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INCORPORATION OF 9-β-D-ARABINOFURANOSYL-2-FLUOROADENINE INTO HL-60 CELLULAR RNA AND DNA*

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Abstract—The incorporation of 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) into HL-60 cellular nucleic acids was monitored by cesium sulfate gradient centrifugation. The results demonstrated that F-ara-A incorporated into both RNA and DNA. These findings are in contrast to those previously obtained with 1-β-D-arabinofuranosylcytosine (ara-C) and 9-β-arabinofuranosyladenine (ara-A) which demonstrated incorporation of these nucleosides only in DNA. F-ara-A inhibited HL-60 proliferation, and the incorporation of F-ara-A into both DNA and RNA correlated with loss of clonogenic survival. Furthermore, cytostatic concentrations of F-ara-A resulted in the appearance of a more mature phenotype, a finding consistent with the effects of other inhibitors of DNA synthesis. The incorporation of F-ara-A into RNA and DNA should provide new insights regarding the mechanism of action of this agent.

Ara-C‡ and ara-A are inhibitors of DNA synthesis [1, 2] with antiviral [3, 4] and antitumor [5, 6] activity. Although ara-C is a clinically useful agent [6], ara-A has not been as effective. The clinical utility of ara-A has been limited by its rapid deamination to the inactive metabolite ara-hyp by ADA [7]. ADA inhibitors, such as DCF [8], prevent deamination of ara-A and prolong the plasma half-life of this agent in humans [9]. DCF, however, also antagonizes the effects of ara-A by enhancing intracellular dATP pools [10]. These findings as well as the undesirable side-effects of DCF [9, 11] have prompted the clinical investigation of F-ara-A, an analog of ara-A that is not a substrate for ADA [12, 13].

The precise mechanism of action of F-ara-A remains unclear. Previous studies with ara-C and ara-A have demonstrated a highly significant relationship between incorporation into DNA and loss of leukemic cell clonogenic survival [14–16]. The incorporated arabinosyl residue alters reactivity of the 3′-terminus and thereby acts as a relative chain terminator [16, 17]. Thus, ara-C and ara-A slow DNA chain elongation [18] and can result in rereplication of certain DNA segments within a single cell cycle [19, 20].

F-ara-A is phosphorylated to the triphosphate active metabolite, F-ara-ATP [21]. F-ara-ATP inhibits DNA polymerase α as well as ribonucleotide reductase [22, 23]. F-ara-A is also a weak inhibitor of *S*-adenosylhomocysteine hydrolase [22]. The relative importance of these and other mechanisms in inducing F-ara-A cytotoxicity is uncertain. The structural similarity between F-ara-A and the other arabinosyl nucleosides has prompted the present investigation which explores the incorporation of F-ara-A into HL-60 nucleic acids. The results demonstrate that F-ara-A, in contrast to ara-C and ara-A, incorporates into both RNA and DNA.

MATERIALS AND METHODS

Cell culture. The HL-60 cells were grown in suspension culture in RPMI-1640 medium (Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY), 100 units of streptomycin/ml, 100 μg penicillin and 1% L-glutamine at 37° in a 5% CO_2 atmosphere.

Incorporation of F-ara-A into nucleic acids. HL-60 cells in logarithmic growth phase were suspended in supplemented RPMI-1640 medium at a concentration of 1×10^6 ml. The cells were incubated with [8-^{3}H]F-ara-A (9 Ci/mmole, Moravek Biochemicals, Brea, CA) at concentrations of 10^{-7} to 10^{-4} M for periods of 3–24 hr. For 10^{-5} and 10^{-4} M concentrations, a concentration of 10^{-6} M [8-^{3}H]F-ara-A was employed and supplemented with unlabeled drug. The nucleic acids were then purified and analyzed by Cs_2SO_4 density centrifugation [14].

Nucleic acid digestion. Nucleic acid extracts were separated by Cs_2SO_4 gradient centrifugation [24]. The DNA and RNA fractions were collected and dialyzed against 0.01 M Tris buffer, pH 7.0, for 24 hr. The DNA fraction was digested to nucleosides with DNase I (50 μg/ml), snake venom phosphodiesterase (0.04 units/ml), and alkaline phosphatase

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‡ Abbreviations: ara-C, 1-β-D arabinofuranosylcytosine; ara-A, 9-β-D-arabinofuranosyladenine; ara-hyp, arabinosylhypoxanthine; ADA, adenosine deaminase; DCF, deoxycoformycin; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; F-Ado, 2-fluoroadenosine; F-Ade, 2-fluoroadenine; and F-dAdo, 2-fluorodeoxydenosine.

(0.06 units/ml) (Sigma, St. Louis, MO). The digest solutions were then precipitated with 0.25 ml of 12% perchloric acid to remove the enzymes, neutralized with saturated $KHCO_3$ and analyzed by high pressure liquid chromatography (HPLC). The RNA fraction was prepared in an analogous fashion, substituting RNase A (50 μg/ml) for the DNase I.

HPLC separations. The nucleotide separation of 5′-monophosphates was performed using a Waters gradient elution system HPLC (Waters Associates, Milford, MA). A Z-module radial compression system with a Radial-PAK SAX cartridge (Waters Associates) was employed at a flow rate of 3.0 ml/min. Buffer A was 0.01 M KH_2PO_4/80% acetonitrile, pH 2.85. Buffer B was 0.01 M KH_2PO_4, pH 2.85. The gradient elution program for buffer B was as follows: (1) 0–40% over 6 min, (2) 40–0% over 6 min. Absorbance was measured at 254 nm. The nucleoside separation was performed using a Varian 5020 instrument (Varian Associates, Palo Alto, CA) and a reverse phase C18, MCH-5 Micro-Pak column at a flow rate of 1.2 ml/min. The isocratic separation was achieved using 0.01 M KH_2PO_4, pH 4.9, with 4% methanol.

Clonogenic survival. Cell suspensions were washed three times with phosphate-buffered saline. Four thousand cells per ml of supplemented RPMI-1640 medium with 0.4% Bacto-Agar (Difco Laboratories, Detroit, MI) were placed in gridded 35-mm petri dishes. The plates were incubated for 7 days at 37° in 5% CO_2. The control plating efficiency was approximately 30%. All colonies of more than 20 cells were counted and all results expressed as a percentage of colonies obtained from the untreated control plates.

Nonspecific esterase activity. Non-specific esterase (NSE) activity was monitored by centrifuging HL-60 cells in a Shandon Elliot Cytospin (Sewickley, PA) for 5 min at 600 rpm and then staining the cells for α-napthyl acetate esterase activity [25]. Percent positive was determined by counting 200 cells in duplicate.

RESULTS

We employed cesium sulfate gradient analysis to monitor the incorporation of F-ara-A into HL-60 cellular nucleic acids. This methodology permits the separation of RNA (banding at density 1.65 g/ml) and DNA (banding at density 1.45 g/ml). Figure 1 shows the incorporation of [^{3}H]F-ara-A and ^{32}P into RNA and DNA during incubation periods of 3, 6, 12 and 24 hr. Significant amounts of tritium were detectable within both the RNA and DNA regions of these gradients. Further, the extent of tritium incorporation into RNA and DNA was dependent on time of exposure. The labeling with ^{32}P serves as a measure of newly synthesized RNA and DNA and the ratio of [^{3}H]F-ara-A/^{32}P serves as a measure of the relative incorporation of F-ara-A into nucleic acids.

It is relevant to ensure that the tritium label detectable in RNA and DNA in fact represents incorporation of [^{3}H]F-ara-A rather than possible metabolites. HL-60 cells were labeled with [^{3}H]F-ara-A, and the nucleic acid fractions resulting from Cs_2SO_4 centrifugation were purified and digested to nucleosides for analysis by reverse phase HPLC. A nucleoside elution pattern of the HL-60 RNA obtained from cells labeled with [^{3}H]F-ara-A is shown in Fig. 2. The results demonstrate that less than 10% of the tritium comigrated with F-Ado. In contrast, over 90% of the tritium radioactivity was found to comigrate with F-ara-A. This finding has been reproduced in multiple experiments.

The nucleoside HPLC elution pattern of DNA purified by Cs_2SO_4 gradient centrifugation from HL-60 cells treated with [^{3}H]F-ara-A is shown in Fig. 3. In this representative experiment, over 80% of the tritium activity comigrated with F-ara-A. In contrast, less than 10% of the radioactivity comigrated with F-Ade and F-dAdo. Further, less than 10% of the tritium comigrated adjacent to F-Ado and represents an uncharacterized metabolite. The deamination product, F-ara-hyp, had a much shorter retention time and was not present to a measurable extent in the nucleic acid digests (data not shown). These findings were confirmed by labeling HL-60 cells with [^{3}H]F-ara-A and then extracting total nucleic acid. The RNA was digested with RNase and the DNA reprecipitated with ethanol. The purified DNA was then digested to 5′-monophosphates and analyzed by anion exchange HPLC. Figure 4 demonstrates that 3% of the tritium label eluted with F-ara-A and

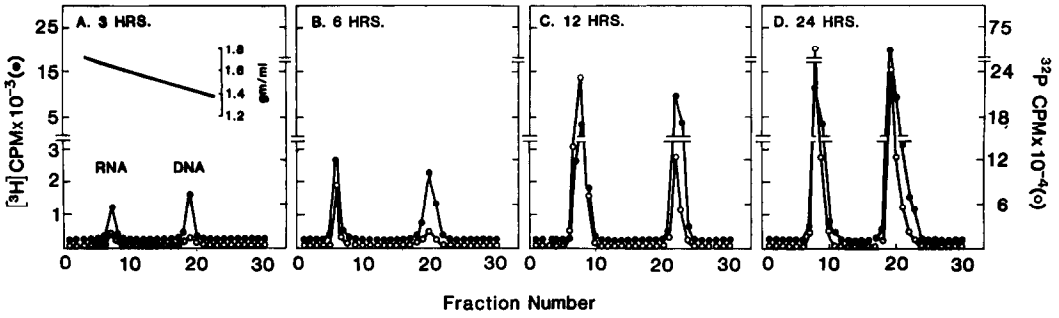

Fig. 1. Cesium sulfate density gradient analysis of nucleic acids from F-ara-A treated HL-60 cells. HL-60 cells in logarithmic growth phase at a concentration of 1×10^6 cells/ml were incubated with 10^{-7} M [^{3}H]F-ara-A and 10 μCi/ml ^{32}P for 3, 6, 12 and 24 hr. The total cellular nucleic acids were purified and analyzed by Cs_2SO_4 density gradient centrifugation. The tritium and ^{32}P counts were determined for the RNA region (density 1.65 g/ml) and DNA region (density 1.45 g/ml) of the gradients.

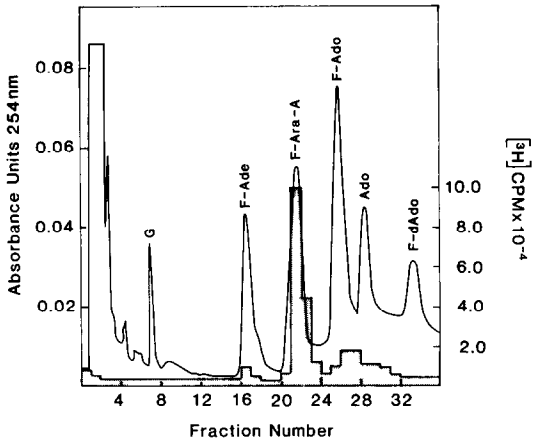

Fig. 2. HPLC elution profile for a nucleoside digest from RNA. HL-60 cells were incubated with 10^{-7} M [^{3}H]F-ara-A for 12 hr. The nucleic acids were extracted and purified by Cs_2SO_4 gradient centrifugation. The RNA fraction was treated with RNase A, snake venom phosphodiesterase, and bacterial alkaline phosphatase. The nucleosides were purified and analyzed by HPLC. Appropriate nucleoside markers were added, and the fractions were collected and assayed for tritium radioactivity.

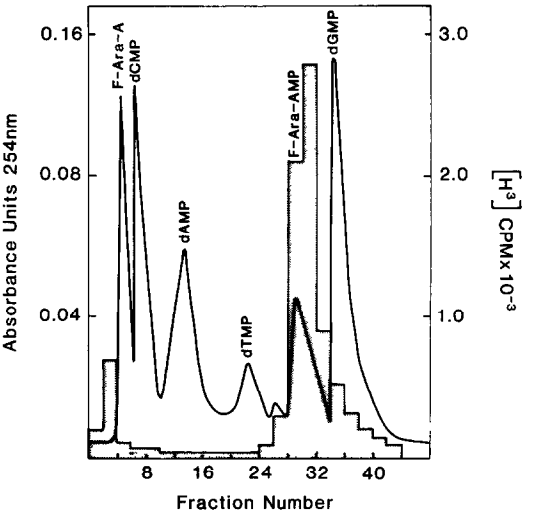

Fig. 4. HPLC elution profile for a nucleotide digest from DNA. HL-60 cells were incubated with 10^{-7} M [^{3}H]F-ara-A for 24 hr and the nucleic acids extracted. RNA was digested with RNase A. The DNA was purified by ethanol precipitation and then digested with DNase I and snake venom phosphodiesterase. Appropriate nucleotide markers were added and the nucleotides separated by anion exchange HPLC. The fractions were collected and assayed for tritium radioactivity.

the remainder comigrated with F-ara-AMP. Further digestion of the 5′-monophosphates to nucleosides with bacterial alkaline phosphatase demonstrated that most of the tritium radioactivity comigrated with F-ara-A (Fig. 5). A small amount of radioactivity was found in the void volume, probably representing nucleoside breakdown during digestion.

The relevance of F-ara-A incorporation into cellular RNA and DNA to the biologic effects of this drug was studied by comparing the amount of F-ara-A incorporation with the clonogenic potential of the cells after drug exposure. HL-60 cells were exposed to concentrations of F-ara-A ranging from 10^{-7} to 10^{-4} M for periods of 3, 6, 12 and 24 hr. As shown in Fig. 6, loss of clonogenicity was a function of both drug concentration and time of exposure at each different concentration.

The relationship between incorporation of F-ara-A into HL-60 cellular DNA with the effect of the drug on clonogenic survival was evaluated by measuring the amount of [^{3}H]F-ara-A incorporation into DNA over a wide range of concentration and time

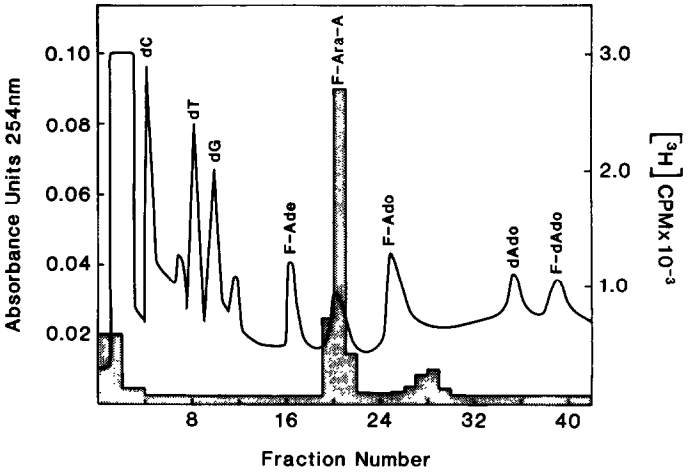

Fig. 3. HPLC elution profile for a nucleoside digest from DNA. HL-60 cells were incubated with 10^{-7} M [^{3}H]F-ara-A for 12 hr. The nucleic acids were extracted and purified by Cs_2SO_4 centrifugation. The DNA fraction was treated with DNase I, snake venom phosphodiesterase, and bacterial alkaline phosphatase. The nucleosides were purified and analyzed by reverse phase HPLC. Appropriate nucleoside markers were added, and the fractions were collected to monitor tritium radioactivity.

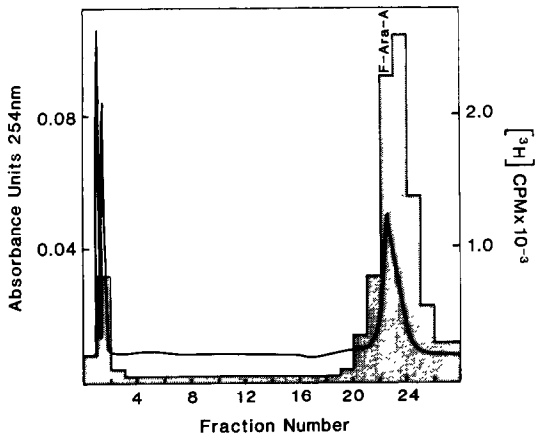

Fig. 5. HPLC elution profile for F-ara-AMP fractions after alkaline phosphatase treatment. The fractions comigrating with F-ara-AMP were pooled and lyophilized. After digestion with bacterial alkaline phosphatase, the digest was analyzed by reverse phase HPLC.

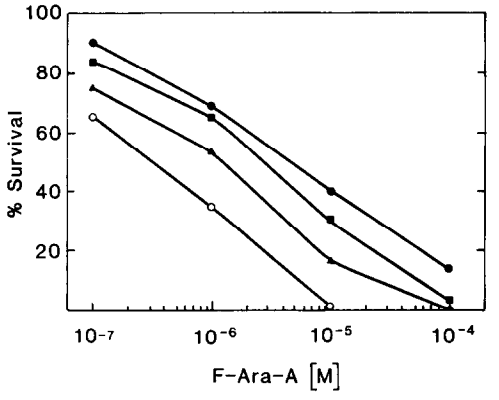

Fig. 6. Clonogenic survival of HL-60 cells after F-ara-A exposure. HL-60 cells (10^6/ml) in logarithmic growth phase were incubated with 10^{-7} to 10^{-4} M F-ara-A for 3 (●), 6 (■), 12 (▲) and 24 (○) hr. The cells were then washed and resuspended in drug-free medium prior to plating in agar. Clonogenic survival was determined after 10 days by scoring colonies greater than 20 cells. Percent survival was determined by the ratio of colonies formed by F-ara-A treated cells compared to untreated cells.

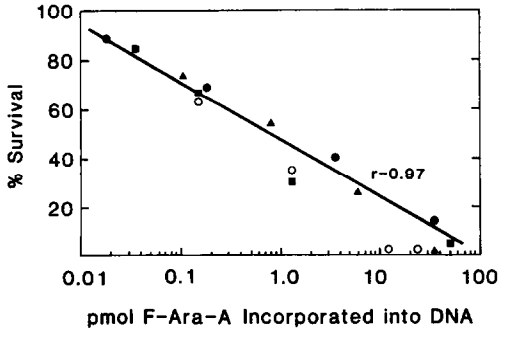

Fig. 7. Relationship between HL-60 clonogenic survival and [^{3}H]F-ara-A incorporation into DNA (pmoles/10^6 cells) at F-ara-A exposures of 3 (●), 6 (▲), 12 (■) and 24 (○) hr.

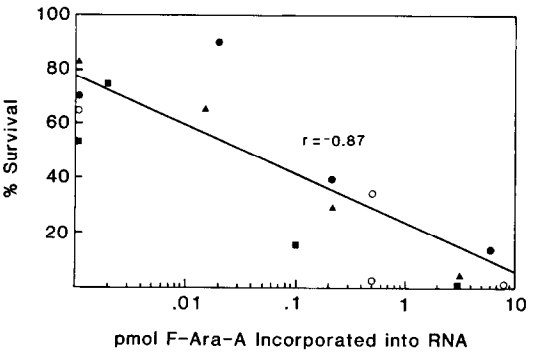

Fig. 8. Relationship between HL-60 clonogenic survival and [^{3}H]F-ara-A incorporation into RNA (pmoles/10^6 cells) and F-ara-A exposures of 3 (●), 6 (▲), 12 (■) and 24 (○) hr.

($C \times T$) values. Incorporation studies were performed at [^{3}H]F-ara-A concentrations ranging from 10^{-7} to 10^{-4} M, and the amounts incorporated into DNA during the incubation periods (3, 6, 12 and 24 hr) were determined by purification of the DNA fraction. The clonogenic survival at each F-ara-A concentration is derived from the cloning data shown in Fig. 6 as well as data derived from experiments performed with a 1-hr incubation. The significant relationship between the probability of survival and incorporation of F-ara-A into DNA is illustrated in Fig. 7. Linear regression analysis of this relationship demonstrated a coefficient of −0.97. The relationship between incorporation of F-ara-A into HL-60 RNA and clonogenicity was also determined using linear regression (Fig. 8). The correlation coefficient for this relationship was −0.87.

Our previous studies with ara-C and ara-A have demonstrated that sublethal drug concentrations result in differentiation of HL-60 cells [26, 27]. HL-60 cells were thus exposed to F-ara-A for 24, 48 and 72 hr and monitored for induction of maturation by determining the relative and absolute number of cells expressing non-specific esterase (NSE). The effects of various concentrations of F-ara-A on the growth of HL-60 cells in suspension are shown in Fig. 9. Figure 10 illustrates the effects of F-ara-A (1×10^{-6}

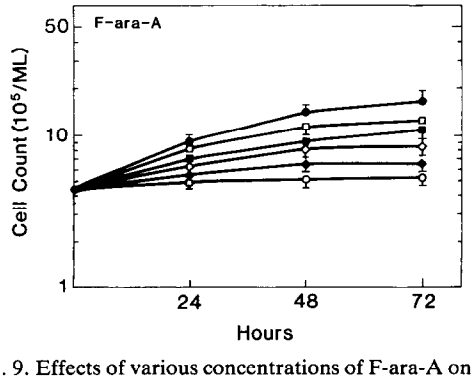

Fig. 9. Effects of various concentrations of F-ara-A on HL-60 cell growth at 24, 48 and 72 hr. Key: no drug (●), 1×10^{-6} M (□), 2×10^{-6} M (■), 3×10^{-6} M (◇), 4×10^{-6} M (◆) and 5×10^{-6} M (○) F-ara-A.

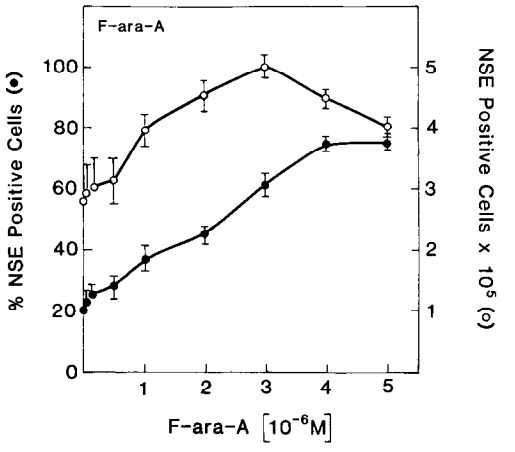

Fig. 10. Expression of NSE activity following F-ara-A exposure. HL-60 cells were exposed to various concentrations of F-ara-A for 72 hr for determination of the percent NSE positive cells (●) and the absolute number of NSE positive cells (○).

to 5×10^{-6} M) on NSE reactivity after 72 hr of drug exposure. Exposure to higher concentrations resulted in an increasing percentage of cells positive for NSE and increases in the cytoplasmic to nuclear ratio. Thus, NSE staining increased directly with drug concentration from control levels of approximately 20% to over 75% following exposure to 5×10^{-6} M F-ara-A. This effect was associated with absolute increases in NSE positive cells up to 3×10^{-6} M, while higher drug concentrations resulted in a slight decrease in absolute numbers of NSE positive cells. This decrease in absolute number reflected the increasing cytotoxicity of higher drug concentration. Taken together, these findings suggested that F-ara-A also induces maturation of HL-60 cells.

DISCUSSION

The demonstration of a highly significant relationship between the extent of incorporation of ara-C into DNA and inhibition of DNA synthesis provided evidence for the incorporation of ara-C into DNA as a mechanism of drug action [17]. We have also demonstrated that ara-A, like ara-C, incorporates specifically in DNA and that the ara-A residue at the 3′-terminus provides a poor primer for elongating strands [16]. Studies with both ara-C and ara-A have demonstrated highly significant relationships between incorporation into DNA and loss of self-renewal capacity, thus suggesting that the incorporation of arabinosyl derivatives into DNA results in either cytotoxicity or terminal differentiation. Furthermore, an evaluation of ara-C DNA incorporation, ara-CTP pools, concentration time products and clonogenic survival of HL-60 cells has demonstrated that incorporation of ara-C into DNA is the best correlate with the loss of clonogenic survival [28].

The present study demonstrated that F-ara-A also incorporated into cellular DNA. Furthermore, the extent of incorporation of F-ara-A into DNA correlated significantly with loss of HL-60 clonogenic survival. These findings are consistent with our previous work with ara-C and ara-A [14–16]. However, in contrast to the specific incorporation of ara-C and ara-A into DNA, the present results demonstrated that F-ara-A was also detectable in RNA. Incorporation of F-ara-A into RNA was also highly correlated with the loss of clonogenic survival. The 2-fluorine modification of ara-A apparently enables RNA polymerase to utilize this arabinosyl derivative as substrate, resulting in this different pattern of nucleic acid incorporation. The correlation between F-ara-A incorporation into DNA and clonogenic survival was significantly better than the correlation between F-ara-A incorporation into RNA and clonogenic survival ($P < 0.10$) [29]. In this logarithmic growth phase cell culture system, the incorporation of F-ara-A into RNA was closely related to DNA incorporation, and the relative importance of either mechanism for inducing cytotoxicity remains unclear. Work is underway to define the effects of isolated RNA incorporation through use of a growth arrested cell system.

F-ara-A, like ara-A, is an inhibitor of *S*-adenosylhomocysteine hydrolase and thus may alter patterns of DNA methylation [22]. Furthermore, F-ara-ATP has been shown to inhibit DNA polymerase α and ribonucleotide reductase [22, 23]. Previous studies with other inhibitors of DNA synthesis, including arabinosyl derivatives, aphidicolin and hydroxyurea, have demonstrated the induction of HL-60 and K562 leukemic cell differentiation [26, 27, 30]. The present studies suggest a similar effect with sublethal concentrations of F-ara-A on the maturation of HL-60 cells. The inhibition of DNA replication by ara-C results in an aberrant form of DNA synthesis with certain segments of DNA being replicated more than once in a single cell cycle [19, 20]. The inhibition of DNA replication by F-ara-A may also result in endoreduplication with alterations in gene expression and loss of clonogenic survival.

These studies have thus demonstrated that F-ara-A, unlike ara-A and ara-C, was incorporated into RNA as well as DNA. This incorporation into nucleic acids was highly correlated with the *in vitro* loss of clonogenic survival and may provide insight into the mechanisms of its antineoplastic effect. If the RNA incorporation has important independent effects, F-ara-A may prove to have an altered clinical profile of toxicity and efficacy when compared to the other clinically useful arabinosyl derivatives.

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