted with 0.4 N $HClO_4$ (4). The amounts of intracellular F-ara-ATP, nucleoside triphosphates, and deoxynucleoside triphosphates were quantitated by HPLC analysis (17).

Incorporation of F-ara-A into RNA. CEM cells in exponential growth phase were incubated with various concentrations of F-[^{3}H]ara-A for the desired periods. The F-[^{3}H]ara-A was purified by HPLC to greater than 99.9% purity and was diluted with unlabeled F-ara-A to a specific activity of 0.45 Ci/mmol. At the end of the incubation period, the cells were washed twice with cold phosphate-buffered saline, and RNA was isolated by CsCl gradient centrifugation (18) and quantitated by UV absorbance at 260 nm. Poly(A)$^+$ RNA was separated from poly(A)$^-$ RNA by chromatography through a column of oligo(dT)-cellulose (18). The incorporated F-[^{3}H]ara-AMP in the respective nucleic acid fractions was quantitated by liquid scintillation counting.

Enzymatic degradation of RNA to internal nucleotides and 3′-terminal nucleosides. RNA isolated from CEM cells that were labeled with F-[^{3}H]ara-A, [^{3}H]uridine, or [^{3}H]adenosine was dissolved in 200 μl of H_2O and denatured by boiling (2 min) and rapid cooling in an ice bath. The nucleic acids were then degraded to internal nucleotides and 3′-terminal nucleosides by the sequential action of micrococcal nuclease and spleen phosphodiesterase (19). The reaction mixture (0.8 ml) contained 100 μg of RNA, 2 mM $CaCl_2$, 2 mM Tris-acetate (pH 9.0), and 100 units of micrococcal nuclease. After incubation at 37° for 3 hr, the reaction mixture was adjusted to pH 6.0 with HCl, and then 1 unit of spleen phosphodiesterase was added. After incubation at 37° for 3 hr, the reaction was stopped by the addition of 0.4 N $HClO_4$. After centrifugation, neutralization of the supernatant with KOH, and removal of $KClO_4$, the 3′-monophosphates and nucleosides were separated by reverse phase HPLC. Radioactivity was quantitated by liquid scintillation counting.

RNA synthesis in cell culture. Cells in exponential growth phase were treated with various concentrations of drugs for appropriate periods. To determine RNA synthesis activity, [5-^{3}H]uridine was added to the cell culture and incubated for 30 min. The cells were collected on a 25-mm glass fiber disc (pre-wet with 1% sodium pyrophosphate) by filtration and then washed three times with 4 ml of cold 0.4 N $HClO_4$ and twice with 2 ml of ethanol. The radioactivity of RNA retained on the filter disc was determined by liquid scintillation counting. Separation of cellular RNA from DNA by Cs_2SO_4 gradient centrifugation demonstrated that 92 ± 6% of the incorporated radioactivity was in the RNA fraction.

RNA synthesis in isolated nuclei. About 2.5 × 10^7 cells were washed with cold phosphate-buffered saline, and nuclei were prepared according to the method previously described (20). The nuclei were suspended in 100 μl of buffer containing 50 mM Tris·HCl (pH 8.0), 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, and 5 mM dithiothreitol. The activity of RNA synthesis in the isolated nuclei was determined by incubation of the nuclei in a 100-μl reaction mixture, containing 300 μM each of ATP, CTP, and GTP, 50 μM [^{3}H]UTP (1 Ci/mmol), 15 μM *S*-adenosylmethionine, 120 mM KCl, and 2.5 mM magnesium acetate, at 25° for the indicated periods. The reaction mixture was spotted onto a Whatman 3 MM filter and washed with 10% trichloroacetic acid and 1% sodium pyrophosphate at 0°. The radioactivity of RNA retained on the filter was determined by liquid scintillation counting.

The relative activities of RNA polymerases I, II, and III in isolated nuclei were determined by their differential sensitivity to α-amanitin, as described previously (20). RNA polymerase II and polymerase III are inhibited by 1 and 200 μg/ml α-amanitin, respectively, whereas polymerase I is not affected at these concentrations. Thus, the activity of RNA polymerase I was determined by measurement of RNA synthesis activity in the presence of 200 μg/ml α-amanitin. RNA polymerase II activity was calculated by subtraction of the RNA synthesis activity in the presence of 1 μg/ml α-amanitin from the total RNA synthesis activity in isolated nuclei. Likewise, RNA polymerase III activity was determined by subtraction of RNA synthesis activity in the presence of 200 μg/ml α-amanitin from the activity detected in the presence of 1 μg/ml α-amanitin. The effect of F-ara-ATP on RNA polymerases I, II, and III was determined by measurement of the RNA synthesis activity in the isolated nuclei in the presence of appropriate concentrations of α-amanitin and F-ara-ATP and was calculated based on the same principle.

***In vitro* transcription.** SV40 DNA cleaved with *Pst*I was used as the template for RNA transcription *in vitro* (21). A CEM cell lysate with transcription activity was prepared as described (21). The reaction mixture (50 μl) contained 2 μg of SV40 DNA (*Pst*I cleaved), 0.14 mM EDTA, 500 μM each of ATP, CTP, and GTP, 50 μM [α-^{32}P]UTP, cell lysate, and desired concentrations of drugs. The reaction was incubated at 30° for 60 min and terminated by the addition of 250 μl of 8 M urea, 0.5% sodium dodecyl sulfate, and 10 mM EDTA. The reaction products were extracted with phenol/chloroform, precipitated with ethanol, treated with glyoxal and dimethyl sulfoxide, and analyzed by electrophoresis through a 1.4% agarose gel as described (21).

***In vitro* translation.** Total cellular RNA and poly(A)$^+$ RNA isolated from CEM cells that were treated with F-ara-A or ara-A were used as templates for protein synthesis *in vitro* by a rabbit reticulocyte lysate translation system (New England Nuclear, Boston, MA). The reaction was constituted under conditions recommended by the manufacturer. Each 50-μl reaction mixture contained 20 μl of rabbit reticulocyte lysate, 10 μl of reaction cocktail, 600 μM magnesium acetate, 75 mM potassium acetate, 1.7–8.5 μg of total RNA or 0.25–1.25 μg of poly(A)$^+$ RNA, and 110 μCi of [^{35}S]methionine (1163 Ci/mmol). The [^{35}S]methionine-labeled products were divided into two portions. One portion was precipitated by 10% trichloroacetic acid, washed, and quantitated by liquid scintillation counting. The other portion was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel) and visualized by autoradiography.

Nucleic acid hybridization. A DNA-excess filter hybridization technique (22) was used to measure DHFR mRNA transcription activity. *Escherichia coli* strains containing the recombinant plasmids pDHFR21, pDHS11, pDRB18, pDH5, and pDB20 were kindly provided by Dr. R. Kellems (Baylor College of Medicine). The plasmid DNA was isolated by CsCl gradient centrifugation and immobilized on nitrocellulose filters (10 μg of DNA/25-mm filter disc, type BA85; Schleicher & Schuell Co.), as described (23). The filters were hybridized with [^{3}H]uridine-labeled RNA (which was isolated from S180-500R cells that were treated with or without F-ara-A), washed, digested with ribonuclease A, washed again, and evaluated for radioactivity as described (22).

Results

Incorporation of F-ara-A into RNA. CEM cells incubated with F-[^{3}H]ara-A incorporated a significant amount of radioactivity into the RNA fraction. As depicted in Fig. 1, incorporation of F-ara-AMP into RNA was concentration dependent

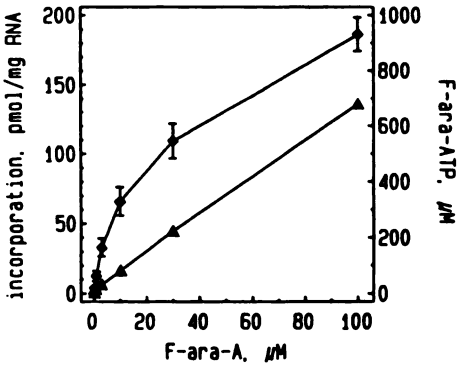

Fig. 1. Formation of intracellular F-ara-ATP and its incorporation into RNA. CEM cells were incubated with the indicated concentrations of either unlabeled F-ara-A or F-[^{3}H]ara-A for 5 hr. The levels of cellular F-ara-ATP and its incorporation into RNA were determined as described in Experimental Procedures. ♦, F-ara-ATP, mean ± standard error of four separate experiments; ▲, incorporation into RNA, average of two determinations.

TABLE 1

Terminal and internal incorporation of F-ara-AMP into RNA in CEM cells

CEM cells were labeled with F-[^{3}H]ara-A or [^{3}H]uridine for 5 hr. RNA was isolated and digested to internal nucleotides and 3′-terminal nucleosides with micrococcal nuclease and spleen phosphodiesterase. The digests were analyzed by HPLC, and the radioactivity associated with the respective nucleotides or nucleosides was quantitated by liquid scintillation counting. Each value represents the mean ± standard deviation of two to four separate experiments.

Sample	Terminal incorporation		Internal incorporation	
	dpm	%	*dpm*	%
F-ara-A				
10 μM	1381 ± 148	78.7	373 ± 44	21.3
30 μM	2034 ± 18	77.9	576 ± 38	22.1
Uridine				
0.5 μCi/ml	398 ± 38	4.9	7751 ± 121	95.1

at F-ara-A concentrations between 1 and 100 μM. The amount of incorporated F-ara-AMP in RNA increased in parallel with intracellular F-ara-ATP levels. Incubation of CEM cells with 100 μM F-ara-A for 5 hr led to an accumulation of 930 μM cellular F-ara-ATP and an incorporation of 138 pmol of F-ara-AMP/mg of RNA. This result contrasts with its incorporation into DNA, which is self-limited at greater than 10 μM F-ara-A (11). HPLC analysis of the enzymatically degraded products of the labeled RNA confirmed that 98.9 ± 1.0% of the total incorporated radioactivity eluted with authentic F-ara-A or F-ara-AMP.

To determine the positions of the F-ara-AMP residues in the RNA, total cellular RNA isolated from CEM cells that were incubated with F-[^{3}H]ara-A was enzymatically degraded to internal nucleotides and terminal nucleosides and analyzed by HPLC. As shown in Table 1, about 78% of the incorporated F-ara-AMP appeared at terminal sites. These results suggest that the F-ara-AMP residues at the 3′ end of the RNA chain cannot be efficiently extended by RNA polymerases; only 22% of the incorporated F-ara-AMP molecules were extended by RNA polymerases, as indicated by their location at internal positions. In a control experiment, cells were incubated with [^{3}H]uridine, and RNA was isolated and degraded under identical conditions. About 5% of the incorporated [^{3}H]uridine was detected as terminal nucleosides, whereas 95% appeared as internal nucleotides, indicating that the assay conditions were appropriate.

The distribution of the incorporated F-ara-AMP residues among different RNA species was investigated further. To elucidate the actions of F-ara-A on specific RNAs, F-[^{3}H]ara-A-labeled RNA isolated from CEM cells was fractionated into poly(A)$^+$ and poly(A)$^-$ RNA, and the specific incorporation (pmol/mg of RNA) in each fraction was determined. As shown in Table 2, F-ara-AMP was preferentially incorporated into poly(A)$^+$ RNA, by a ratio of 12 to 1 over incorporation into poly(A)$^-$ RNA. A similar incorporation ratio was obtained in experiments using S180-500R cells (data not shown). The greater incorporation into poly(A)$^+$ RNA was not due to the rapid turnover rate of this RNA species, because a longer incubation period (20 hr) did not change the ratio. A parallel experiment in which RNA was labeled with [^{3}H]adenosine demonstrated that the incorporation ratio of adenosine was 1.2 to 1, only slightly in favor of poly(A)$^+$ RNA, under identical culture conditions.

In addition to RNA polymerase II, poly(A) polymerase is also required for the synthesis of poly(A)$^+$ RNA. This enzyme adds about 200 AMP residues to the free 3′-OH end of the mRNA. To evaluate the possibility that the high level of incorporation of F-ara-A into poly(A)$^+$ RNA might be due to its incorporation into the poly(A) tail, poly(A)$^+$ RNA labeled with either F-[^{3}H]ara-A or [^{3}H]adenosine was hybridized with an excess amount of poly(dU) and then digested with S1 nuclease, which degraded single-stranded RNA. The poly(A) tails protected by poly(dU) were precipitated with ethanol, and the radioactivity in the supernantants and pellets was quantitated. These experiments demonstrated that 8.2 ± 0.2% (three experiments) of the total incorporated F-[^{3}H]ara-A was located in poly(A) tails; this value was 10.8 ± 0.4% for [^{3}H]adenosine incorporation. Thus, it appeared that F-ara-A was not preferentially incorporated into the poly(A) tail by poly(A) polymerase.

Inhibition of RNA synthesis. To determine the action of F-ara-ATP on RNA synthesis, cells were incubated with various concentrations of F-ara-A for 5 hr, to accumulate different levels of cellular F-ara-ATP, and RNA synthesis activity was determined by pulsing with [^{3}H]uridine. As shown in Fig. 2, RNA synthesis activity decreased as the cellular F-ara-ATP level increased, suggesting that F-ara-ATP is involved in the inhibition of RNA synthesis. In contrast, incubation with ara-A, an analogue of deoxyadenosine that is not incorporated into RNA (13–15), did not affect RNA synthesis (data not shown).

TABLE 2

Preferential incorporation of F-ara-A into poly(A)$^+$ RNA in CEM cells

Exponentially growing CEM cells were labeled with the indicated concentrations of either F-[^{3}H]ara-A or [^{3}H]adenosine for the indicated time periods. Total cellular RNA was isolated and poly(A)$^+$ RNA was separated from poly(A)$^-$ RNA. RNA was quantitated by UV spectrophotometry, and the incorporated radioactivity was detected by liquid scintillation counting. The numbers in parentheses indicate the amount of RNA (μg/10^8 cells) isolated in the experiments.

Sample	Incorporation into RNA		Ratio of (A)$^+$/(A)$^-$
	Poly(A)$^+$	Poly(A)$^-$	
	pmol/mg of RNA		
F-ara-A			
10 μM, 6 hr	212.3 (25.2)	17.6 (1,074)	12.1
10 μM, 20 hr	305.5 (20.9)	23.0 (1,012)	13.3
20 μM, 20 hr	510.2 (17.3)	45.6 (901)	11.2
	dpm/μg of RNA		
Adenosine			
0.5 μCi/ml, 20 hr	44,200 (45.7)	36,977 (1,035)	1.20
1.0 μCi/ml, 20 hr	50,797 (50.4)	40,505 (1,197)	1.25
2.0 μCi/ml, 20 hr	72,315 (51.1)	63,819 (994)	1.13

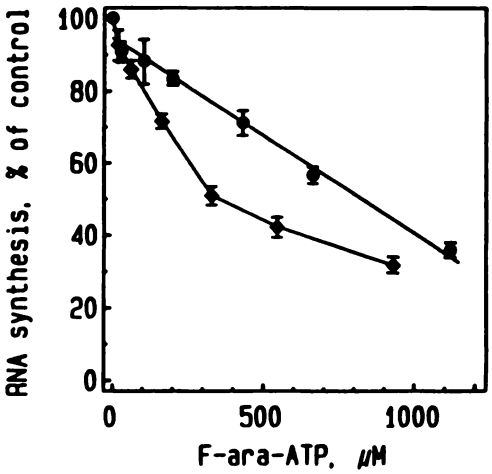

Fig. 2. Action of F-ara-ATP on RNA synthesis in CEM cells and isolated nuclei. CEM cells were incubated with various concentrations of F-ara-A to accumulate different levels of cellular F-ara-ATP. At 4.5 hr, [^{3}H]uridine was added to the culture, which was incubated for an additional 30 min. The incorporation of [^{3}H]uridine into RNA and cellular F-ara-ATP levels were determined as described in Experimental Procedures. RNA synthesis activity was plotted against cellular F-ara-ATP (♦) (control value, 1.09 × 10^5 dpm/10^6 cells); the action of F-ara-ATP on RNA synthesis in isolated nuclei was determined as described in Experimental Procedures (●) (control value, 5268 dpm/filter). Each *point* is the mean ± standard deviation of three separate experiments.

HPLC analysis of the cell extracts demonstrated no change in the specific activity of cellular [^{3}H]UTP after treatment with either F-ara-A or ara-A.

To examine the direct effect of F-ara-ATP on RNA transcription, nuclei were isolated from CEM cells and incubated with 300 μM each of ATP, CTP, and GTP, [^{3}H]UTP, and various concentrations of F-ara-ATP. As demonstrated in Fig. 2, the inhibition of RNA synthesis in isolated nuclei was linearly correlated with F-ara-ATP concentration (r = 0.993), suggesting a direct inhibitory action of F-ara-ATP on the process of RNA transcription. About 850 μM F-ara-ATP was required to inhibit RNA synthesis by 50% in isolated nuclei. In whole cells, however, the same degree of inhibition was achieved by incubation with 10 μM F-ara-A for 5 hr, which resulted in an accumulation of 330 μM cellular F-ara-ATP. This difference suggests that F-ara-A might act by additional mechanisms to inhibit RNA synthesis in whole cells. Quantitation of cellular ribonucleoside triphosphate revealed that 10 μM F-ara-A caused a decrease of the ATP pool to 70% during a 5-hr incubation period, whereas cellular CTP, GTP, and UTP levels remained unchanged (data not shown). Possible compartmentation of the cellular F-ara-ATP pool might also account for the apparently more potent inhibition on cellular RNA synthesis.

To evaluate the effect of F-ara-A on the synthesis of different RNA species, CEM cells were incubated with 30 μM F-ara-A for 6 hr, and RNA was isolated. Poly(A)$^+$ RNA was separated from poly(A)$^-$ RNA and quantitated as described in Experimental Procedures. The newly synthesized poly(A)$^+$ RNA and poly(A)$^-$ RNA, as measured by [^{3}H]uridine incorporation, were inhibited by 54 and 49%, respectively. Absolute cellular content of poly(A)$^+$ RNA decreased from 47.2 μg/10^8 cells to 25.2 μg/10^8 cells (53% of the control) during the 6-hr incubation period. However, poly(A)$^-$ RNA remained unchanged by the end of the 6-hr incubation (control, 1035 μg/10^8 cells; F-ara-A-treated, 1074 μg/10^8 cells), probably due to its greater stability.

Mammalian RNA polymerases I, II, and III are the enzymes responsible for the synthesis of rRNA, mRNA, and tRNA, respectively (24). We employed α-amanitin to evaluate the action of F-ara-ATP on the activity of these RNA polymerases in isolated nuclei. The relative enzyme activities were 24, 73, and 3% for RNA polymerases I, II, and III, respectively (Table 3). Incubation with 850 μM F-ara-ATP, the concentration that reduced [^{3}H]uridine incorporation by 50% in isolated nuclei (Fig. 2), inhibited total cellular RNA synthesis by 47% in this assay system. RNA polymerase I and polymerase II were inhibited by 24 and 56%, respectively, but no detectable inhibition of polymerase III was observed. Under these experimental conditions, F-ara-ATP reduced the total RNA synthesis activity by 5,909 dpm (from 12,748 dpm to 6,839 dpm). The inhibition of RNA polymerase II activity by 5,224 dpm (from 9,280 dpm to 4,050 dpm) represented 88% of the total inhibition.

Partial termination of RNA chains. A eukaryotic transcription system with SV40 DNA as a template (21) was used to further study the action of F-ara-ATP on RNA synthesis. Cordycepin triphosphate (3′-dATP), a nucleotide analogue that is incorporated into RNA and that terminates RNA chain elongation (25), was used in a parallel experiment for comparison. Fig. 3 illustrates the effects of F-ara-ATP and 3′-dATP (500 μM each) on the *in vitro* RNA synthesis by HeLa cell transcription lysate (Bethesda Research Laboratories) in the presence of 500 μM each of four nucleoside triphosphates. The amount of high molecular weight RNA transcripts (>6 kilobases) was substantially reduced, whereas the smaller size RNAs (<6 kilobases) were only slightly affected. Densitometric scanning showed that F-ara-ATP reduced the high molecular weight and small size RNAs to 45 and 85% of the control, respectively. In the reaction with 3′-dATP, the high molecular weight RNA and small size RNA were 35 and 93% of the control, respectively. The same effect was observed in experiments using transcription lysates prepared from CEM cells (data not shown). These results support the hypothesis that F-ara-ATP acted as a partial RNA chain terminator, as indicated by the incorporation experiments in whole cells (Table 1). Such a mechanism suggests that the probability of F-ara-AMP incorporation increased as the RNA transcripts lengthened; thus, synthesis of the high molecular weight RNA chains was decreased.

The action of F-ara-A on the transcription of a specific mRNA was evaluated in intact cells. The mouse sarcoma S180-500R cell line, which has an amplified expression of DHFR

TABLE 3

Action of F-ara-ATP on RNA polymerase activities in isolated nuclei

Nuclei were isolated from CEM cells, and the enzyme activities of RNA polymerases I, II, and III were determined as described in Experimental Procedures. The relative activity of each RNA polymerase and the extent of inhibition by 850 μM F-ara-ATP were calculated based on the differential action of α-amanitin on RNA polymerases. Each value represents the mean ± standard error of two separate experiments, with triplicate samples.

RNA polymerase	Enzyme activity			
	Control		+850 μM F-ara-ATP	
	dpm/disc	%	*dpm/disc*	*% of control*
Total	12,748 ± 860	100	6,839 ± 719	53
I	3,050 ± 513	24	2,317 ± 673	76
II	9,280 ± 528	73	4,056 ± 25	44
III	418 ± 38	3	466 ± 73	111

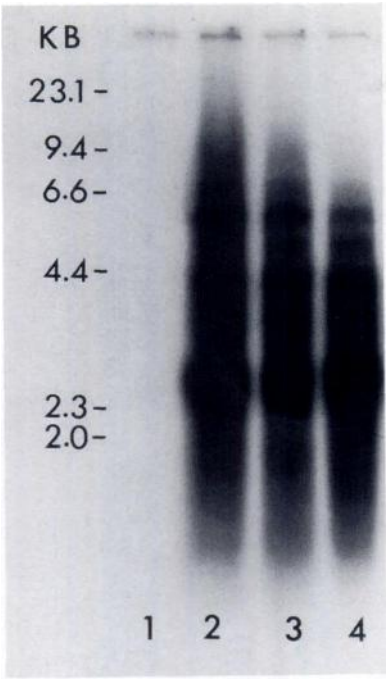

Fig. 3. Termination of RNA transcription by F-ara-ATP and 3′-dATP. The ability of HeLa cell lysates to transcribe SV40 DNA was examined in the presence of F-ara-ATP or 3′-dATP, as described in Experimental Procedures. An equal volume of the reaction mixture was processed and loaded onto a 1.4% agarose gel. *Lane 1*, reaction with HeLa cell lysate without exogenous SV40 DNA; *lane 2*, HeLa cell lysate with SV40 DNA; *lane 3*, same as *lane 2* plus 0.5 mM F-ara-ATP; *lane 4*, same as *lane 2* plus 0.5 mM 3′-dATP.

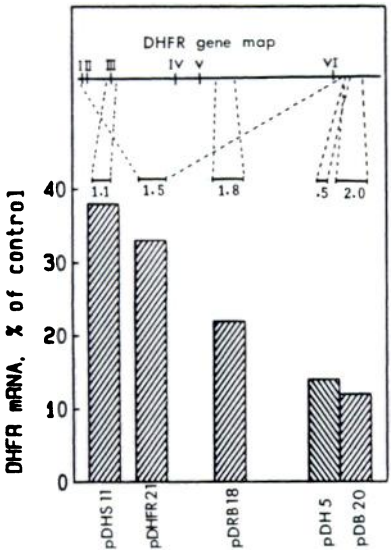

Fig. 4. Effect of F-ara-A on the transcription of DHFR mRNA in S180-500R Cells. Cells were incubated with 200 μM F-ara-A for 4 hr and then labeled with [^{3}H]uridine for 1 hr. Total cellular RNA was isolated and hybridized to a collection of probes spanning the *DHFR* gene, shown above the histogram. The pDHFR21 is a cDNA probe; pDHS11, pDRB18, pDH5, and pDB20 are genomic probes (27).

mRNA (26), was incubated with 200 μM F-ara-A for 4 hr and then labeled with [^{3}H]uridine for 1 hr. Total RNA was isolated from the cells, and the same amount of RNA was hybridized to a series of probes specific for discrete sequences of the *DHFR* gene (27). Fig. 4 illustrates the map of the *DHFR* gene, the locations of the probes, and the radioactivity detected by each probe, relative to controls, after incubation of cells with F-ara-A. Using RNA from S180-500R cells that were not treated with F-ara-A as a control, a graded decrease of RNA signal was detected in the F-ara-A-treated cells as the probes used in hybridization extended farther toward the 3′-end of the gene. This result suggests that F-ara-A caused premature termination of RNA transcription, probably resulting from the partial chain termination effect of F-ara-AMP incorporation.

In a separate experiment, S180-500R cells were incubated with 200 μM F-ara-A for 4 hr, labeled with [^{3}H]uridine for 1 hr, and then chased in drug-free medium containing unlabeled uridine. The results demonstrated that the DHFR mRNA elimination rates ($t_{1/2}$) for the control cells and F-ara-A-treated cells were 5.6 and 6.4 hr, respectively. This finding suggests that F-ara-AMP incorporation into RNA did not change the overall stability of this specific RNA transcript. Thus, the reduction of long length DHFR RNA transcripts was most likely due to the chain termination effect of the incorporated F-ara-AMP in RNA.

Action on protein synthesis. The effect of F-ara-A on protein synthesis was first evaluated in intact cells by measurement of [^{3}H]leucine incorporation. Incubation of CEM cells with 10, 30, and 100 μM F-ara-A reduced the protein synthesis activity by 39, 48, and 54%, respectively. In contrast, treatment with ara-A under identical conditions did not decrease the incorporation of [^{3}H]leucine into protein (data not shown).

Because F-ara-AMP is preferentially incorporated into mRNA, it is possible that this perturbation may affect the function of mRNA as the template for protein synthesis. To investigate this possibility, total cellular RNA and poly(A)$^+$ RNA isolated from CEM cells that were treated with F-ara-A were used as the templates for *in vitro* translation. RNA isolated from cells that were incubated with ara-A was used in parallel experiments for comparison. Table 4 shows that the translation activity of the total cellular RNA from F-ara-A-treated cells was 68% of the control, whereas the translation activity of the poly(A)$^+$ RNA from F-ara-A treated cells was not significantly different from that of the control RNA. Both total cellular RNA and poly(A)$^+$ RNA from ara-A-treated cells had the same translational capacity as the control RNA. These data suggest that F-ara-A treatment decreased the number of functional mRNA copies in a given amount of total cellular RNA, which thus had lower translation activity than control RNA. On the other hand, isolation of poly(A)$^+$ RNA by chromatography

TABLE 4

Translation capacity of RNA from cells treated with F-ara-A or Ara-A

Total cellular RNA (4 μg) or poly(A)$^+$ RNA (0.75 μg) isolated from CEM cells that were treated with F-ara-A or ara-A was used as the template for protein synthesis *in vitro*. The [^{35}S]methionine-labeled products were precipitated with 10% trichloroacetic acid, washed, and quantitated by liquid scintillation counting. Each value represents the mean ± standard error of two to four separate experiments with quadruplicate samples. The translational activity in the control samples was 3.68 × 10^5 dpm/μg of total cellular RNA and 3.58 × 10^6 dpm/μg of poly(A)$^+$ RNA.

Template	Translation capacity	*p* value
	% of control	
Total cellular RNA		
F-ara-A-treated	68 ± 5	<0.01
ara-A-treated	101 ± 5	>0.2
poly(A)$^+$-RNA		
F-ara-A-treated	95 ± 8	>0.2
ara-A-treated	102 ± 1	>0.2

through oligo(dT)-cellulose resulted in a specific recovery of full length RNA with a 3′ poly(A) tail. The same amount of poly(A)$^+$ RNA from either the control or F-ara-A-treated cells contained an equal number of full length RNA copies and, thus, had the same translation capacity. This result also suggested that the internally incorporated F-ara-AMP residues in the RNA chain did not affect the rate of overall translation.

The *in vitro* translation products (using total RNA as the template) labeled with [^{35}S]methionine were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiography revealed no specific inhibition of a particular protein band by F-ara-A treatment. Only a general decrease in the density of the protein bands, especially in the high molecular weight region, occurred (data not shown). This result further supports the existence of a partial RNA chain termination mechanism, which would have a greater likelihood of affecting longer RNA transcripts.

Discussion

The ability of F-ara-A to be incorporated into RNA is one property that distinguishes this compound from other arabinosyl nucleosides in general and from ara-A in particular. It has been demonstrated that ara-A is incorporated exclusively into DNA (13–15). Thus, it seems that the presence of fluorine at the 2-carbon of the purine ring makes F-ara-A an alternative substrate of RNA polymerases. Fluorine, which is electrophilic, may cause a shift of the electron cloud of the F-ara-A molecule so that its configuration fits the binding site of some RNA polymerases. It would be interesting to examine whether other molecules with a similar structural modification (e.g., 2-fluoro-2′-deoxyadenosine) are able to be incorporated into RNA. The preferential incorporation of F-ara-AMP into poly(A)$^+$ RNA species, however, suggests that F-ara-ATP may be a better alternate substrate for RNA polymerase II than for RNA polymerases I and III.

The incorporation of F-ara-AMP into RNA resulted in a partial chain termination. About 78% of the incorporated F-ara-AMP in total cellular RNA was located at terminal positions, and another 22% was extended by RNA polymerases (Table 1). We postulate that, during RNA transcription, the polymerase may pause after incorporation of the F-ara-AMP molecule. Subsequently, the polymerase may either proceed, leaving F-ara-AMP in an internal position, or fall off, releasing a prematurely terminated RNA transcript. The structural change caused by the incorporated F-ara-AMP in the RNA chain may create an obstacle for the cellular transcription complex. This hypothesis is supported by a variety of experimental evidence. *In vitro* transcription studies demonstrated that high molecular weight RNAs were affected more than small size RNAs (Fig. 3), because longer RNA chains had a higher probability of incorporating the analogue. This action was further confirmed in experiments using intact cells (Fig. 4). The results of *in vitro* translation experiments also indicated that high molecular weight RNA templates decreased in cells treated with F-ara-A. The partial RNA chain termination effect of F-ara-AMP contrasts with its more potent chain termination activity for DNA synthesis (11).

Total cellular RNA isolated from cells pretreated with F-ara-A had significantly lower template capacity for translation than did normal RNA from control cells (Table 4), suggesting that fewer functional RNA templates existed in the F-ara-A-treated cells. That result is consistent with the partial RNA chain termination activity of F-ara-AMP, which may lead to the production of functionally inactive premature RNA transcripts. Interestingly, it appeared that the internally incorporated F-ara-AMP residues in mRNA had no effect on overall protein translation, because the full length poly(A)$^+$ RNA, which contained internal F-ara-AMP, had the same translation efficiency as the control RNA (Table 4).

In addition to terminating the RNA chain, F-ara-ATP may directly inhibit RNA polymerases without being incorporated. There is a linear correlation between the F-ara-ATP concentration and the inhibition of RNA synthesis in isolated nuclei (Fig. 2). Furthermore, induction of a decrease in the cellular ATP pool may also contribute to the inhibition of RNA synthesis in whole cells.

With respect to cytotoxicity, the contribution of F-ara-AMP incorporation into RNA and its inhibitory effect on RNA synthesis is not presently clear. We and others have demonstrated that the loss of clonogenicity in exponentially growing cells is linearly correlated with the amount of F-ara-A incorporated into DNA (10, 11). Experiments using HL-60 cells indicated that the incorporation of F-ara-A into RNA is also correlated, but less strongly, with cytotoxicity (10). Given that F-ara-A is a much more potent chain terminator for DNA synthesis than for RNA synthesis and that the IC_{50} value is 1 μM for DNA synthesis (11) and 10 μM for RNA synthesis in CEM cells, we speculate that the incorporation of F-ara-A into DNA and termination of DNA synthesis may be the key mechanism of cytotoxicity in these cells. However, in certain types of cells, such as peripheral lymphocytes from patients with chronic lymphocytic leukemia, which are inactive in DNA synthesis (28), the action of F-ara-A on RNA metabolism might be important in causing cell death. Further studies are needed to elucidate the mechanism of the cytotoxic effects of F-ara-A in such cells.

Studies with partially purified DNA primase from acute lymphocytic leukemia cells (29) and chronic myelogenous leukemia cells (12) demonstrated that F-ara-ATP inhibited the incorporation of ribonucleotides into the RNA primer synthesized by this enzyme. This inhibitory effect was also observed in experiments with CEM cell lysates (30). The K_i value for F-ara-ATP inhibition of DNA primase was about 25 μM, which is approximately 10 times the K_i value for F-ara-ATP inhibition of DNA polymerases α and δ (11, 12, 31, 32). Thus, the contribution of the action of F-ara-ATP on DNA primase to cytotoxicity may be secondary to its DNA chain termination effect after incorporation by DNA polymerases.

In summary, the substitution of the 2-hydrogen with a fluorine provides F-ara-A with new properties, such as resistance to adenosine deaminase and the ability to be incorporated into RNA. The present studies demonstrated the inhibitory action of F-ara-A on RNA metabolism. Cells were able to incorporate F-ara-A into RNA, preferentially into the poly(A)$^+$ RNA fraction; incorporation resulted in partial RNA chain termination and impaired the functioning of the RNA transcripts as templates for protein synthesis. F-ara-ATP may also inhibit RNA synthesis by direct interaction with RNA polymerases. Two other compounds, 2-fluoroadenosine and 2-fluorodeoxyadenosine, have the same structural modification of the purine ring as F-ara-A. A detailed comparison of the biological activities and mechanisms of actions of F-ara-A, ara-A, 2-fluoroadeno-

sine, 2-fluorodeoxyadenosine, and their respective triphosphates would further our understanding of the relationship between the chemical structure and function of these compounds.

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References

1. Brink, J. J., and G. A. LePage. Metabolic effects of 9-D-arabinosylpurines in ascites tumor cells. *Cancer Res.* **24**:312–318 (1964).
2. Plunkett, W., and S. S. Cohen. Two approaches that increase the activity of analogs of adenine nucleosides in animal cells. *Cancer Res.* **35**:1547–1554 (1975).
3. Brockman, R. W., F. M. Schabel, Jr., and J. A. Montgomery. Biologic activity of 9-β-D-arabinofuranosyl-2-fluoroadenine, a metabolically stable analog of 9-β-D-arabinofuranosyladenine. *Biochem. Pharmacol.* **26**:2193–2196 (1977).
4. Plunkett, W., S. Chubb, L. Alexander, and J. A. Montgomery. Comparison of the toxicity and metabolism of 9-β-D-arabinofuranosyl-2-fluoroadenine and 9-β-D-arabinofuranosyladenine in human lymphoblastoid cells. *Cancer Res.* **40**:2349–2355 (1980).
5. 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).
6. Avramis, V. I., and W. Plunkett. Metabolism and therapeutic efficacy of 9-β-D-arabinofuranosyl-2-fluoroadenine against murine leukemia P388. *Cancer Res.* **42**:2587–2591 (1982).
7. Grever, M. R., K. J. Kopecky, C. A. Coltman, J. C. Files, B. R. Greenberg, J. J. Hutton, R. Talley, D. D. Von Hoff, and S. P. Balcerzak. Fludarabine monophosphate: a potentially useful agent in chronic lymphocytic leukemia. *Nouv. Rev. Fr. Hematol.* **30**:457–459 (1988).
8. Keating, M. J., H. Kantarjian, M. Talpaz, J. Redman, C. Koller, B. Barlogie, W. Velasquez, W. Plunkett, E. J. Freireich, and K. B. McCredie. Fludarabine: a new agent with major activity against chronic lymphocytic leukemia. *Blood* **74**:19–25 (1989).
9. Plunkett, W., and S. Chubb. Toxicity, metabolism and incorporation into DNA and RNA of arabinosyl-2-fluoroadenine (F-ara-A). *Proc. Am. Assoc. Cancer Res.* **26**:19 (1985).
10. Spriggs, D., G. Robbins, T. Mitchell, and D. Kufe. Incorporation of 9-β-D-arabinofuranosyl-2-fluoroadenine into HL-60 cellular RNA and DNA. *Biochem. Pharmacol.* **35**:247–252 (1986).
11. Huang, P., S. Chubb, and W. Plunkett. Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine: a mechanism for cytotoxicity. *J. Biol. Chem.* **265**:16617–16625 (1990).
12. Parker, W. B., A. R. Bapat, J.-X. Shen, A. J. Townsend, and Y.-C. Cheng. Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. *Mol. Pharmacol.* **34**:485–491 (1988).
13. Plunkett, W., L. Lapi, P. J. Ortiz, and S. S. Cohen. Penetration of mouse fibroblasts by the 5′-phosphate of 9-β-D-arabinofuranosyladenine and incorporation of the nucleotide into DNA. *Proc. Natl. Acad. Sci. USA* **71**:73–77 (1974).
14. Pelling, J. C., J. C. Drach, and C. Shipman, Jr. Internucleotide incorporation of arabinosyladenine into herpes simplex virus and mammalian cell DNA. *Virology* **109**:323–335 (1981).
15. Kufe, D. W., P. P. Major, D. Monroe, M. Egan, and D. Herrick. Relationship between incorporation of 9-β-D-arabinofuranosyladenine in L1210 DNA and cytotoxicity. *Cancer Res.* **43**:2000–2004 (1983).
16. Novotny, L., and W. Plunkett. Synthesis of 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate, in *Nucleic Acid Chemistry* (L. B. Townsend and R. S. Tipson, eds.), Part 4. John Wiley and Sons, New York, 337–340 (1990).
17. Gandhi, V., and W. Plunkett. Modulation of arabinosyl-nucleoside metabolism by arabinosylnucleotides in human leukemia cells. *Cancer Res.* **48**:329–334 (1988).
18. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning, a Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 382–389 (1982).
19. Plunkett, W., and S. S. Cohen. Metabolism of 9-β-D-arabinofuranosyladenine by mouse fibroblasts. *Cancer Res.* **35**:415–422 (1975).
20. Marzluff, W. F., and R. C. C. Huang. Transcription of RNA in isolated nuclei, in *Transcription and Translation* (B. D. Hames and S. J. Higgins, eds.). IRL Press, Oxford, UK, 89–129 (1984).
21. Handa, H., R. J. Kaufman, J. Manley, M. Gefter, and P. A. Sharp. Transcription of Simian virus 40 DNA in a HeLa whole cell extract. *J. Biol. Chem.* **256**:478–482 (1981).
22. Leys, E. J., and R. E. Kellems. Control of dihydrofolate reductase messenger ribonucleic acid production. *Mol. Cell. Biol.* **1**:961–971 (1981).
23. Melli, M., G. Spinelli, and E. Arnold. Synthesis of histone messenger RNA of HeLa cells during the cell cycle. *Cell* **12**:167–174 (1977).
24. Darnell, J., H. Lodish, and D. Baltimore. *Molecular Cell Biology.* Scientific American Books, Inc., New York, 305–369 (1986).
25. Shiokawa, K., and Y. Misumi. Effects of cordycepin on the synthesis of nuclear and cytoplasmic RNAs in embryonic cells of *Xenopus laevis*. *Cell Struct. Funct.* **8**:347–356 (1984).
26. Alt, F. W., R. E. Kellems, and R. T. Schimke. Synthesis and degradation of folate reductase in sensitive and methotrexate-resistant lines of S-180 cells. *J. Biol. Chem.* **251**:3063–3074 (1976).
27. Frayne, E. G., E. J. Leys, G. F. Crouse, A. G. Hook, and R. E. Kellems. Transcription of the mouse dihydrofolate reductase gene proceeds unabated through seven polyadenylation sites and terminates near a region of repeated DNA. *Mol. Cell. Biol.* **4**:2921–2924 (1984).
28. Andreeff, M. Cell kinetics of leukemia. *Semin. Hematol.* **23**:300–314 (1986).
29. Parker, W. B., and Y.-C. Cheng. Inhibition of DNA primase by nucleoside triphosphates and their arabinofuranosyl analogs. *Mol. Pharmacol.* **31**:146–151 (1987).
30. Catapano, C. V., K. B. Chandler, and D. J. Fernandes. Effects of anticancer agents on primer RNA formation in human leukemia cells. *Proc. Am. Assoc. Cancer Res.* **31**:420 (1990).
31. White, E. L., S. C. Shaddix, R. W. Brockman, and L. L. Bennett, Jr. Comparison of the actions of 9-β-D-arabinofuranosyl-2-fluoroadenine on target enzymes from mouse tumor cells. *Cancer Res.* **42**:2260–2264 (1982).
32. Tseng, W. C., D. Derse, Y.-C. Cheng, R. W. Brockman, and L. L. Bennett, Jr. *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. *Mol. Pharmacol.* **21**:474–477 (1982).

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