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2′-Fluoro-2′,3′-dideoxyarabinosyladenine: A Metabolically Stable Analogue of the Antiretroviral Agent 2′,3′-Dideoxyadenosine

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SUMMARY

In this report, we have compared the uptake, metabolism, and relevant enzymology of a novel anti-acquired immunodeficiency syndrome drug, 2′-fluoro-2′,3′-dideoxyarabinosyladenine (2′-F-dd-ara-A) with the corresponding properties of its parent compound 2′,3′-dideoxyadenosine (2′,3′-ddAdo) in three human T cell lines, MOLT-4, ATH8, and CEM. In previous communications, we have reported that the primary route of metabolism of 2′,3′-ddAdo in human T lymphoblasts is catabolic, i.e., deamination to 2′,3′-dideoxyinosine (2′,3′-ddIno). At this point, the metabolic pathway diverges, to result in either cleavage and inactivation of 2′,3′-ddIno by purine nucleoside phosphorylase or in 5′-phosphorylation by a phosphotransferase, a reaction that generates 2′,3′-inosine monophosphate and ultimately the putative active metabolite 2′,3′-dideoxy-ATP. Studies with kinase-deficient mutant CEM lines indicate, however, that 2′-F-dd-ara-A favors a more direct anabolic route toward formation of 2′-fluoro-dideoxynucleotides, catalyzed initially by 2′-deoxycytidine kinase. In MOLT-4 cells, amounts of 2′-fluoro-dideoxyarabinosyladenine di- and triphosphate formed were approximately 20-fold and 5-fold greater than the respective accumulation of 2′,3′-dideoxy-ADP and 2′,3′-dideoxy-ATP over the same time of exposure. This metabolic profile was supported by enzymological studies, which revealed that 2′-F-dd-ara-A is deaminated 10 times less rapidly than ddAdo and that the resulting deaminated product is resistant to hydrolysis by purine nucleoside phosphorylase. Under similar conditions, ddAdo was rapidly degraded through cleavage of its deamination product ddIno. Like ddAdo, 2′-F-dd-ara-A was found to be transported by passive diffusion and does not enter cells via the purine nucleoside transport carrier system. However, the rate of entry of 2′-F-dd-ara-A was about half that of ddAdo (9.7 pmol/10^6 cells/min for 2′-F-dd-ara-A versus 18.4 pmol/10^6 cells/min for ddAdo). This investigation, therefore, demonstrates that, under the conditions studied, 2′-F-dd-ara-A and its deamination product 2′-fluoro-2′,3′-dideoxyarabinosylhypoxanthine have metabolic properties that differ significantly from those of their parent compounds ddAdo and ddIno. These properties, combined with the previously reported resistance of the fluorinated nucleosides to acid degradation, make these compounds interesting candidates for further study as orally administered agents for the inhibition of human immunodeficiency virus replication in patients with acquired immunodeficiency syndrome.

The dideoxypurine nucleosides ddAdo and ddIno appear to have an *in vivo* virustatic effect and to improve immune function in patients with AIDS and severe AIDS-related complex (1). Further testing to define the efficacy and safety of ddIno is currently under way. However, because of the extreme acid lability of their glycosylic bonds, these compounds require administration either with antacids or in an enteric-coated formulation in order to be orally bioavailable. With this in mind, Marquez and his colleagues (2) recently synthesized two dideoxypurine nucleoside analogues resistant to degradation at acidic pH; these were 2′,3′-dideoxy-2′-fluororibosyladenine and 2′-F-dd-ara-A (Fig. 1). Of these two purine nucleosides, only 2′-F-dd-ara-A was effective in controlling the replication of HIV in the ATH8 test system. A similar result for these two 2′-fluoro analogues of ddAdo was obtained by Herdewijn and his colleagues (3) in the MT-4 test system. In previous communications, we reported that ddAdo and ddIno are metabolically activated to yield the putative active metabolite ddATP (4, 5). However, ddAdo is more extensively metabolized than other anti-HIV dideoxynucleosides such as 2′,3′-dideoxycytidine (6, 7) and 3′-azidothymidine (8), being deaminated and cleaved, with the resulting purine base (hypoxanthine) being

ABBREVIATIONS: ddAdo, 2′,3′-dideoxyadenosine; 2′-F-dd-ara-A, 2′-fluoro-2′,3′-dideoxyarabinosyladenine; PNP, purine nucleoside phosphorylase; ddIno, 2′,3′-dideoxyinosine; 2′-F-dd-ara-Hx, 2′-fluoro-2′,3′-dideoxyarabinosylhypoxanthine; 2′-F-dd-ara-ATP, 2′-fluoro-2′,3′-dideoxyarabinosyladenosine triphosphate; ddATP, 2′,3′-dideoxyadenosine triphosphate; ddADP, 2′,3′-dideoxyadenosine diphosphate; 2′-F-dd-ara-IMP, 2′-fluoro-2′,3′-dideoxyarabinosylinosine monophosphate; 2′-F-dd-ara-ADP, 2′-fluoro-2′,3′-dideoxyarabinosyladenosine diphosphate; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; 2′-F-dd-ara-AMP, 2′-fluoro-2′,3′-dideoxyarabinosyladenosine monophosphate.

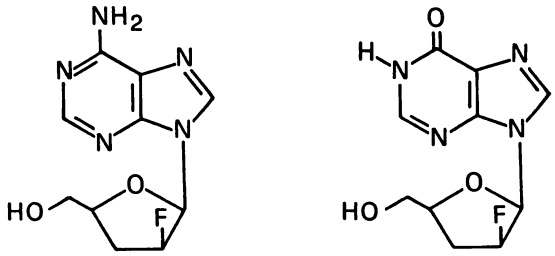

Fig. 1. Structures of 2′-F-dd-ara-A and 2′-F-dd-ara-Hx.

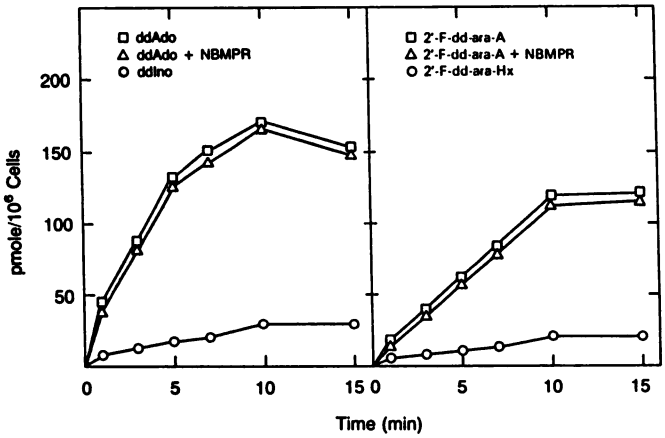

Fig. 2. A, Accumulation of [^{3}H]ddAdo (10 μM) with or without nitrobenzylthioinosine (*NBMPR*) (50 μM) and [^{3}H]ddIno (10 μM) by MOLT-4 cells; B, accumulation of 2′-F-[^{3}H]dd-ara-A (10 μM) with or without nitrobenzylthioinosine (50 μM) and 2′-F-[^{3}H]dd-ara-Hx (10 μM) by MOLT-4 cells. Studies were carried out as described in Experimental Procedures. The radioactivity recovered in the cell pellet was converted to molarity by using the specific activity of the incubated substrate. Similar results were obtained in two separate experiments.

TABLE 1

Comparison of formation of phosphorylated metabolites of 2′-F-dd-ara-A versus ddAdo (5 μM) in MOLT-4 cells

MOLT-4 cells were treated with either [^{3}H]ddAdo or 2′-F-[^{3}H]dd-ara-A for 5 hr (5 μM). After incubation, extracts of the cell pellets were analyzed by ion exchange chromatography (Partisil 10-SAX), as described in Experimental Procedures. Each data point represents the mean of duplicate analyses with less than 5% variation. Similar results were obtained in three separate experiments.

	Phosphorylated metabolites		Metabolite ratio (2-F-dd-ara-A:ddAdo)
	2′-F-dd-ara-A	ddAdo	
	(*pmol/10⁶ cells*)		
ddNDP[a]	0.65	0.03	21.7
ddNTP	0.86	0.18	4.8

[a] ddNDP, 2′,3′-dideoxynucleotide diphosphates; ddNTP, 2′,3′-dideoxynucleotide triphosphates.

anabolized to ribonucleotides. In view of the favorable therapeutic index of the purine dideoxynucleoside series of analogues, we have sought in the present communication to compare the metabolic stability of 2′-F-dd-ara-A with that of ddAdo in human T cell (MOLT-4) extracts and to characterize rates of deamination, phosphorolysis, and, most importantly, conversion to dideoxynucleotides in human lymphocytes. The results of these studies lead to the conclusion that the title compound is, in fact, a potential candidate for preclinical development as an orally administered anti-AIDS agent. A preliminary account of some of these studies has appeared (9).

TABLE 2

Comparative metabolism of 2′-F-dd-ara-A versus ddAdo in ATH8 cells

ATH8 cells were grown as previously described (6, 12) and were incubated with [^{3}H]ddAdo or 2′-F-[^{3}H]dd-ara-A for 5 or 24 hr. Methanolic extracts (60%) of the cell pellets were subjected to ion exchange Partisil 10-SAX chromatography, as described in Experimental Procedures. Values shown are the mean of duplicate assays.

	Metabolite formed	
	5 hr	24 hr
	pmol/10⁶ cells	
2′-F-dd-ara-ADP	0.19	0.35
2′-F-dd-ara-ATP	0.20	0.42
ddADP	0.02	0.05
ddATP	0.04	0.10

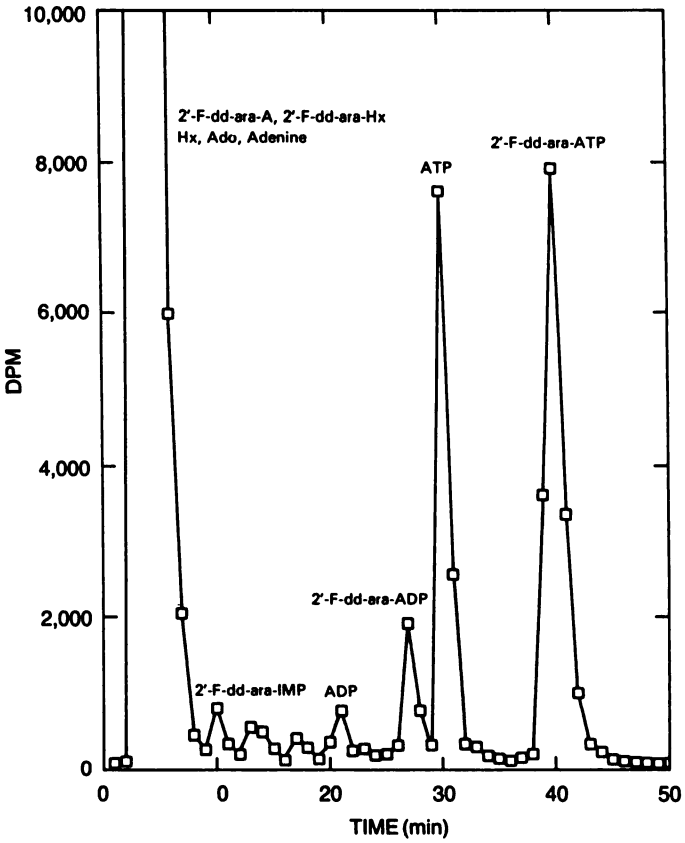

Fig. 3. Ion exchange (Partisil 10-SAX) high pressure liquid chromatography elution profile of MOLT-4 cells incubated for 5 hr with 2′-F-[^{3}H]dd-ara-A (5 μM). Buffer A: 0.03 M ammonium phosphate, pH 4.8; buffer B: 9 parts 0.7 M ammonium phosphate, pH 4.6, and 1 part 100% ethanol. The following elution program was used: 5 min of buffer A, followed by 10 min of highly convex gradient to 75% buffer A/25% buffer B, followed by 15 min of slightly convex gradient to 100% buffer B, and finally followed by a 15-min isocratic elution with buffer B.

Experimental Procedures

Materials

Isotopes. 2′-F-[^{3}H]dd-ara-A (25 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO); [2′,3′-^{3}H]-ddAdo (30 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). 2′-F-[^{3}H]dd-ara-Hx (Fig. 1) and [^{3}H]ddIno (the latter compound labeled in the 2′- and 3′-positions of the dideoxyribose moiety) were prepared by means of enzymatic deamination of 2′-F-[^{3}H]dd-ara-A and

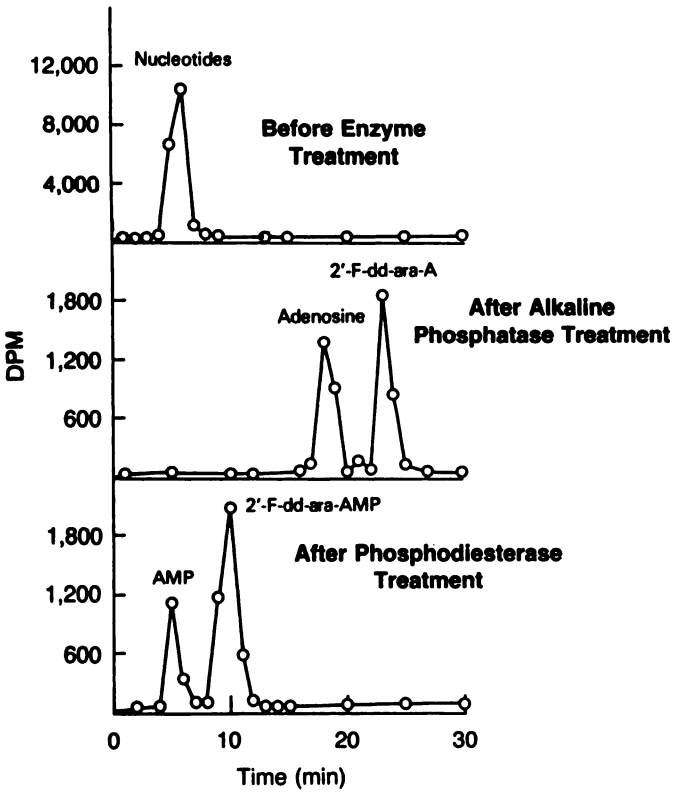

Fig. 4. Effect of alkaline phosphatase and phosphodiesterase on nucleotides derived from 2′-F-dd-ara-A. MOLT-4 cells were treated with 2′-F-[^{3}H]dd-ara-A (5 μM; 5 μCi/ml) for 5 hr and the cellular nucleotides were analyzed by reverse phase chromatography (fractions 4–8). The nucleotide fraction was freeze dried and the reconstituted aliquots were treated either with alkaline phosphatase or with snake venom phosphodiesterase, as described in Experimental Procedures, followed by reanalysis on a reverse phase C-18 column (ODS-Ultrasphere).

TABLE 3

Effect of L-alanosine and deoxycytidine on 2′-F-dd-ara-A metabolism in MOLT-4 cells

MOLT-4 cells were incubated for 5 hr with 2′-F-[^{3}H]dd-araA (5 μM; 3 μCi/ml), after 0.5-hr preincubation with either normal saline (control), 25 μM L-alanosine, or 200 μM deoxycytidine. Methanolic extracts (60%) were prepared and analyzed by ion exchange high pressure liquid chromatography (Partisil 10-SAX), as described in Experimental Procedures. Each data point represents the mean of duplicate analyses with less than 5% variation from the values given. Similar results were obtained in three separate experiments.

Treatment	Metabolite formed		
	2′-F-dd-ara-IMP	2′-F-dd-ara-ADP	2′-F-dd-ara-ATP
	pmol/10^6 cells		
Control	0.22	0.65	0.86
L-Alanosine	0.97	0.35	0.31
Deoxycytidine	0.21	0.45	0.27

[^{3}H]ddAdo, respectively, utilizing calf intestinal adenosine deaminase (Sigma Chemical Co., St. Louis, MO).

Chemicals. ddADP and ddATP were purchased from Pharmacia (Piscataway, NJ), 2′-F-dd-ara-AMP was synthesized by the general method of Yoshikawa and Takenishi (10), and 2′-F-dd-ara-ATP was synthesized by the general method of Kovács and Ötvös (11). ddAdo (NSC 98700) and 2′-F-dd-ara-A (NSC 613792) were obtained from the Drug Synthesis and Chemistry Branch (National Cancer Institute, National Institutes of Health). All other nucleoside/nucleotide standards were purchased from Sigma.

Enzymes. AMP deaminase (rabbit muscle) (60 units/mg), PNP (calf spleen) (27 units/mg), and alkaline phosphatase (*Escherichia coli*; 45 units/mg) were purchased from Sigma; adenosine deaminase (calf intestine) (200 units/mg) and snake venom phosphodiesterase (*Crotalus durissus*; 1.5 units/mg) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Cells. MOLT-4 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (56°, 30 min), 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM L-glutamine, at 37° in a humidified atmosphere of 95% air/5% CO_2. The deamination of 2′-F-[^{3}H]dd-ara-A in medium was much slower than that of the parent compound ddAdo ($t_{1/2}$ = 251 and 31 min, respectively, at 37°); the only detectable metabolite arising in cell-free medium on incubation with 2′-F-dd-ara-A was 2′-F-dd-ara-Hx, the deamination product arising from the action on the compound of residual adenosine deaminase present in the bovine serum-supplemented cell culture medium (see below). ATH8 and CEM cells (including CEM mutant lines) were grown as previously described (12–14). Cells were verified to be in logarithmic growth at the time of use.

Methods

Uptake studies. Molt-4 cells growing in log phase (<1.0 × 10^6 cells/ml) were centrifuged and the pellet was resuspended in fresh RPMI 1640 medium at a concentration of 2–3 × 10^6 cells/ml. One-half milliliter of the cell suspension was added to Eppendorf polypropylene tubes containing 5 nmol of the respective radiolabeled substrate (5 μCi/vessel of ddAdo, 2′-F-dd-ara-A, ddIno, or 2′-F-dd-ara-Hx). In addition, uptake of [^{3}H]ddAdo and 2′-F-[^{3}H]dd-ara-A was determined in the presence or absence of 50 μM concentrations of the nucleoside transport-inhibitor nitrobenzylthioinosine. All studies were carried out at 37°. At time intervals of 1, 3, 5, 7, 10, and 15 min, cells were loaded onto 0.5 ml of silicon oil, prewarmed to 37° (Versilube F50, GE, Waterford, NY) in the microcentrifuge tubes, and centrifuged immediately at 12,000 rpm for 20 sec. Centrifuged samples were placed in powdered dry ice in order to freeze the upper layer of medium. The lower portion of the microcentrifuge tubes, containing the cellular pellet, was cut by using a hot wire. Oil from above the cell pellet was drained by gentle tapping and after vigorous agitation the pellet was digested overnight in 1 ml of 1 N NaOH; 1 ml of equimolar HCl was added to the digested samples and the radioactivity was determined using scintillation counting.

Metabolism studies. Commercially obtained 2′-F-[^{3}H]dd-ara-A (98% pure) was further purified by reverse phase chromatography, using a prestandardized 5-μ Ultrasphere-ODS column (4.6 mm × 25 cm; Beckman). Elution was carried out with a linear gradient of H_2O (solvent A) and 50% acetonitrile (solvent B) (1.33% increase in solvent B/min, up to 60 min) at a flow rate of 1 ml/min. 2′-F-[^{3}H]dd-ara-A eluted in fractions 40–45 (1 min/fraction). Fractions containing peak activity were pooled, freeze dried, and used for the metabolism studies. Purity of the chromatographed material exceeded 99.8%.

For metabolism studies, cells (MOLT-4, CEM, or ATH8) growing in log phase (<1 × 10^6/ml) were incubated with either [^{3}H]ddAdo or 2′-F-[^{3}H]dd-ara-A (10 μM final concentration, with 5 μCi/ml of cell suspension). After 5 hr of incubation, cells were centrifuged and the pellets were washed with 1 ml of cold normal saline and extracted with 0.4 ml of 60% methanol. After centrifugation, 200 μl of the supernatant were subjected to chromatography on an ion exchange Partisil 10-SAX column (4). One-minute fractions were collected and radioactivity was determined.

For the experiments on the rate of decay of 2′-F-dd-ara-ADP and 2′-F-dd-ara-ATP after removal of the parent nucleoside, MOLT-4 cells growing in log phase were treated with 2′-F-[^{3}H]dd-ara-A (5 μM, 5 μCi/ml) for 12 hr. After incubation, cells were washed twice with RPMI 1640 medium and resuspended in fresh medium at 37°. Aliquots were taken over the next 24 hr and extracts were subjected to ion exchange Partisil 10-SAX chromatography, as described above.

Additional metabolism studies were carried out in CEM cells and CEM mutant lines. Human CEM cells or variants deficient in either deoxycytidine kinase or adenosine kinase or in both enzyme activities

TABLE 4

Phosphorylation of 2′-F-dd-ara-A by kinase-deficient CEM cells

Studies with kinase-deficient CEM mutants were carried out as described in Experimental Procedures. Each data point represents the mean of duplicate analyses.

Cell line	Deoxycoformycin (5 μM)	2′-F-dd-ara-ADP	2′-F-dd-ara-ATP	Total
			pmol/10⁶ cells	
Wild-type cells (CEM)	−	1.30	2.14	3.44
	+	1.29	2.36	3.65
Deoxycytidine kinase-deficient	−	0.84	1.09	1.93
	+	0.35	<0.10	0.35
Adenosine kinase-deficient	−	0.59	2.48	3.07
	+	0.83	2.78	3.61
Deoxycytidine kinase-deficient and adenosine kinase-deficient	−	0.24	1.60	1.84
	+	<0.10	<0.10	<0.10

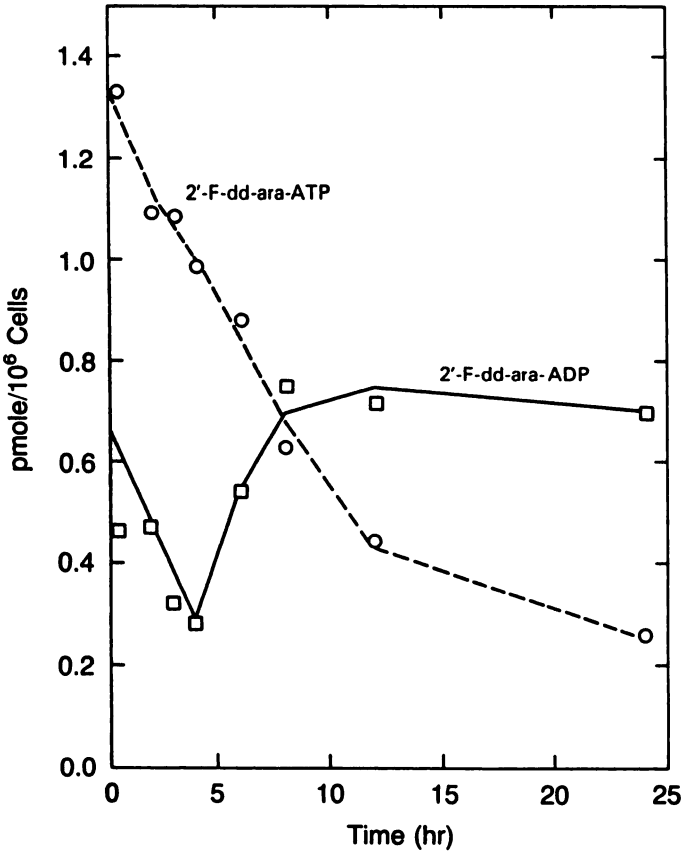

Fig. 5. Intracellular levels of 2′-F-dd-ara-A nucleotides after incubation with 2′-F-[³H]dd-ara-A. MOLT-4 cells were treated with 2′-F-[³H]dd-ara-A (5 μM; 5 μCi/ml) for 12 hr; the cells were then resuspended in fresh drug-free medium and aliquots were removed for determination of nucleotide (5′-diphosphate and 5′-triphosphate) levels, at the intervals shown, for the next 24 hr. Levels of 2′-F-[³H]dd-ara-ADP and 2′-F-[³H] dd-ara-ATP were determined by ion exchange chromatography (Partisil 10-SAX) as described in Fig. 3.

were incubated at 37° at a density of 1 × 10⁶ cells/ml in the presence or absence of 5 μM deoxycoformycin for 10 min before addition of 10 μM 2′-F-dd-ara-A. After 5 hr, the nucleotides in the methanol-soluble extracts were determined as described above.

Enzymology. Adenosine deaminase. Using a purified enzyme preparation from calf intestine or a crude extract of MOLT-4 cells (12,000 × *g* supernatant), deamination of 2′-F-dd-ara-A was compared with that of ddAdo by the spectrophotometric assay of Agarwal *et al.* (15); thus, 10 μmol of ddAdo or 2′-F-dd-ara-A in an assay volume of 1 ml were treated with the purified adenosine deaminase (0.1 μg of protein) or the cell extract (0.125 mg of protein) and the rate of decrease in absorbance at 265 nm was used as a measure of enzyme activity.

PNP. PNP susceptibility of 2′-F-dd-ara-A was compared with that of ddAdo, using a commercial enzyme preparation from calf spleen as well as a homogenized MOLT-4 cell extract. The assay mixture contained 50 μl of 0.05 M potassium phosphate, pH 7.4, with a 1:100 dilution of purified calf intestine adenosine deaminase, 20 μl of 5 mM [³H]ddAdo (1 μCi) or 2′-F-[³H]dd-ara-A (1 μCi), 30 μl of MOLT-4 extract (0.45 mg of protein), purified PNP (0.132 mg of protein), or distilled water. After a 30-min incubation at 37°, the assay was terminated by heating for 1 min at 95°. Aliquots of the samples were analyzed on a prestandardized C-18 column (ODS-Ultrasphere) using ion-pairing chromatography (5) capable of separating hypoxanthine, adenine, adenosine, ddAdo, ddIno, 2′-F-dd-ara-A, and 2′-F-dd-ara-Hx. Radioactivity was determined in the column eluate by a continuous flow automated radioisotope analyzer (Ramona, IN/US).

AMP deaminase. The rate of deamination of ddAMP and 2′-F-dd-ara-AMP was determined using 10 μmol of the respective substrate and 100 μg of either purified AMP deaminase from rabbit muscle or MOLT-4 cell extract (0.5 mg of protein), in an assay volume of 1 ml, by the spectrophotometric method of Zielke and Suelter (16). The decrease in absorbance at 265 nm was used as a measure of enzyme activity.

Results

Uptake of ddAdo and 2′-F-dd-ara-A. As shown in Fig. 2, the rate of entry of ddAdo (18.4 pmol/10⁶ cells/min) is almost twice that of 2′-F-dd-ara-A (9.7 pmol/10⁶ cells/min); similarly, the rate of accumulation of the deaminated analog ddIno (1.9 pmol/10⁶ cells/min) is greater than that of 2′-F-dd-ara-Hx (1.12 pmol/10⁶ cells/min). Significantly lower rates of accumulation of the deaminated moieties are compatible with their lower lipid solubility (5). That passive diffusion seems to be the major entry mechanism for these dideoxy nucleosides is supported by the observation that the nucleoside transport inhibitor nitrobenzylthioinosine, even at 50 μM, failed to inhibit the entry of either ddAdo or 2′-F-dd-ara-A (Fig. 2).

Metabolism of ddAdo and 2′-F-dd-ara-A. Metabolism of ddAdo versus 2′-F-dd-ara-A was compared in MOLT-4 and ATH8 cells (Tables 1 and 2 and Fig. 3). Radiolabeled ddAdo or 2′-F-dd-ara-A was incubated with the cells and the resulting radiolabeled metabolites were separated by ion exchange chromatography. The identity of the radiolabeled peaks was established by comparison with the elution times of the unlabeled standards, ADP, ATP, ddADP, ddATP, 2′-F-dd-ara-AMP, and 2′-F-dd-ara-ATP. The identity of apparent fluorodideoxynucleotide peaks was confirmed by enzymatic means (see below). It was observed that 2′-F-dd-ara-A is markedly less susceptible

to catabolism than is ddAdo. The relative amounts of fluorodideoxynucleotides generated from 2′-F-dd-ara-A were significantly greater than the corresponding dideoxynucleotides generated from ddAdo. In MOLT-4 cells, 2′-F-dd-ara-ADP and 2′-F-dd-ara-ATP accumulated to levels about 22-fold and 5-fold greater than ddADP or ddATP, respectively (Table 1). On the other hand, incorporation of radioactivity into ADP and ATP from 2′-F-dd-ara-A treatment was only about 2% of that observed after the ddAdo treatment (data not shown). It is possible that ADP and ATP detected in 2′-F-dd-ara-A-pulsed cells may be attributable to trace contamination of ^{3}H-labeled 2′-F-dd-ara-A by residual [^{3}H]adenine (even after chromatographic repurification), rather than to enzymatic cleavage and reutilization of the purine moiety of 2′-F-dd-ara-A. In ATH8 cells, as was the case with MOLT-4 cells, the conversion of 2′-F-dd-ara-A to the corresponding 5′-di- and triphosphates was more efficient than seen with the parent compound ddAdo, when determined at either 5 or 24 hr exposure to these compounds (Table 2).

Enzymatic confirmation of identity of fluoro-dideoxynucleotides. MOLT-4 cells (40 ml of 10^{6} cells/ml) were exposed to purified 2′-F-[^{3}H]dd-ara-A for 5 hr. Methanolic extracts of the cellular pellets were pooled and freeze dried. The reconstituted sample was loaded onto a reverse phase ODS-Ultrasphere column and cellular nucleotides, which eluted in fractions 4-8 (4–8 min), were pooled and freeze dried (Fig. 4, *top*). Aliquots of the samples were treated with alkaline phosphatase (pH 9) or phosphodiesterase (pH 7.4). Analysis of samples by reverse phase high pressure liquid chromatography revealed the presence of 2′-F-dd-ara-A and also adenosine in the alkaline phosphatase-treated sample (Fig. 4, *middle*); similarly, 2′-F-dd-ara-AMP and AMP (confirmed by coelution with authentic standards) were produced in phosphodiesterase-treated samples (Fig. 4, *lower*).

Effects of L-alanosine and 2-deoxycytidine and of the mutational loss of 2′-deoxycytidine kinase and adenosine kinase on 2′-F-dd-ara-A metabolism. We reported previously (4, 5) that activation of ddAdo to its putative active metabolite ddATP is carried out in T lymphocytes both by a direct route of successive phosphorylation involving deoxycytidine kinase and/or adenosine kinase and, more significant quantitatively, by an indirect route, via deamination to ddIno with consequent phosphorylation to 2′,3′-dideoxyinosine monophosphate and reamination to ddAMP in a reaction catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase. To assist in the elucidation of the activation routes followed by 2′-F-dd-ara-A, we determined the metabolic profile of the drug in T lymphoblasts on pretreatment with either L-alanosine (25 μM), a well known inhibitor of adenylosuccinate synthetase, or 2′-deoxycytidine (200 μM), the preferred substrate of deoxycytidine kinase. As shown in Table 3, pretreatment with L-alanosine inhibited the accumulation of 2′-F-dd-ara-ADP and 2′-F-dd-ara-ATP by 46% and 64%, respectively, with a concomitant increase in 2′-F-dd-ara-IMP of 4.4-fold. On the other hand, the presence of 2′-deoxycytidine during the incubation did not cause any increase in accumulation of 2′-F-dd-ara-IMP but caused a marked reduction (approximately 70%) in the accumulation of 2′-F-dd-ara-ATP. These inhibitor studies indicate that, like ddAdo, 2′-F-dd-ara-A can be activated in T lymphocytes utilizing both direct and indirect routes. To elucidate the relative contributions of these pathways by an alternate method, the anabolism of 2′-F-dd-ara-A was also studied in mutant T cell lines (CEM) deficient in 2′-deoxycytidine kinase, adenosine kinase, or both of these enzymes. As shown in Table 4, phosphorylation of 2′-F-dd-ara-A to its nucleotides decreased about 45% in the 2′-deoxycytidine kinase deficient cells and in the double mutant lacking both 2′-deoxycytidine kinase and adenosine kinase activities but was unchanged from the wild type in the adenosine kinase-deficient variant. The presence of the adenosine deaminase inhibitor 2′-deoxycoformycin in the incubation was found to eliminate almost completely the capacity of either the 2′-deoxycytidine kinase-deficient or the double mutant to accumulate 2′-F-dd-ara-ATP from 2′-F-dd-ara-A, whereas 2′-deoxycoformycin was without effect on the adenosine kinase-deficient mutant. These results indicate that, under conditions which do not inhibit deamination, 2′-F-dd-ara-A is anabolized in cells by a combination of both direct phosphorylation via 2′-deoxycytidine kinase and indirectly via the intermediate formation of 2′-F-dd-ara-Hx and subsequent conversion to 2′-F-dd-ara-ATP. Previous studies (17) with CEM cells suggest that a 5′-nucleotidase converts ddIno, the deaminated form of ddAdo, to its monophosphate derivative and the same or a similar enzyme may be required for 2′-F-dd-ara-Hx phosphorylation.

Disappearance of intracellular 2′-F-dd-ara-ATP. The decay profile of 2′-F-dd-ara-ADP and 2′-F-dd-ara-ATP after removal of the parent nucleoside is shown in Fig. 5. The $t_{1/2}$ for the corresponding 5′-diphosphate was considerably longer; the apparent slow clearance of the latter may be a reflection, however, of continuing new formation of the 5′-diphosphate by dephosphorylation of the 5′-triphosphate form of the drug.

Enzymology. We first examined the relative efficiency of 2′-F-dd-ara-A and ddAdo toward deamination by either purified adenosine deaminase or a crude extract of the enzyme from MOLT-4 cells. Deamination of 2′-F-dd-ara-A proceeded at a rate only about 10% of that of ddAdo with either of these enzyme preparations (Table 5). Similarly, in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum, the rate of deamination of 2′-F-dd-ara-A due to residual adenosine deaminase in the medium was only 12.4% of that seen for ddAdo (see Experimental Procedures). Such metabolic stability could contribute in an important way towards the net accumulation of the respective 2′-fluorodideoxynucleoside triphosphate.

Since the metabolic profile of 2′-F-dd-ara-A indicated stability of the molecule to cleavage although not to deamination (i.e., compared with the nonfluorinated parent compound), the relative phosphorylytic susceptibility of the deamination products of ddAdo and 2′-F-dd-ara-A was, therefore, examined using a purified preparation of PNP from calf spleen, as well as a crude extract from MOLT-4 cells. As shown in Table 5, deaminated 2′-F-dd-ara-A (i.e., 2′-F-dd-ara-Hx) was found not to be a substrate of this enzyme, whereas under the identical conditions ddIno was rapidly degraded to hypoxanthine. Even in the presence of xanthine oxidase, where the reaction was forced in the forward direction, catabolism of 2′-F-dd-ara-Hx was not observed (results not shown). These experiments demonstrate resistance of 2′-F-dd-ara-Hx to phosphorylytic cleavage by PNP.

The 5′-monophosphate of ddAdo as well as of 2′-F-dd-ara-A showed relatively poor substrate activity toward AMP deaminase (Table 5). In comparison, AMP was approximately 800-

TABLE 5

Comparative enzyme susceptibility of ddAdo versus 2′-F-dd-ara-A

Enzymes	Substrate	Rate with purified enzyme[a]	Rate with MOLT-4 extract[b]
PNP (nmol/mg/hr)	ddIno	7640.0	31.0
	2′-F-dd-ara-Hx	<0.1	<0.1
Adenosine deaminase (μmol/mg/min)	ddAdo	72.0	0.080
	2′-F-dd-ara-A	8.5	0.007
AMP deaminase (nmol/mg/min)	ddAMP	80.0	2.0
	2′-F-dd-ara-AMP	60.0	1.7

[a] PNP from calf spleen; adenosine deaminase from calf intestine; AMP-deaminase from rabbit muscle.

[b] For PNP activity, MOLT-4 cell homogenate in 0.01 M Tris, pH 7.4, was used. For adenosine deaminase, 12,000 × *g* supernatant of the above homogenate was used. Enzyme activities were determined as given in Experimental Procedures. Each data point represents the mean of duplicate analyses, with less than 5% variation from the values given. Similar results were obtained in two separate experiments.

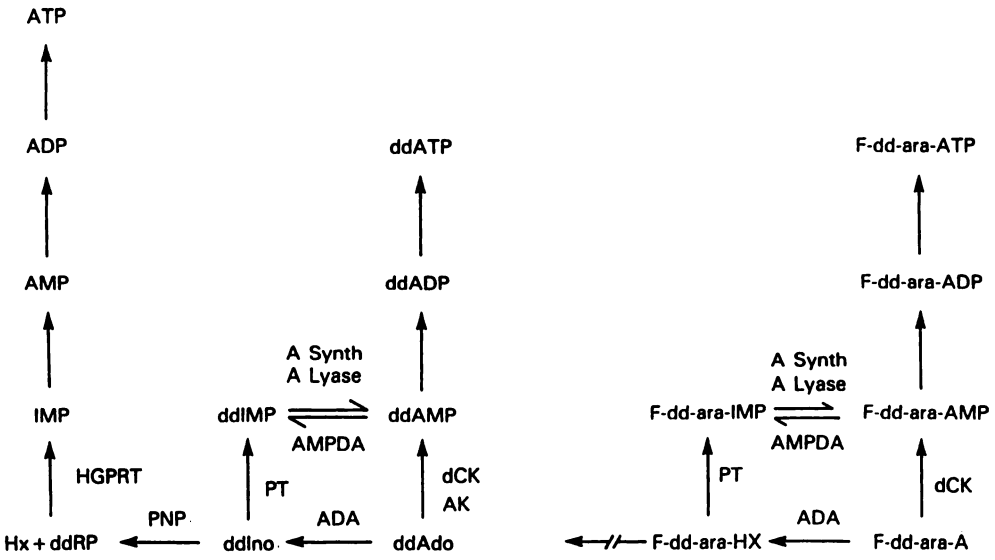

Fig. 6. Outline of metabolic pathways for ddAdo (*left*) and 2′-F-dd-ara-A (*right*).

PNP: purine nucleoside phosphorylase; ADA: adenosine deaminase; HGPRT: hypoxanthine: guanine phosphoribosyltransferase; dCK: 2′-deoxycytidine kinase; AK: adenosine kinase; A Synth: adenylosuccinate synthetase; A Lyase: adenylosuccinate lyase; AMPDA: adenylate deaminase. PT: phosphotransferase|(5′-nucleotidase).

to 1000-fold more susceptible to deamination under these conditions (results not shown).

Discussion

Among the 2′,3′-dideoxynucleosides studied to date, the purine nucleoside analogues ddAdo and ddIno exhibit the most favorable therapeutic ratios in the ATH8 test system (12). The latter compound is presently undergoing extensive Phase I clinical trials (1). However, its practical therapeutic usefulness, particularly for oral administration, may ultimately be limited by its acid lability and catabolic profile (2, 4, 5). A 2′-fluoroarabinosyl substitution in ddAdo, however, renders the molecule acid stable without loss of antiretroviral activity or potency in the ATH8 test system (2). ddAdo and 2′-F-dd-ara-A are also equipotent in inhibiting HIV replication in the human monocyte/macrophage test system in vitro (data not shown). In this study we have determined the biochemical and metabolic consequences of this modification by comparing 2′-F-dd-ara-A with its parent molecule, ddAdo.

Not only is 2′-F-dd-ara-A much less susceptible to enzymic deamination (Table 5), its deamination product, 2′-F-dd-ara-Hx, is resistant to phosphorolytic cleavage by PNP. Both properties distinguish 2′-F-dd-ara-A from ddAdo. Commercial PNP, as well as MOLT-4 cell extracts, were ineffective in cleaving 2′-F-dd-ara-Hx in the presence of phosphate.

In order to determine how this catabolic stability would influence the metabolism of 2′-F-dd-ara-A, the metabolic profile of 2′-F-[^{3}H]-dd-ara-A was compared with that of [^{3}H] ddAdo in MOLT-4 cells. These studies showed that the putative active metabolite, 2′-F-dd-ara-ATP, accumulates at concentrations approximately 5-fold greater than those of ddATP in this cell line. Moreover, 2′-F-dd-ara-ADP was shown to accumulate to concentrations 20-fold greater than those for ddADP. Previously, we showed that ddADP could be generated via two metabolic pathways, i.e., directly by 2-deoxycytidine kinase and/or adenosine kinase and indirectly via the intermediation of ddIno, with the latter route being quantitatively predominant in human T cells (5, 13, 17). Because of the relatively low deamination rate of 2′-F-dd-ara-A, a greater intracellular half-life of the latter compound is anticipated. That 2′-F-dd-ara-A favors the direct (i.e., deoxycytidine kinase) route, appears to be the case, on the basis of the studies described here utilizing CEM mutant lines deficient in adenosine kinase, 2′-deoxycytidine kinase, or both of these enzymes. The use of inhibitors and preferred substrates of these pathways has also provided some insight into the relative contributions of the direct and indirect routes. A greater than 50% inhibition, but not total

elimination, of the 2′-F-dd-ara-A di- and triphosphates by either 2′-deoxycytidine or L-alanosine indicates that both direct and indirect pathways are involved. Reduction in 2′-F-dd-ara-ADP or 2′-F-dd-ara-ATP by L-alanosine is thought to be mediated by the known mechanism of action of the latter drug (i.e., inhibition of adenylosuccinate synthetase by L-alanosine-5′-amino-4-imidazole carboxylic acid ribotide (L-alanosine AICOR). An accumulation of 2′-F-dd-ara-IMP with concomitant reduction of 2′-F-dd-ara-ADP and 2′-F-dd-ara-ATP supports the indirect pathway of 2′-F-dd-ara-A activation. However, compared with the effect of L-alanosine on ddAdo metabolism (5), the reduction in 2′-F-dd-ara-ADP and 2′-F-dd-ara-ATP is considerably less marked. On the other hand, the effect of 2′-deoxycytidine in inhibiting the accumulation of these nucleotides is more pronounced in the case of 2′-F-dd-ara-A than in that of ddAdo. Viewed in concert, these results suggest that direct phosphorylation plays a more prominent role in 2′-F-dd-ara-A activation than in ddAdo activation. A diagrammatic representation of the proposed metabolic pathways for 2′-F-dd-ara-A, in contrast to those for its parent compound ddAdo, is given in Fig. 6.

In conclusion, 2′-F-dd-ara-A and/or 2′-F-dd-ara-Hx, in view of their ability to form the active antiviral 5′-triphosphate anabolite 2′-F-dd-ara-ATP and their lesser susceptibility to either acidic hydrolysis or enzyme-catalyzed phosphorolysis (via the adenosine deaminase:PNP route), may offer possible advantages over the corresponding compounds ddAdo and ddIno, as potential anti-HIV agents, particularly when considered as candidates for oral administration. On the other hand, the susceptibility of ddIno to metabolic cleavage (and thus to pharmacological inactivation) may be in part responsible for the high therapeutic index and low toxicity profile of the latter drug (1) and thus represents a favorable characteristic in terms of its clinical application. A definitive resolution of the relative merits of ddIno and its 2′-fluoro-arabinosyl analogue is, thus, not feasible on the basis of the cellular pharmacology studies described here. These results would appear, however, to warrant *in vivo* toxicology studies and possible clinical trial of the fluoro analogue.

Note added in proof. In studies subsequent to those presented in this paper, we have determined the K_i of 2′-F-dd-ara-ATP vs. recombinant HIV reverse transcriptase (a generous gift of Dr. J. McMahon, NCI, NIH). Using a poly d(A–T)-d(A–T) template primer, the K_i of 2′-F-dd-ara-ATP was 1 μM, a value ten times higher than the K_i of ddATP (0.1 μM) in this system. In both cases, inhibition was formally competitive with the variable substrate, dATP.

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