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PHOSPHOROLYTIC CLEAVAGE OF 2-FLUOROADENINE FROM 9-β-D-ARABINOFURANOSYL-2-FLUOROADENINE BY *ESCHERICHIA COLI*

A PATHWAY FOR 2-FLUORO-ATP PRODUCTION*

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Abstract—2-Fluoroadenine (F-Ade) is a metabolite of 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) that may be involved in the development of toxic side effects from this anticancer drug. The liberation of F-Ade from F-ara-A has been examined in different biological systems. Extracts of *Escherichia coli* but not mammalian cells or tissues catalyzed the conversion of F-ara-A to F-Ade with apparent K_m and V_{max} values of 1350 μM and 7.7 nmol/min/mg protein respectively. This reaction depended on the presence of phosphate and was inhibited by purine nucleosides in a competitive manner, indicating that the enzyme responsible for the conversion is purine nucleoside phosphorylase. After incubation of intact bacteria with 100 μM [^{3}H]F-ara-A, [^{3}H]F-Ade was the same percentage of cellular radioactivity as in the medium, but it was only one-tenth the concentration of F-ara-A in the cells. In contrast, the cellular concentration of 2-fluoro-ATP was 20-fold greater than that of F-ara-A-5′-triphosphate. These results suggest that F-ara-A entered the bacteria intact and was phosphorolytically cleaved to liberate F-Ade, which would have been either anabolized to the toxic triphosphate or excreted. The latter pathway would provide a route by which F-Ade might be absorbed into the host circulation.

F-ara-A‡ is a purine nucleoside analog with potent antitumor activity in experimental systems [1–4]. This drug is phosphorylated to F-ara-ATP, an active metabolite which mediates cytotoxicity [1, 2, 4], presumably by inhibition of DNA polymerase and ribonucleotide reductase [5, 6]. Recent studies have shown that F-ara-A is incorporated into both DNA and RNA [7, 8] and exerts inhibitory effects on transcription and translation [9]. Since F-ara-A is relatively resistant to deamination by adenosine deaminase [1, 2, 10], it overcomes the drawback of ara-A, whose therapeutic activity is limited owing to rapid deamination by this enzyme [11, 12].

F-ara-AMP, the more soluble monophosphate formulation for clinical use, appears to be an active antitumor drug [13, 14]. However, clinical trials have shown that administration of F-ara-AMP may produce severe central nervous system toxicity, elevation of liver enzyme levels, and transient somnolence [15–18], all of which are dose dependent. Although the precise mechanism responsible for these undesirable side effects is not known, it is possible that F-Ade, a metabolite that is known to be liberated from F-ara-A [19, 20] and to accumulate as the toxic triphosphate F-ATP [21], may be involved. However, the metabolic pathway by which F-Ade is generated from F-ara-A is unclear. The objective of this study was to determine the mechanism by which F-Ade is liberated from F-ara-A in different biological systems.

MATERIALS AND METHODS

Materials. F-ara-A was provided by Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Adenosine, 2′-deoxyadenosine, and adenine were purchased from the Sigma Chemical Co., St. Louis, MO. [8-^{3}H]F-ara-A was prepared by Moravek Biochemicals, Inc., Brea, CA (sp. Act. 11 Ci/mmol). [2,8-^{3}H]Adenosine was the product of ICN, Chemical & Radioisotope Division, Irvine, CA (sp. act. 40 Ci/mmol). Radioactive F-ara-A was purified by HPLC [4, 21] to more than 99.9% purity and then mixed with unlabeled F-ara-A to the desired specific activity before each experiment. F-Ade and 2-fluoroadenosine were provided by Dr. T. Krenitsky (Wellcome Research Laboratories, Research Triangle Park, NC). F-ara-ATP was chemically synthesized in our laboratory by Dr. Ladislav Novotný. F-ATP was obtained by incubation of CCRF-CEM cells with 10 μM 2-fluoroadenosine for 2 hr and 0.4 N PCA extraction. Following neutralization with KOH, this preparation

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‡ Abbreviations: F-Ade, 2-fluoroadenine; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-AMP, 9-β-D-arabinofuranosyl-2-fluoroadenine-5′-monophosphate; F-ara-ATP, 9-β-D-arabinofuranosyl-2-fluoroadenine-5′-triphosphate; F-ATP, 2-fluoroadenosine-5′-triphosphate; PCA, perchloric acid; and PNPase, purine nucleoside phosphorylase.

was used as a marker of F-ATP for HPLC analysis [21].

Bacterial culture and cell extract preparation. The *Escherichia coli* strain W3110 (Trp^-, Val^-) was provided by Dr. Priscilla Saunders of this department. Bacteria were grown in liquid medium containing 0.2% $(NH_4)_2SO_4$, 1.4% KH_2PO_4, 0.6% K_2HPO_4, 0.1% sodium citrate, 0.0075% $MgSO_4$, 0.5% glucose, 0.004% tryptophan, and 0.004% valine. The cells were grown to late exponential phase, harvested by centrifugation, washed with 50 mM Tris–HCl (pH 7.4), and resuspended in 4 vol. of either 50 mM Tris–HCl buffer (pH 7.1) or phosphate buffer (33 mM NaH_2PO_4, 67 mM Na_2HPO_4, pH 7.1). The suspension was sonicated and then centrifuged at 12,000 *g* for 15 min at 4°. The supernatant fraction was dialyzed against either Tris–HCl buffer (50 mM, pH 7.1) or phosphate buffer (100 mM, pH 7.1) for 16 hr at 4°. This preparation was used in enzyme assays. Protein concentrations in the cell extracts were determined by the method of Bradford [22], using bovine serum albumin as a standard.

Preparation of mammalian cell extracts. Human leukemia cells (line CCFR-CFM) were grown to a density of 5×10^5 cells/ml, washed twice with cold phosphate buffer, resuspended in 3 vol. of phosphate buffer, sonicated in an ice bath, and then centrifuged at 12,000 *g* for 15 min. The supernatant fraction was used in enzyme assays. Fresh livers and kidneys were taken from mice and rats immediately after the animals were killed. The tissues were washed with phosphate buffer, mechanically homogenized in 4 vol. of phosphate buffer, sonicated, and centrifuged at 12,000 *g* for 15 min at 4°. The supernatant fractions were used in enzyme assays.

Enzyme assays. The ability to liberate F-Ade and adenine from F-ara-A and adenosine, respectively, was determined in extracts of bacteria. Except where otherwise indicated, assays contained in a volume of 1 ml: 30 mM sodium phosphate buffer, pH 7.1, 10–150 μg *E. coli* extract protein, various concentrations of substrates (F-ara-A or adenosine), and certain inhibitors as specified in the text. In some experiments, phosphate buffer was replaced with 50 mM Tris–HCl buffer (pH 7.1) in the reaction mixture. Reaction mixtures were incubated at 37°, and the reactions were stopped by addition of 10 N PCA to a final concentration of 0.4 N. After being chilled in an ice bath for 10 min, the acid-soluble material was recovered by centrifugation and was neutralized with KOH. The $KClO_4$ precipitate was removed by centrifugation, and the supernatant fraction was analyzed for F-Ade and F-ara-A or adenine and adenosine by HPLC as described below. A computer-assisted program was used to determine the enzyme kinetic parameters [23]. Student's *t*-test for the comparison of two slopes was employed to evaluate the effect of adenosine on the kinetics of F-ara-A phosphorolysis.

HPLC. The purine bases, purine nucleosides, and their analogs were separated using a Waters Associates ALC-204 high-pressure liquid chromatograph equipped with two model 6000A pumps, a model 660 solvent programmer, and a column of μBondapak C_{18}. A concave gradient (curve 9 on the solvent programmer) from 10% to 25% methanol was run at a flow rate of 1 ml/min for 20 min, and then a 5-min isocratic elution with 25% methanol was performed. Eluted compounds were detected by their absorbance at 254 nm by the model 440 detector. The eluate was fractionated at 1-min intervals and collected in scintillation vials containing 10 ml of Safety Solve (Research Products International Corp., Mount Prospect, IL). The radioactivity in each vial was determined in a Packard liquid scintillation spectrometer at an average counting efficiency of 50%.

Determination of F-ara-ATP and F-ATP levels. E. coli cultures were grown to late exponential phase, incubated with [^{3}H]F-ara-A (100 μM, sp. act. 2.27×10^4 dpm/nmol) at 37° for 60 min, and harvested by centrifugation. The culture medium was precipitated with 0.4 N PCA, neutralized with KOH, and analyzed for F-Ade and F-ara-A by HPLC as described above. The cell pellet was washed twice with phosphate buffer, resuspended in 3 vol. of water, sonicated, extracted with 0.4 N PCA, and neutralized with KOH. This cell extract was analyzed for F-Ade and F-ara-A by HPLC as described above. The amounts of F-ara-ATP and F-ATP in the extract were determined by HPLC using an anion-exchange column of Partisil 10-SAX (Whatman, Inc., Clifton, NJ). Nucleotides were eluted from the column initially with 60% 0.005 M $NH_4H_2PO_4$ (pH 2.8) and 40% 0.75 M $NH_4H_2PO_4$ (pH 3.5) at a flow rate of 2 ml/min for 10 min, and then an increasing gradient (curve 7 on the solvent programmer) to 100% 0.75 M $NH_4H_2PO_4$ (pH 3.5) was run over 30 min. The eluted nucleotides were detected by their absorbance at 254 nm by the model 440 detector. The retention times for F-ATP and F-ara-ATP were 32.9 and 35.6 min, respectively, whereas the bordering peaks of ATP and GTP eluted at 28.8 and 37.6 min. The HPLC eluate was collected in scintillation vials at 0.5-min intervals, and the radioactivity was determined by liquid scintillation counting.

RESULTS

The liberation of F-Ade from F-ara-A was first tested by incubating extracts of CCRF-CEM cells, liver, and kidney with [^{3}H]F-ara-A (100 μM) in 30 mM phosphate buffer (pH 7.1). After incubation periods up to 60 min, the reaction mixtures were extracted with 0.4 N PCA and analyzed for [^{3}H]F-Ade. No significant production of F-Ade was detected in any cell or tissue extracts (data not shown). The lower limits of detection were 2.6, 1.1, and 0.8 pmol of F-Ade evolved/min/mg protein for the CCRF-CEM cells, liver and kidney extracts respectively.

Because it was possible that an enterohepatic secretion and reabsorption route that made the drug available to bacteria could generate F-Ade, we next investigated the metabolism of F-ara-A by a common strain of intestinal bacteria. An exponentially growing culture of *E. coli* was incubated with 100 μM [^{3}H]F-ara-A for 60 min. HPLC analysis was used to detect F-Ade in the culture medium and in the extracts of bacteria. The HPLC separation of F-ara-A and F-Ade, and the radioactivity associated with each peak are shown in Fig. 1. In the *E. coli* extract,

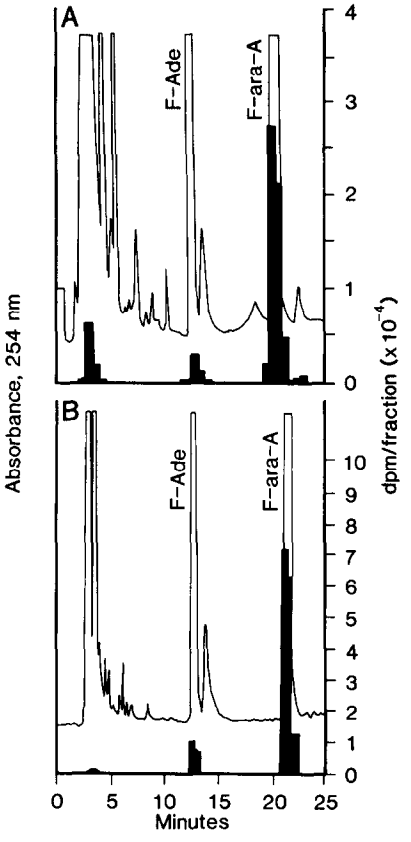

Fig. 1. HPLC separation of F-Ade and F-ara-A from *E. coli* (A) and the culture medium (B). A culture of *E. coli* was labeled with [^{3}H]F-ara-A (100 μM, sp. act. 2.27×10^4 dpm/nmol) for 1 hr, washed and harvested. The culture medium and bacteria were extracted with PCA and separated by HPLC as described in Materials and Methods. Fractions of the eluate were collected, and radioactivity was quantitated by liquid scintillation counting.

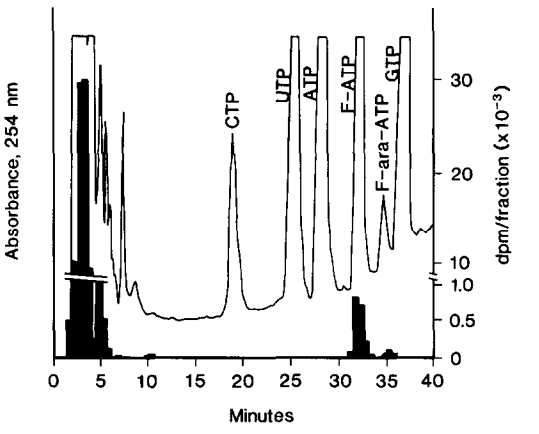

Fig. 2. HPLC separation of nucleotides from extracts of *E. coli*. A culture of *E. coli* was labeled with [^{3}H]F-ara-A, washed, and extracted as described in Fig. 1. The extract was separated by HPLC, and the radioactivity in the eluate was determined by liquid scintillation counting.

Table 1. Formation of F-Ade, F-ATP, and F-ara-ATP from F-ara-A by *E. coli**

Compound	Amount formed (nmol/ml extract)	Ratio
F-ara-ATP	0.04 ± 0.01	0.002†
F-ara-A	24.06 ± 0.35	
F-ATP	0.78 ± 0.11	0.448‡
F-Ade	1.75 ± 0.09	

* After a culture of bacteria was incubated with 100 μM [^{3}H]F-ara-A for 1 hr, cells were extracted with 0.4 N PCA, and 100 μl of the extract was analyzed for metabolites by HPLC as described in Materials and Methods. Each value (mean ± SD) is the average of three determinations.

† Ratio of F-ara-ATP to F-ara-A.

‡ Ratio of F-ATP to F-Ade.

7% of the total radioactivity coeluted with authentic F-Ade (Fig. 1A), while 8.9% of the total radioactivity in the culture medium was associated with F-Ade (Fig. 1B). When the same *E. coli* extract was analyzed further for F-ara-ATP and F-ATP by HPLC, F-ATP was found in 20-fold excess over F-ara-ATP (Fig. 2). In an equal amount of *E. coli* extract, the ratio of F-ATP to F-Ade was 0.448, whereas that of F-ara-ATP to F-ara-A was 0.002 (Table 1). These results suggest that, following entry of F-ara-A into the cells, the liberation of F-Ade was the primary metabolic pathway by which F-ATP was generated, whereas the route that involved direct phosphorylation of the nucleoside to the monophosphate was much less active. It is of importance that substantial quantities of F-Ade were excreted by the cell into the medium.

The ability of *E. coli* extracts to catalyze the conversion of F-ara-A to F-Ade was evaluated further by *in vitro* experiments. Various amounts of extract were added to the enzyme assay system in the presence of phosphate and the concentrations of F-ara-A that ranged between 10 and 400 μM. The production of F-Ade increased in proportion to the amount of extract protein added to the reaction mixture, the F-ara-A concentrations, and the incubation times up to 45 min (data not shown).

To evaluate the possibility that liberation of F-Ade proceeded by a phosphorolytic mechanism, the phosphate requirement of the reaction was investigated (Table 2). When phosphate was eliminated from *E. coli* extract by extensive dialysis against Tris–HCl buffer and no phosphate was added to the reaction mixture, no significant production of F-Ade was detected. However, enzymatic activity was restored when phosphate was added to the reaction mixture containing the dialyzed extract. Varying the concentrations of phosphate in the assay system indicated that the optimal phosphate concentration for the reaction was between 10 and 30 mM (data not shown). When the same extract was analyzed for ability to catalyze phosphorolysis of adenosine to adenine, significant production of adenine was also detected (data not shown).

Kinetic studies of the reaction indicated apparent K_m values for F-ara-A and adenosine to be 1.35 and 0.10 mM, whereas the apparent V_{max} values were 7.7 and 253.6 nmol/min/mg for the respective

Table 2. Phosphate dependence of the conversion of F-ara-A to F-Ade by *E. coli* extract*

Sample No.†	F-ara-A‡ (μl)	*E. coli* extracts§ (μl) A	*E. coli* extracts§ (μl) B	Phosphate buffer‖ (μl)	Tris–HCl¶ (μl)	F-Ade (nmol/tube)
1	30	100		870		2.898
2	30		100		870	0.096
3	30		100	300	570	1.732
4	30			970		0.058

* The incubation time was 60 min.
† The volume of each reaction mixture was 1 ml/tube.
‡ The final concentration of F-ara-A was 42 μM.
§ The *E. coli* extracts were dialyzed against either phosphate buffer (A) or Tris–HCl buffer (B). The final concentration of *E. coli* protein in the reaction mixture was 188 μg/ml.
‖ Phosphate buffer, 50 mM, pH 7.1.
¶ Tris–HCl buffer, 50 mM, pH 7.1.

Table 3. Conversion of F-ara-A to F-Ade in the presence of other nucleosides*

Sample	Concn. (μM)	F-Ade produced (nmol/min/mg protein)	% of Control
Control (1)		0.2261	100
Adenosine	50	0.0938	41.5
	100	0.0436	19.3
Control (2)		0.2490	100
2′-Deoxyadenosine	50	0.1266	50.8
	100	0.0796	32.0
Adenine	50	0.1266	50.8
	100	0.0884	35.5
Control (3)		0.2259	100
Guanosine	50	0.1353	59.9
	100	0.0695	30.8
2′-Deoxyguanosine	50	0.1276	56.5
	100	0.1102	48.8
Inosine	50	0.1352	59.8
	100	0.0643	28.5

* The reaction mixtures contained 40 μM F-ara-A as a substrate, 30 mM phosphate, *E. coli* extract (150 μg protein), and inhibitors as indicated above.

substrates. The abilities of various natural nucleosides to inhibit the reaction that generates F-Ade were determined in order to define better the nature of this reaction. The conversion of 40 μM F-ara-A to F-Ade by *E. coli* extract in the presence of 50 or 100 μM adenosine, 2′-deoxyadenosine, adenine, guanosine, 2′-deoxyguanosine, and inosine is shown in Table 3. The reaction was inhibited in a dose-dependent fashion. Among the compounds tested, adenosine was the most potent inhibitor. In contrast, various concentrations of F-ara-A (up to 260 μM) did not inhibit significantly the phosphorolysis of 20 μM adenosine to adenine (data not shown).

To further understand the nature of these inhibitory effects, the action of adenosine on the kinetics of F-Ade formation was studied (Fig. 3). Kinetic analysis showed that, in the presence of 50 μM adenosine, the apparent V_{max} was 9.41 nmol/min/mg and the apparent K_m was 4.00 mM, whereas in the presence of 100 μM adenosine the apparent V_{max} was 7.50 nmol/min/mg, and the apparent K_m was 5.16 mM. Without inhibitor, the V_{max} was 7.74 nmol/min/mg, and the K_m was 1.35 mM. These parameters strongly suggest that adenosine is a competitive inhibitor, since the apparent K_m was increased significantly ($P < 0.001$) with increasing concentrations of inhibitor, while the apparent V_{max} remained unchanged ($P > 0.7$).

DISCUSSION

The objective of this investigation was to elucidate the metabolic pathway by which F-Ade is evolved. We [21] and others [6] have reported that incubation of F-ara-A with partially purified purine nucleoside phosphorylase from mammlian cells did not result in the liberation of F-adenine. This paper further shows that, although extracts of eukaryotic cells and mouse tissues failed to catalyze the conversion of F-ara-A to F-Ade *in vitro*, preparations from *E. coli* exhibited significant enzyme activity in this reaction. We postulate that the activity responsible is the bacterial enzyme, PNPase. Our findings that the activity depended on the presence of phosphate, and that the reaction was inhibited by purine nucleosides in a competitive manner, are consistent with this

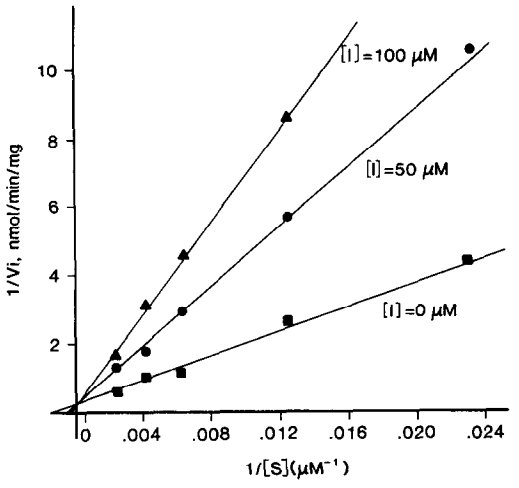

Fig. 3. Kinetics of conversion of F-ara-A to F-Ade in the presence or absence of adenosine. A double-reciprocal plot of reaction velocity versus the substrate (F-ara-A) concentrations. V_i, initial velocity; [*S*], substrate concentration; [*I*], adenosine concentration.

hypothesis. Metabolic studies using mutants that lack this enzyme would provide further evidence to define this possibility.

We reported previously that F-ATP was detected in CCRF-CEM cells after incubation with [^{3}H]F-ara-A [21]. However, this result could not be reproduced in the present study which employed [^{3}H]F-ara-A that had been extensively purified immediately prior to use. It is possible that the [^{3}H]F-ara-A used in making the previous observations was contaminated with traces of [^{3}H]F-Ade that were converted to F-ATP. Evaluations of this cell line for mycoplasma contamination throughout the period spanning the two studies have been negative [24].

The findings that extracts of bacteria but not mammalian cells catalyzed the conversion of F-ara-A to F-Ade are not surprising. It has been documented that PNPase from *E. coli* and *Salmonella typhimurium* is capable of using a broad spectrum of purine nucleosides, including adenosine, guanosine, and inosine as substrates [25, 26]. PNPase from *Enterobacter aerogenes* uses purine arabinosides as well as purine ribosides as substrates [27]. On the other hand, adenosine is not a substrate for PNPase from eukaryotic cells [28, 29]. Attempts to generate F-Ade from F-ara-A by incubation with mammalian PNPase have not been successful [6, 21]. Therefore, it is not difficult to imagine that F-ara-A may serve as a substrate for PNPase from *E. coli*, but not for the enzyme of mammalian cells.

The transport and catabolism of purine nucleosides in bacteria are complicated events. It has been suggested that in membrane vesicles of *E. coli* the PNPase may act as mediator for the transport of purine nucleosides by cleaving purine nucleosides to purine bases which are then transported into the cell by membrane phosphoribosyltransferase [30]. However, recent studies with *E. coli* mutants that lack PNPase have shown that transport occurs prior to phosphorolysis [31, 32]. Our observation that F-ara-A was in 13-fold excess over F-Ade (Table 1) in the *E. coli* extract is consistent with the evidence indicating that phosphorolysis and transport of F-ara-A are independent events. The fact that F-ATP is the predominant phosphorylated metabolite suggests that the phosphorolysis of the nucleoside and subsequent salvage of the base is a more efficient process than is direct phosphorylation of the nucleoside. However, excretion of the base by bacteria with competent phosphoribosyltransferase indicates that the pathway may not be tightly coupled at this point. Excretion of F-Ade from bacteria may be the route by which this toxic base analogue is presented for reabsorption by the host.

Generation of F-Ade from F-ara-A may be of clinical importance. F-Ade is an extremely toxic agent [33, 34]. The maximum dose tolerated by mice on a single dose schedule is 11 mg/kg [20] compared to a value of 1500 mg/kg for F-ara-AMP [35, 36]. Accumulation of F-Ade in kidney, liver, gastrointestinal tract, and other tissues has been found in experimental animals [20]. Kidney and bladder toxicity has been related to the accumulation of F-Ade in these tissues in dogs administered with therapeutic doses of F-ara-A [19]. Recently, F-Ade has been detected in human urine during phase I trials [37]. Although mammalian cells themselves may not possess enzymes to catalyze the phosphorolysis of F-ara-A, F-Ade may arise and become available by an enterohepatic circulation. Secretion of F-ara-A in bile may provide access of the drug to bacteria flora which may then metabolize it and excrete free F-Ade. The F-Ade released from bacteria may be absorbed into the bloodstream and carried to other tissues where it could be converted to the monophosphate by adenine phosphoribosyltransferase [34] and subsequently accumulate as the toxic F-ATP [21]. Further experiments in animals are required to substantiate this hypothesis. In the meantime, consideration should be given to the utility of gut sterilization prior to clinical use of F-ara-AMP as a possible means of reducing toxic side effects of this promising anticancer drug [38, 39].

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