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2-FLUORO-ATP: A TOXIC METABOLITE OF 9-β-D-ARABINOSYL-2-FLUOROADENINE

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SUMMARY: Murine P388 cells incubated in vitro with the anticancer drug arabinosyl 2-fluoroadenine accumulate its 5'-triphosphate, F-araATP, as the major phosphorylated metabolite. A new chromatographically separate metabolite that accumulated to levels 10% of that of F-araATP was identified as 2-fluoro-ATP, by the following criteria. 1. The metabolite coeluted with the authentic compound on anion-exchange HPLC. 2. Dephosphorylation of the metabolite yielded a compound that was chromatographically identical to 2-fluoroadenosine. 3. The compound was sensitive to $NaIO_4$ oxidation. Cellular incubation experiments indicated that 2-fluoroadenine, but not arabinosyl 2-fluorohypoxanthine, was the likely intermediate in the formation of 2-fluoro-ATP.

F-araA[2] and its soluble 5'-monophosphate derivative F-araAMP are two new compounds with promising antitumor activity (1-4). The active metabolite, F-araATP is known to inhibit both ribonucleotide reductase and DNA polymerase α (5,6) and is incorporated into DNA [3]. Studies on F-araA administered to monkeys, dogs, and mice demonstrated not only the deamination of the analog to F-araHyp, but also the production of F-Ade (7,8). Because F-Ade and its nucleoside 2-fluoroadenosine are extremely toxic compounds (9,10) we sought to investigate the biological significance of the catabolism of F-araA. Our results demonstrated that F-ATP is accumulated by P388 murine leukemia cells incubated with either F-araA or F-araAMP in vitro or after treatment in

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[2] Abbreviations used are: F-araA, 9-β-D-arabinofuranosyl-2-fluoroadenine; F-araATP, 9-β-D-arabinofuranosyl 2-fluoroadenine 5'-triphosphate; F-araHyp, 9-β-D-arabinofuranosyl 2-fluorohypoxanthine; F-Ade, 2-fluoroadenine; F-ATP, 2-fluoroadenosine 5'-triphosphate; TCNR, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine.

[3] Manuscript in preparation.

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vivo and that a catabolic pathway that gives rise to F-Ade is the most likely route of F-ATP formation (12).

MATERIALS AND METHODS

Materials. F-Ade, F-Ado and F-dAdo were kindly provided by Dr. T.A. Krenitsky, The Wellcome Research Laboratories Research Triangle Park N.C.; F-araA and 2'-deoxycoformycin were supplied by Drug Synthesis and Chemistry Branch Division of Cancer Treatment, National Cancer Institute. [8-^{3}H]F-araA was purchased from Moravek Biochemicals Inc. Brea, CA. and purified 2 or 3 times by HPLC to greater than 99.9% homogeniety before use. No radioactivity greater than background was detected in the region of the last chromatogram where F-Ade would have eluted. [8-^{3}H]F-araHyp was produced enzymatically by incubation of [8-^{3}H]F-araA with 2 units/ml calf intestinal adenosine deaminase (Sigma Chemical Co., St. Louis, MO), in 0.05 M K_2HPO_4, pH 7.5, for 18 hr followed by HPLC purification of the product. Dr. Roland K. Robins generously provided 1-β-D-ribofuranosyl-1,2,4-triazole3-carboxamidine (TCNR). The effect of TCNR on purine nucleoside phosphorylase was determined spectrophotometrically as described by Kalckar (13).

Cells. A line of P388 murine leukemia cells was maintained in mice as previously described (4). For short-term incubations in vitro, the cells were aspirated from the host peritoneal cavity, washed, and resuspended in RPMI 1640 medium containing 5% fetal calf serum (Grand Island Biological Co., Grand Island, NY) in plastic flasks. Cell number and the mean cell volume were determined by a model ZBI Coulter Counter equipped with a model C-1000 Channelyzer.

HPLC of Nucleotides and Nucleosides. Cellular nucleotides were extracted with $HClO_4$ and after separation by HPLC were quantitated as described (1) except that the pH of the high concentration buffer was 3.5. Periodate oxidation of nucleotides was conducted by the procedure of Neu and Heppel (14,15) and products were separated by HPLC as described above. Nucleosides produced by dephosphorylation with alkaline phosphastase (E. coli, Type III Sigma Chemical Co) were separated on a column of μBondapak C_{18} (Waters Associates, Milford, MA) by first eluting for 10 min with 0.5 M CH_3COONH_4, pH 6.5 before being run in a 30-min linear gradient to 20% methanol in the same buffer at 1 ml/min.

RESULTS AND DISCUSSION

When P388 cells were incubated in vitro with [^{3}H]F-araA a new major UV absorbing peak appeared in the triphosphate region of the HPLC chromatogram at 34 min (Fig 1B). Analysis of the column eluate indicated that radioactivity was associated with this peak at a specific activity similar to that of the exogenous [^{3}H]F-araA. These facts are consistent with previous observations that this peak is F-araATP (1,2,4), and that the smaller amounts of radioactivity eluting at about 7 min and 18 min are associated with F-araAMP and F-araADP, respectively. In addition, a smaller UV-absorbing peak eluting in the triphosphate region at 32 min was observed. This compound coeluted with ATP under the chromatographic conditions conducted at pH 3.7 described

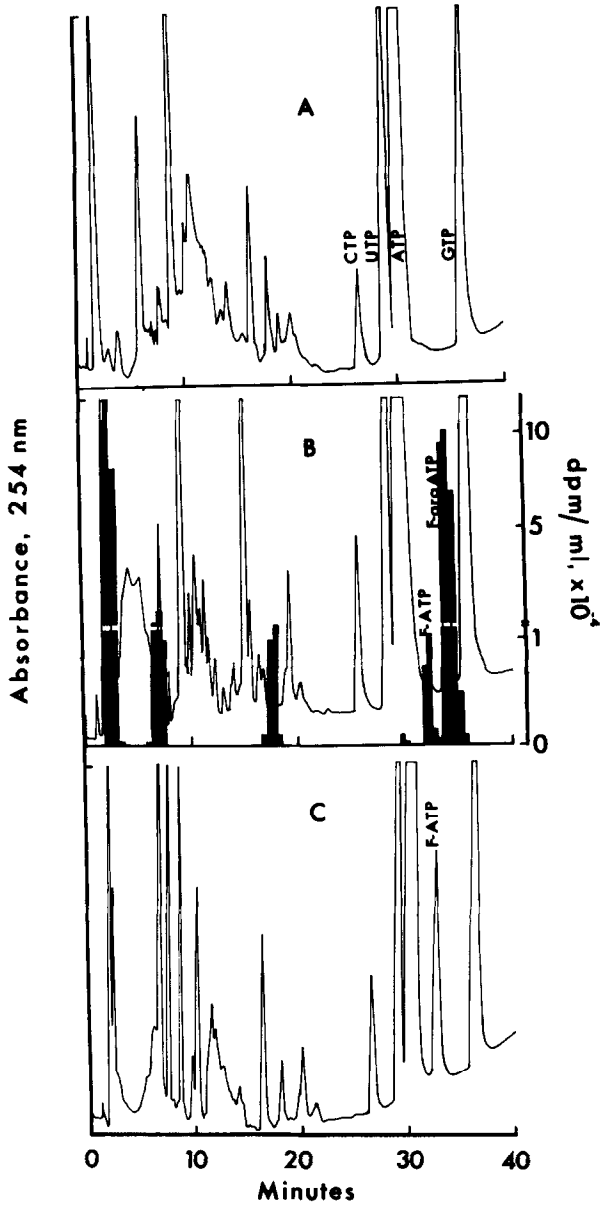

Figure 1. HPLC separation of P388 cell nucleotides. A. Chromatogram of nucleotides from 2.5 x 10^6 untreated cells. B. Separation of nucleotides from 5.0 x 10^6 P388 cells extracted after incubation with 400 μM [^{3}H]F-araA (specific activity, 1.24 x 10^5 dpm/nmol) for 4 hr. The radioactivity in fractions collected at 0.5-min intervals was determined by liquid scintillation counting. C. Separation of nucleotides extracted from 2.5 x 10^6 P388 cells after incubation with 2.5 μM F-Ado for 1 hr. Full-scale absorbance, 0.01 AU.

by Brockman et al (1), but was seen as a separate peak under the present conditions of pH 3.5 (Fig 1B). The compound accumulated to about 10% of the

cellular F-araATP level in P388 cells after they were incubated *in vitro* or treated *in vivo* with either F-araA or F-araAMP. A similar peak has been observed in extracts of host bone marrow and intestinal mucosa and has also been characterized in CCRF-CEM human lymphoblastoid cells after incubation with F-araA. P388 cells incubated with either F-Ade (not shown) or F-Ado (Fig 1C) readily been accumulated F-ATP, a compound that coeluted with the unknown radioactive peak in [^{3}H]F-araA-treated cells. Cells incubated with F-dAdo accumulated the respective 5'-triphosphate that eluted between F-araATP and GTP (not shown) under the present chromatographic conditions.

To evaluate the possibility that the unknown peak was F-ATP, a portion of an acid extract from a culture of cells incubated with [^{3}H]F-araA was treated with $NaIO_4$ in a manner known to destroy ribose-containing nucleotides without affecting deoxyribonucleotides and arabinosylnucleotides (2,14) (Table I). Subsequent chromatographic analysis of the periodate reaction products indicated that greater than 94% of the F-araATP was recovered, whereas less than 5% of the radioactivity originally associated with the unknown peak remained. This result is consistent with the likelihood that the carbohydrate portion of the unknown nucleoside triphosphate was ribose, as is the case for F-ATP.

Evidence for the identity of the base portion of the compound was obtained after collection of those portions of the chromatographic eluate associated with the unknown compound and with F-araATP, adsorption onto acti-

TABLE 1

Compound	dpm/10^7 cells before $NaIO_4$	after $NaIO_4$	recovery
F-araATP	100,856	94,871	94.1%
F-ATP	10,792	451	4.2%

Effect of $NaIO_4$ oxidation on nucleotides in cell extracts. P388 cells were incubated for 1 hr with 400 μM [^{3}H]F-araA (spec act 1.24 x 10^5 dpm/nmol). Cellular nucleotides were extracted and separated by HPLC before and after destruction of ribonucleotides by treatment with $NaIO_4$ (14). The radioactivity associated with the column eluate containing F-araATP and F-ATP was determined at 0.5-min intervals by liquid scintillation counting. The total radioactivity associated with each compound is presented.

vated charcoal to remove phosphate buffer, and dephosphorylation of the compounds with alkaline phosphatase. Subsequent separations of the dephosphorylation products of each compound by reverse-phase HPLC are shown in Fig 2. The column eluate associated with F-araATP (Fig 2B) resulted in a nucleoside product that coeluted with authentic F-araA (33 min). Radioactivity eluting between 10 and 13 min may have been either F-araAMP, an indication that the dephosphorylation reaction was not quantitative, or F-araHyp, a suggestion of the presence of adenosine deaminase in the alkaline phosphatase preparation. Dephosphorylation of the unknown peak resulted in a nucleoside product that coeluted with F-Ado (Fig 2C). We believe that the peak that eluted at 3 min is tritiated H_2O based on its absence after evaporation of the sample. The evidence that the radioactive metabolite of F-araA that accumulated in cells exposed to F-araA or F-araAMP and that eluted at 32 min was F-ATP may be summarized as follows. In our experience the compound arose only in cells exposed to fluoroadenine-containing compounds; cells treated similarly with arabinosyladenine did not accumulate a compound with similar properties. It eluted by anion-exchange HPLC in a region that is characteristic of triphosphates and coeluted with authentic F-ATP. The compound was sensitive to periodate oxidation, indicating that F-araA was metabolized by a pathway by which the arabinose moiety was either removed and subsequently replaced by ribose or, less likely, underwent isomerization to the riboside. Finally, analysis of the dephosphorylation product yielded a compound that coeluted with authentic F-Ado.

In contrast to expectations expressed early in the investigations of the metabolism of F-araA (1), subsequent studies of the disposition and metabolism of F-araA in mice, dogs and monkeys demonstrated that F-araA undergoes extensive catabolism (7,8). Prominent among metabolites were the product of the action of adenosine deaminase, F-araHyp, and also F-Ade. We have obtained similar results in mice and have detected both compounds after incubation of F-araA with homogenates of kidney, liver, and P388 cells. To evaluate pathways by which F-ATP might arise, P388 cells were incubated with either [^{3}H]-

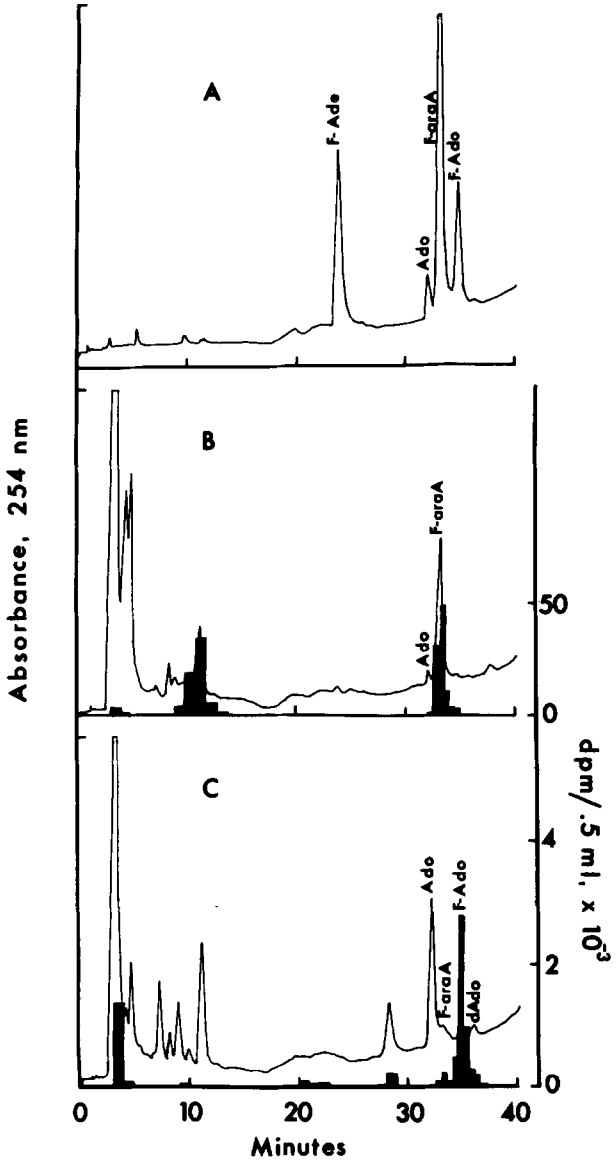

Figure 2. HPLC separation of alkaline phosphatase reaction products. P388 cells were incubated 4 hr with 400 μM [^{3}H]F-araA (specific activity, 1.24 x 10^5 dpm/nmol). Cellular nucleotides were separated by HPLC and the column eluates containing F-araATP and F-ATP were collected. Nucleotides were adsorbed to activated charcoal to remove inorganic phosphate (70% recovery) and dephosphorylated over 2 hr in reactions containing 0.4 units/ml alkaline phosphatase and 0.1 mmol/ml glycine, pH 9.0. Reaction products were separated by reverse phase HPLC as described in MATERIALS AND METHODS and radioactivity was quantitated by liquid scintillation counting A. Standard compounds. B. F-araATP dephosphorylation products. C. F-ATP dephosphorylation products. Full scale absorbance, 0.05 AU.

F-araHyp or F-Ade and the formation of F-ATP was monitored by HPLC (Table 2). A one-hour incubation with F-Ade resulted in the accumulation of 1800 pmol/10^7

TABLE 2

Compound	Concentration, μM	pmol of F-ATP/10^7 cells
F-Ade	20	1800
F-araHyp	30	< 4
	60	< 4

Accumulation of F-ATP by P388 cells. Cells were incubated with F-Ade, or [^{3}H]F-araHyp (spec act 7.5 x 10^3 dpm/nmol) for 1 hr. Nucleotides were extracted and separated by HPLC. The amount of F-ATP accumulated was quantitated by UV absorbance (F-Ade) or by collection of radioactivity ([^{3}H]F-araHyp).

cells, whereas, higher concentrations of F-araHyp were unable to support the production of any detectable F-ATP. Although no radioactivity was detected in the region of the chromatogram where F-ATP eluted at either concentration of [^{3}H]F-araHyp, if one assumes 30 dpm as a background level indicative of a lower limit of detection, less than 4 pmoles of F-ATP were formed. Thus, F-Ade supported the accumulation of more than 450 times the F-ATP that could be derived from F-araHyp. It is unclear whether 2-fluorohypoxanthine can be liberated from F-araHyp by purine nucleoside phosphorylase (16) or if 2-fluorohypoxanthine can serve as a substrate for hypoxanthine-guanine phosphoribosyl transferase (17). In parallel studies (Table 3), similar amounts of F-ATP accumu-

TABLE 3

Compound	Concentration, μM	pmol of F-ATP/10^7 cells[1]
F-araA	400	97.9± 4.3
F-araA plus 2'-deoxycoformycin	400 2	78.9±14.9
F-araA plus TCNR	400 1000	107.2± 4.5

Accumulation of F-ATP by P388 cells. Cells were incubated with [^{3}H]F-araA (spec. act., 2.2 x 10^4 dpm/nmol) alone or in the presence of either 2'-deoxycoformycin or TCNR for 1 hr. Control studies indicated that 2 μM 2-deoxycoformycin inhibited the deamination of arabinosyladenine by P388 cell homogenate by greater than 99.9% and that 1000 μM TCNR inhibited phosphorolysis of inosine by P388 cell homogenates by greater than 98.2% F-ATP was quantitated as described in Table 2. Repeated analyses of the medium after incubation with [^{3}H]F-araA without cells for 1 hr did not detect the presence of radioactive F-Ade.

[1] n = 3, mean ± SD. The means were not statistically different by 2-tailed t-test.

ulated in P388 cells incubated with [^{3}H]F-araA alone or in the presence of the adenosine deaminase inhibitor, 2'-deoxycoformycin. This result also suggests that F-ATP formation is independent of a pathway that involves F-araHyp.

Our results are consistent with the likelihood that F-ATP arises from a pathway that involves F-Ade. Purine nucleoside phosphorylase has only weak activity against adenine nucleosides (16), but the possibility exists that the fluorine at C-2 might alter the electronic properties of the purine ring of F-araA in such a way that the nucleoside analog could be a substrate for this enzyme. To investigate this possibility, TCNR, an inhibitor of purine nucleoside phosphorylase (18) was added to F-araA in the incubation mixture and F-ATP accumulation by P388 cells was determined. As shown in Table 3, no change was observed in the cellular concentration of F-ATP. Furthermore, prolonged incubation of F-araA with partially purified enzyme by us and others (6) did not result in the liberation of F-Ade.

The principal pathway for the formation of adenine in the cells is by the action of methylthioadenosine phosphorylase on methylthioadenosine, a byproduct of polyamine metabolism (19). However, the fact that CCRF-CEM cells that lack this enzyme (20) also accumulate F-ATP casts doubt on the importance of this reaction in F-Ade production. S-adenosylhomocysteine hydrolase may liberate some free base from nucleosides (21), but White et al (6) were unable to demonstrate the formation of F-Ade from F-araA by the enzyme from L1210 cells. Thus, although F-Ade is found in biological systems after treatment with F-araA and can serve as a precursor for F-ATP, the mechanism(s) by which F-Ade is evolved from F-araA remain(s) unknown. Similarly, further studies are needed to identify the contribution of F-ATP (22) to the cytotoxicity and therapeutic activity of F-araA.

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