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# IDENTIFICATION OF METABOLITES OF 9-β-D-ARABINOFURANOSYL-2-FLUOROADENINE, AN ANTITUMOR AND ANTIVIRAL AGENT*

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**Abstract**—Analysis of blood from a dog given a 400 mg/m$^2$ dose of 9-β-D-arabinofuranosyl-2-fluoroadenine (2-F-araA) led to the identification of parent drug and a major metabolite, 9-β-D-arabinofuranosyl-2-fluorohypoxanthine. 2-Fluoroadenine, a toxic derivative of 2-F-araA, was not detected in blood within the limits of detection, suggesting that parent drug was absorbed and distributed without systemic exposure to this toxic derivative. Parent drug, 2-fluoroadenine, and 9-β-D-arabinofuranosyl-2-fluorohypoxanthine were identified in urine of dog, monkey, and mouse.

9-β-D-Arabinofuranosyladenine (araA) is an effective antiviral agent [1], but it has demonstrated little activity against experimental tumors [2]. AraA is a good substrate for adenosine deaminase [3], which converts it *in vivo* to the tumor-inactive 9-β-D-arabinofuranosylhypoxanthine (araH) [2]. However, in combination with 2′-deoxycoformycin (2′-DCF), an effective inhibitor of adenosine deaminase *in vivo* [4], araA becomes an active antitumor agent [5], and both antitumor activity and inhibition of DNA synthesis correlate with increased levels of araATP in leukemia cells in mice treated with this combination [6].

The observation that 2-fluoroadenosine [7] is not a substrate for adenosine deaminase [3] led to the synthesis of 9-β-D-arabinofuranosyl-2-fluoroadenine (2-F-araA) [8, 9], an agent that is a poor substrate for adenosine deaminase but is comparable in experimental antitumor activity to the combination of araA plus 2′-DCF [10].

2-F-AraA is phosphorylated by deoxycytidine kinase [11], and levels of 2-F-araATP in L1210 cells *in vivo* are comparable to, or higher than, levels of araATP in cells treated with araA plus 2′-DCF [6, 10]. Because of the potential clinical utility of 2-F-araA as an alternative to the combination of araA and 2′-DCF, an investigation of the metabolism of 2-F-araA in several mammalian species was performed. Its disposition in mice, dogs, and monkeys was reported recently [12].

## MATERIALS AND METHODS

Radioactive samples were counted in Aquasol (New England Nuclear Corp., Boston, MA) in a Packard Tri-Carb model 3315 liquid scintillation spectrometer with external standard. Thin-layer plates (analytical and preparative) precoated with silica-gel G were purchased from Analtech, Newark, DE, and were activated for 1 hr at 100° and stored in a dessicated cabinet. Radiochromatogram scanning was performed with a Packard model 7220/21 scanner.

2-Fluoroadenine (2-F-Ad), 2-fluorohypoxanthine and 2-F-araA were obtained as reported previously [8, 9, 13]. Labeling of 2-F-araA was performed by the New England Nuclear Corp. by exposure to tritium gas in the presence of rhodium catalyst. Purification was accomplished in our laboratories by preparative thin-layer chromatography (TLC) in chloroform–methanol (3:1, v/v). A sample of greater than 98% chemical and radiochemical purity was obtained as determined by high-pressure liquid chromatography (HPLC) and TLC respectively. Specific activity was 6.8 mCi/mmole.

Methylation of blood metabolites was performed by addition of excess ethereal diazomethane to a methanol extract of blood, allowing the solution to stand for 5 min at room temperature, and evaporating it to dryness in a stream of nitrogen in a hood. Ethereal diazomethane was prepared from Diazald (Aldrich Chemical Co., Milwaukee, WI) by a procedure supplied with the reagent.

Animals used in this study were male BDF$_1$ mice weighing 25–31 g, female beagle dogs weighing 9.7–10.3 kg, and female rhesus monkeys weighing 3.9–4.6 kg. All animals were denied food overnight but allowed water *ad lib.* prior to administration of the drug. A dose of 400 mg/m$^2$ of [$^3$H]-2-F-araA was injected intravenously (i.v.) into the tails of mice and into the brachial or saphenous veins of dogs and monkeys. Urine from mice, dogs, and monkeys was collected by catheter; blood was collected from the cephalic, brachial, and saphenous veins of dogs. Freezing and storage had no effect on the formation of metabolites or the degradation of the parent compound.

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High-pressure liquid chromatography was performed on a Waters Associates (Walthan, MA) ALC-242 chromatograph with a u.v. detector at 254 nm using a 3.9 mm × 30 cm Waters reverse-phase μBondapak $C_{18}$ column (10 μm particle size) with a linear-convex gradient (Waters gradient 9) of 0.01 M $NH_4H_2PO_4$ (pH adjusted to 5.1 with $NH_4OH$) to 0.01 M $NH_4H_2PO_4$ (pH 5.1)–methanol (75:25, v/v) in 20 min at ambient temperature at a flow rate of 1 ml/min. Ultraviolet spectra were recorded at pH 5.1 on a Beckman model 25 spectrophotometer equipped with a Waters microcell. When HPLC fractionation was performed, 1-ml fractions were collected.

Mass spectra were obtained with a Varian MAT 311A spectrometer equipped with a combination electron impact/field ionization/field desorption ion source.

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-100 spectrometer.

Rabbit muscle adenosine deaminase (Schmidt's deaminase) was obtained from the Sigma Chemical Co., St. Louis, MO. This preparation had a specific activity of 61 units/mg protein.

Antitumor evaluation was performed according to protocols established by the National Cancer Institute.

## RESULTS AND DISCUSSION

After i.v. administration of [$^3$H]-2-F-araA to a dog, total radioactivity in blood remained essentially constant for the first 3 hr. For detection of possible metabolites, blood (1 ml) collected 1 hr after drug administration was triturated with 10 ml methanol and was filtered, giving an extract and a residue containing 95% and 5% of the total radioactivity respectively. The methanol extract (100 μl) was fractionated by HPLC, and radioassay of the fractions indicated six radioactive components. However, only two of the six amounted to greater than 0.5% of the total, and these two components accounted for over 98% of the total radioactivity in the blood extract, as shown in Table 1. HPLC analysis of methanol extracts of blood taken at times up to 8 hr revealed this same qualitative pattern of metabolites, with a decrease in the major component (peak 6) and a relative increase in peak 3 while the other radioactive components remained < 0.5%. The major component (76.8%) was identical, by HPLC retention time, to starting material. The other component was suspected of being a modified nucleoside or possibly a nucleotide. Nucleotides were eliminated from consideration by the observation that HPLC retention time of the major blood metabolite was not altered upon treatment with the methylating agent diazomethane. A remaining, reasonable candidate for the structure of the metabolite was 9-β-D-arabinofuranosyl-2-fluorohypoxanthine (2-F-araH), arising through deamination of the starting material.

A sample of this proposed metabolite was synthesized by dropwise addition of sodium nitrite (200 mg) in 1 ml water to 2-F-araA (100 mg) in 5 ml of 4 N hydrochloric acid at 0°. The mixture was stirred for 5 min in the cold, neutralized with aqueous potassium hydroxide to pH 7, stirred for 30 min at room temperature, and centrifuged to precipitate a solid. Lyophilization of the supernatant solution gave a solid, which was extracted with methanol (10 ml) and filtered. Evaporation of the filtrate gave crude 2-F-araH, which was purified by preparative TLC in acetone–chloroform (3:1, v/v) ($R_f$ 0.0) to remove 2-F-araA followed by chloroform–methanol (1:1) ($R_f$ 0.8). Isolation from the silica gel plates in both separations was accomplished by elution with methanol of the appropriate u.v.-absorbing, TLC bands, yielding 10 mg of 2-F-araH. The $^1$H-NMR$_{DMSO}$ spectrum (ppm) of the synthetic product was in agreement with that expected for 2-F-araH: 3.5–3.9 (multiplet; 3-H; $C_5'$-$CH_2$, $C_4'$-CH), 4.0–4.4 (multiplet; 2-H; $C_2'$-CH, $C_3'$-CH), 6.11 (doublet; 1-H; $C_1'$-CH; $J_{1',2'}$ = 4 Hz), 8.25 (singlet, 1-H, $C_8$-CH).

Biosynthesis of 2-F-araH was accomplished in quantitative yield by reaction of 10 mg of 2-F-araA with adenosine deaminase (0.35 unit) in water for 48 hr at room temperature, yielding a single product of 99% purity as determined by HPLC analysis. Methanol extraction (2 × 20 ml) of the lyophilized reaction mixture served to solubilize the product and denature the enzyme protein, which was then removed by filtration. Evaporation of the extracts gave a white powder (10 mg). This procedure was performed on a larger scale (500 mg 2-F-araA, 2000 units of adenosine deaminase) to produce sufficient material (350 mg) for tumor evaluation *in vivo*. The $^1$H-NMR spectrum of the biosynthetic product was identical to that of the synthetic product described above. In addition, a $^{13}$C-NMR$_{DMSO}$ spectrum was also consistent with that expected for 2-F-araH: 60.82 ($C_5'$), 74.97 and 75.58 ($C_2'$ and $C_3'$), 83.51 and 83.95 ($C_4'$ and $C_1'$), 120.91 ($C_5$, $^4J_{C_5F}$ = 8 ± 3Hz), 138.22 ($C_8$, J not resolved), 149.58 ($C_4$, $^3J_{C_4F}$ = 23 ± 3Hz), 157.27 ($C_2$, $^1J_{C_2F}$ = 215 ± 3 Hz), 163.60 ($C_6$, $^3J_{C_6F}$ = 12 ± 3 Hz). Mass spectral analysis of the biosynthetic product confirmed the structure as 2-F-araH. The field desorption mass spectrum showed two

Table 1. Radioassay of HPLC fractions of a methanol extract of blood from a dog treated with a therapeutic dose (400 mg/m$^2$) of [$^3$H]-2-F-araA

| Radioactive peak | Retention time (min) | Percent total | Blood concn (μg/ml) |
|---|---|---|---|
| 1 | 4.44 | 0.37 | 0.06 |
| 2 | 5.81 | 0.33 | 0.05 |
| 3 | 10.13 | 21.82 | 3.5 |
| 4 | 18.40 | 0.33 | 0.05 |
| 5 | 23.46 | 0.34 | 0.05 |
| 6 | 26.10 | 76.81 | 12.0 |

peaks, one of *m/e* 286, corresponding to the molecular ion ($M^+$); and one of *m/e* 287, corresponding to $(M + 1)^+$; relative abundance of the two peaks was identical. Since it was possible that the peak of *m/e* 286 was arising from trace contamination with 2-F-araA, its field desorption mass spectrum was recorded. The results indicated that 2-F-araA yields *m/e* 285 ($M^+$) as the dominant ion and that the $(M + 1)^+$ ion is only one-third as abundant as $M^+$. Consequently, the absence of *m/e* 285 in the mass spectrum of 2-F-araH indicated that *m/e* 286 is characteristic of 2-F-araH.

Comparison of HPLC retention times of synthetic 2-F-araH, biosynthetic 2-F-araH, and the major metabolite in dog blood indicated that the three samples were identical. Further confirmation was provided by u.v. analysis of the HPLC peak of each of the three samples. Each u.v. spectrum revealed a maximum at 252 nm and a shoulder of 260 nm.

Urine collected during the period of 3–4 hr after administration of 2-F-araA to a dog was clarified by filtration through Celite filter aid. TLC of whole urine was used to isolate 2-F-Ad, 2-F-araA, and 2-F-araH for confirmation of HPLC retention times. Development along with synthetic standards in acetone–chloroform (3:1) to 10 cm moved 2-F-Ad to $R_f$ 0.65 and 2-F-araA to $R_f$ 0.45 while 2-F-araH remained at the origin (Fig. 1A). Development of the same TLC plate in methanol to 3 cm moved 2-F-araH to 2 cm and left only a trace of activity at the origin (Fig. 1B). The three components, which had $R_f$ values identical to the synthetic standards, were isolated by elution of the appropriate band with methanol. HPLC analysis confirmed the identity of retention times of the isolated fractions with those of the corresponding synthetic standards. An electron-impact mass spectrum of the urine fraction of 2-F-Ad was identical to that of the standard. 2-F-Ad gave *m/e* 153 ($M^+$) as the base peak with a major fragment of *m/e* 133 ($[M\text{-}HF]^+$) of 52% relative abundance. These data demonstrate that this metabolite was 2-F-Ad and not 2-fluorohypoxanthine, which yielded *m/e* 154 ($M^+$) and *m/e* 134 ($[M\text{-}HF]^+$) upon mass spectral analysis, HPLC retention time of 5.81 min compared to 23.46 min for 2-F-Ad under conditions described in Materials and Methods, and a u.v. maximum of 257 nm compared to 262 nm for 2-F-Ad at pH 5.1.

2-F-araH and 2-F-Ad were also identified by this procedure in urine from a monkey and from a mouse given 2-F-araA. Identification was based on TLC, HPLC, and u.v. analysis.

In terms of the total dose (400 mg/m$^2$) excreted by a dog during the 3 to 4-hr collection period (8.5%), this corresponds to 6.5% 2-F-araH, 1.9% 2-F-araA, and 0.1% 2-F-Ad. For 24-hr collection, 73.6% of the total dose was excreted with 51.9% as 2-F-araH, 12.8% as 2-F-araA, and 8.9% as 2-F-Ad. Total excretion (24 hr) for mice and monkeys of this high dose was 13.4% and 24.8% 2-F-araH, 63.0% and 58.3% 2-F-araA, and 0.4% and 0.1% 2-F-Ad, respectively.

To determine if 2-F-araH contributes to the antileukemic effect of 2-F-araA, 2-F-araH was evaluated in $CDF_1$ mice against an inoculum of $10^5$ L1210 leukemia cells at a dose of 200 mg per kg per dose by the intraperitoneal route on days 1–9. No antitumor activity and no gross toxicity (weight loss) were observed. 2-F-AraA under identical conditions gave > 100% increase in life span and 10–20% survivors.

Data obtained from this and our companion study [12] indicate that some deamination of 2-F-araA to 2-F-araH occurs in tissues of dogs, mice, and monkeys. Studies to identify the enzyme responsible for the deamination of 2-F-araA and the cleavage of 2-F-araA to 2-F-Ad are in progress. Although 2-F-Ad was not present in dog serum in high concentration (< 0.5%, 50 ng/ml), the toxicity of 2-F-Ad to a mammalian cell line ($ED_{50}$ = 5ng/ml) [14] is such that this metabolite may contribute to the overall toxicity of the drug, such as that described below. Deglycosidation of 2-F-araA in the dog apparently occurs only in the kidney. As shown in our earlier report [12] which describes the quantitative aspects of 2-F-araA deamination and urinary excretion, and

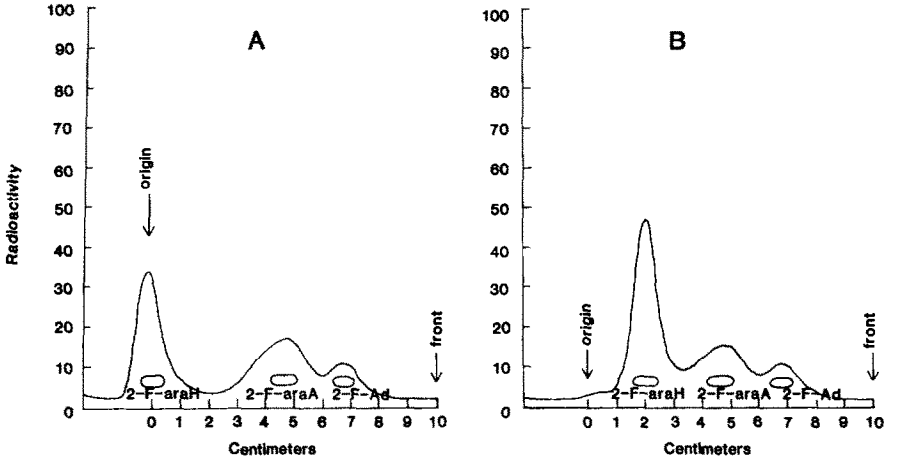


Fig. 1. Radiochromatogram scans of TLC of dog urine (3–4 hr) in acetone–chloroform (3:1, 10 cm) (A) followed by methanol (3 cm) (B).

as illustrated above, dogs consistently metabolized more 2-F-araA than mice and monkeys and excreted much higher levels of 2-F-Ad in urine. Consequently, production of 2-F-Ad and attendant exposure of kidney and bladder to this metabolite is of greater importance for dogs than for the other species used in this study. It is not yet known whether humans would metabolize 2-F-araA more like dogs or like monkeys. Of interest is the observation that one dog, administered a therapeutic dose (400 mg/m$^2$) of 2-F-araA, displayed evidence of bladder toxicity but recovered without special treatment. In another dog, treated with the same dose and killed for pathological observation, toxicity consisted of epithelial vacuolar degeneration and extensive exfoliation; moderate submucosal edema with scattered, small hemorrhagic foci were observed.

The rates of deamination of araA in the species of animals used in this study have not been reported. A study in humans, however, revealed a rate faster than that observed for 2-F-araA in animals. At a dose of 250 mg/m$^2$ given by rapid i.v. administration to two patients, plasma levels of araA were 5 and 1 $\mu$g/ml, and levels of araH were 6 and 7 $\mu$g/ml, respectively [15], 4 hr after treatment.

In spite of the appreciable deamination of 2-F-araA, it is an active antitumor agent [10] while araA is not [2]. 2-F-AraA as its 5′-phosphate has been selected by the National Cancer Institute for clinical development.

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