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Synthesis and Biological Effects of 2′-Fluoro-5-Ethyl-1-β-D-Arabinofuranosyluracil

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2′-Fluoro-5-ethyl-1-β-D-arabinofuranosyluracil (FEAU) was synthesized, and its biological activities were compared with those of 2′-fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU). Earlier studies indicated that both compounds showed potent anti-herpes simplex virus activity, with a 50% effective dose (ED_{50}) of <0.25 μM. In the present study the cell growth inhibitory activity of FEAU (ED_{50}, 200 to 2,060 μM) was found to be about 100-fold less than that of FMAU. With an ED_{50} ranging from 630 to 3,700 μM, FEAU only weakly inhibited thymidine incorporation into DNA, as compared with FMAU with an ED_{50} of 9 to 28 μM. Following exposure to [2-^{14}C]FEAU (100 μM), 0.48 pmol/10^6 cells per h was incorporated into the DNA of herpes simplex virus type 1-infected Vero cells, whereas no detectable incorporation was found in uninfected Vero cells or L1210 cells. The K_i of FEAU for thymidine kinase purified from human leukemic cells was >150 μM. For herpes simplex virus type 1- and 2-encoded thymidine kinases, the K_is were 0.6 and 0.74 μM, respectively. Both FEAU and FMAU were relatively nontoxic for mice, with a 50% lethal dose of >800 mg/kg per day (four intraperitoneal doses). However, the lethal dose of FEAU for dogs was 100 mg/kg per day (10 intravenous doses), a dose which is 40- to 80-fold greater than the toxic dose of FMAU. These results suggest that FEAU is a worthy candidate for further development as an antiherpetic agent.

A series of studies on the structure-activity relationships of pyrimidine nucleoside analogs indicated that the anti-herpes simplex virus effect was greatly increased by 2′-fluoro-arabinosyl group substitution and by 5-iodo or 5-methyl group substitution (7, 11, 13). 2′-Fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU) emerged as the most potent anti-herpes simplex virus agent in a mouse model (13). However, subsequent studies indicated that FMAU caused neurotoxicity in humans (6) and was incorporated into mammalian cell DNA as well as into viral DNA (8).

Earlier studies of FMAU showed that the 50% effective doses (ED_{50}s) for inhibiting herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) replication in vitro are 0.006 and 0.023 μM, respectively, whereas the ED_{50} for inhibiting the growth of Vero cells is 2.8 μM (1). Preliminary studies of 2′-fluoro-5-ethyl-1-β-D-arabinofuranosyluracil (FEAU) indicated that the ED_{50}s for the inhibition of HSV-1 and HSV-2 replication are 0.024 and 0.24 μM, respectively. However, the ED_{50} for the inhibition of Vero cell growth by FEAU is >200 μM (7, 10, 13). FMAU, therefore, is 4- and 10-fold more potent than FEAU against HSV-1 and HSV-2 replication, respectively, but 71-fold more toxic than FEAU toward host cells.

In this study, FEAU is compared with FMAU for cytotoxicity in a variety of mammalian cells, kinetic constants for host and viral thymidine kinases, metabolic properties which may be modulated to reduce toxicity toward host cells, and toxicity in several animal species. The detailed synthesis procedure for FEAU is also reported.

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MATERIALS AND METHODS

Synthesis of FEAU. The chemical synthesis of FEAU was performed by two different procedures (11, 13): (i) catalytic reduction of 5-vinyl-1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) uracil and (ii) condensation of 5-ethyluracil with 3-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-D-arabinofuranosyl bromide. The latter method is more practical than the former. It should be noted that condensation of the silylated 5-ethyluracil with the 2-fluoro-arabinosyl bromide without the Lewis acid catalyst in methylene chloride at room temperature yielded and desired β-nucleoside as the major product, and very little formation of the α-isomer was observed. Saponification of the condensation product with methanolic ammonia yielded FEAU, which was directly crystallized from a mixture of chloroform and acetone.

A solution of 1,3-di-*O*-acetyl-5-*O*-benzoyl-2-deoxy-2-fluoro-D-ribofuranose (11.9 g, 35 mmol) in dry CH_2Cl_2 (50 ml) was chilled in an ice bath, and HBr was bubbled in for 30 min. The mixture was kept at 4°C overnight, and the solvent was removed in vacuo. Traces of acetic acid were removed by several coevaporations with C_6H_6, and the residue was dissolved in CH_2Cl_2 (200 ml). This solution was added to 5-ethyl-2,4-bis-(trimethylsiloxy)pyrimidine [freshly prepared by refluxing 5-ethyluracil (4.9 g, 35 mmol) in hexamethyldisilazane (20 ml) in the presence of 10 mg of $(NH_4)_2SO_4$ until a clear solution was obtained; excess hexamethyldisilazane was removed in vacuo], and the mixture was stirred for 7 days at room temperature. Aqueous $NaHCO_3$ (30 ml) was slowly added to the mixture with vigorous stirring, and the resulting suspension was filtered through a Celite pad. The filtered solid was thoroughly washed with CH_2Cl_2. The organic layer was washed with 100 ml of water two times,

TABLE 1. ED_{50}s of FEAU and FMAU for inhibiting cell growth and for inhibiting [^{3}H]dThd incorporation into DNA in different cells[a]

Expt	ED_{50} (μM) of:		FEAU/FMAU ED_{50} ratio
	FEAU	FMAU	
Cell growth			
HL-60 cells	2,060	15.4	133.8
Vero cells[b]	200	2.8	71.4
[^{3}H]dThd incorporation[c]			
P815 cells	700	14.0	50.0
L1210 cells	630	28.0	22.5
Rat bone marrow cells	3,700	8.9	415

[a] The ED_{50}s were obtained from the median-effect plot (5) by using a computer program (2).
[b] Data are from reference 11.
[c] [^{3}H]dThd (1 μCi, 0.5 μM) was incubated with various concentrations of FEAU or FMAU by previously described method (3).

dried with Na_2SO_4, and concentrated to a syrup which was then purified on a silica gel column with *n*-hexane–ethyl acetate (1:1) as the eluent. After concentration of the major fraction, the residue (10.7 g) was dissolved in ammonia-saturated methanol (500 ml), and the mixture was kept at room temperature for 20 h. The solvent was removed by evaporation, and the residue was crystallized from $CHCl_3$-Me_2CO to yield 5.09 g (53.1%) of FEAU; the mp of 163 to 164°C remained unchanged by admixture with an authentic sample (13). [2-^{14}C]FEAU (1.31 μCi/mmol) was synthesized from α-bromo-FMAU (10) by treatment with [^{14}C]CH_3Li. Experimental details will be reported elsewhere.

Cell lines and viruses. HL-60 cells (a human promyelocytic cell line) were obtained from R. C. Gallo of the National Cancer Institute, Bethesda, Md. L1210 and P815 cells (murine leukemic cell lines) were maintained by serial transplantation in BD_2F_1 mice. Rat bone marrow cells were obtained from the femora of CD rats. Vero cells (African green monkey kidney cell line) were obtained from the American Type Culture Collection, Rockville, Md., and maintained in RPMI 1640 medium with 10% fetal calf serum. Vero cells containing 1 to 1.5 PFU of HSV-1 (strain KOS) or HSV-2 (strain 333) per cell at 12 h postinfection were used.

Cell growth inhibition studies. HL-60 cells (1.5×10^5/ml) were grown in RPMI 1640 medium containing 10% fetal calf serum, 100 μg of streptomycin per ml, and 100 U of penicillin per ml at 37°C in humidified 5% CO_2. Different drug concentrations were added for a continuous 72-h exposure. Viable cells were counted by the trypan blue exclusion method.

Incorporation of labeled precursor into DNA. The ED_{50} for inhibiting the incorporation of [*methyl*-^{3}H]thymidine ([^{3}H]dThd) (1 μCi, 0.15 nmol/ml) into a perchloric acid-insoluble fraction of DNA was measured by a previously described method (3). L1210, P815, or rat bone marrow cells were used. For calculating the K_i for natural nucleoside precursor incorporation into DNA, constant amounts of radioactivity in the form of [^{3}H]dThd (1 μCi), [6-^{3}H]uridine ([^{3}H]dUrd) (1.0 μCi), or [5-^{3}H]cytidine ([^{3}H]dCyd) (0.5 μCi) were added to various amounts of the corresponding unlabeled compound to make five to six concentrations of substrate. FEAU (5 μM) or FMAU (5 μM) was then added to measure the rates of incorporation inhibition. Incubation was carried out at 37°C for 15 min. The incubation period was 2 h for the incorporation of [2-^{14}C]FEAU (10 μM, 0.013 μCi/ml, or 100 μM, 0.13 μCi/ml) and [2-^{14}C]FMAU (10 μM, 0.12 μCi/ml) into the DNAs of L1210 cells, normal Vero cells, and HSV-1-infected Vero cells. The phenol extract procedure (8) was used to measure the amount of radioactivity incorporated into the DNA.

TABLE 2. Inhibition constants of FEAU and FMAU for inhibiting precursor incorporation into DNA in L1210 cells[a]

Substrate varied	K_i (μM) of:		FEAU/FMAU K_i ratio
	FEAU	FMAU	
[^{3}H]dThd	1,220	20.0	61
[^{3}H]dUrd	676	6.1	111
[^{3}H]dCyd	332	41.0	8.1

[a] The inhibitor concentration was 5 μM for either FEAU or FMAU, whereas the substrate precursor concentrations were varied. Incubation was carried out at 37°C for 15 min in the presence of 5×10^6 cells per ml of incubation mixture. The Lineweaver-Burk plot showed that FEAU was noncompetitive with respect to [^{3}H]dThd and was a competitive inhibitor with respect to [^{3}H]dUrd or [^{3}H]dCyd, whereas FMAU was a competitive inhibitor with respect to [^{3}H]dThd, [^{3}H]dUrd, and [^{3}H]dCyd.

Thymidine kinases. Thymidine kinases from human acute myelocytic leukemic cells (cytosolic and mitochondrial) and from HSV-1- and HSV-2-infected cells were prepared by previously described methods (1, 9). The ion-exchange paper disk method (9) was used for the thymidine kinase assays.

Datum analysis. The ED_{50}s and K_is were obtained from the median-effect plot (5) and from the K_i equation (4) by using a computer program designed to do automated analysis (2).

Animals. Male CD mice (20 to 23 g), male or female mongrel dogs (10 to 14 kg), and male cynomolgus monkeys (4 to 5 kg) were purchased from Charles River Breeding Laboratories, Inc., Kingston, N.Y., Quaker Farm Kennels, Quakertown, Pa., and Primate Imports, Port Washington, N.Y., respectively.

RESULTS

The ED_{50} of FEAU for inhibiting the growth of HL-60 cells was 2,060 μM, making FEAU 134-fold less toxic than FMAU (Table 1). This result compared favorably with those of earlier studies, in which the ED_{50}s were 2.8 μM for FMAU and >200 μM for FEAU (7, 11, 12). The ED_{50}s of FEAU against HSV-1 and HSV-2 were 0.024 and 0.24 μM, respectively. Thus, FEAU is only 4- to 10-fold less potent than FMAU. These results suggest that FEAU has a favorable therapeutic index when compared with FMAU.

The ED_{50}s of FEAU for inhibiting [^{3}H]dThd incorporation into murine leukemic cell DNA were 50-fold higher in P815 cells and 23-fold higher in L1210 cells than those of FMAU. In normal rat bone marrow cells, the ED_{50} for inhibition by

TABLE 3. Incorporation of [2-^{14}C]FEAU or [2-^{14}C]FMAU into DNA in HSV-1-infected and uninfected cells[a]

Cells	Incorporation (pmol/10^6 cells per h) of:	
	[2-^{14}C]FEAU	[2-^{14}C]FMAU
L1210	ND[b]	0.69
Vero	ND[b]	1.30
HSV-1-infected Vero	0.48	3.40

[a] The [2-^{14}C]FEAU (0.013 μCi/ml) or [2-^{14}C]FMAU (0.12 μCi/ml) concentration was 10 μM. The incubation conditions were the same as those described in Table 2, footnote *a*, except that the incubation time was 2 h.
[b] ND, Not detectable; also not detectable at 100 μM [2-^{14}C]FEAU (0.13 μCi/ml).

TABLE 4. FEAU and FMAU as inhibitors and as substrates for thymidine kinases purified from different sources

Enzyme source	K_i (μM) with respect to [^{3}H]dThd of:		FEAU/FMAU K_i ratio	% Phosphorylation relative to [^{3}H]dThd of:		FEAU/FMAU % phosphorylation ratio
	FEAU	FMAU		[2-^{14}C]FEAU	[2-^{14}C]FMAU	
Human acute myelocytic leukemic cells						
Cytosolic	150	100	1.5	1.0	81.7	
Mitochondrial	176	100	1.7	ND[a]	219.0	
HSV-1 (strain KOS)	0.60	0.59	1.0	82.7	42.0	1.97
HSV-2 (strain 333)	0.74	2.50	0.3	203.2	146.6	1.39

[a] ND, Not detected.

FEAU was 415-fold higher than for inhibition by FMAU, suggesting that FEAU will not cause bone marrow depression (Table 1). Cellular studies of natural precursor incorporation into the DNA of L1210 cells showed that FEAU had an 8- to 111-fold lower rate of inhibition of DNA synthesis than did FMAU (Table 2). While FMAU inhibited [^{3}H]dUrd incorporation more efficiently than [^{3}H]dCyd incorporation, FEAU inhibited [^{3}H]dCyd incorporation more efficiently than [^{3}H]dUrd incorporation or [^{3}H]dThd incorporation. These results also indicate that FEAU is a weaker inhibitor of natural nucleoside anabolism than is FMAU in mammalian cells.

There was no detectable [2-^{14}C]FEAU radioactivity incorporated into either L1210 cell DNA or Vero cell DNA, but substantial quantities of [2-^{14}C]FMAU were incorporated into the DNA of L1210 cells and into the DNA of Vero cells (Table 3). When HSV-1-infected Vero cells were exposed to [2-^{14}C]FEAU (10 μM), however, 0.48 pmol of [2-^{14}C]FEAU was incorporated into the DNA per 10^6 cells per h. Under the same conditions, 7.1-fold more [2-^{14}C]FMAU than [2-^{14}C]FEAU was incorporated into the DNA of HSV-1-infected Vero cells.

FEAU, like FMAU, exhibited a higher affinity for HSV-1 and HSV-2 thymidine kinases (K_i, 0.6 to 2.5 μM) than it did for host cell enzymes (K_i, >150 μM). Although FEAU was a very poor substrate for cytosolic thymidine kinase, it served as a good substrate for the thymidine kinases produced by both HSV-1 and HSV-2 (Table 4). In contrast, FMAU served as a good substrate for mammalian cell thymidine kinases as well as for the enzymes from HSV-1 and HSV-2 (Table 4).

In vivo experiments (Table 5) indicated that FEAU and FMAU have different species-related risks of toxicity. In phase I clinical trials an FMAU dose as low as 0.8 mg/kg per day (five intravenous doses) caused encephalopathy with extrapyramidal dysfunction (6); the toxicity of FEAU in humans is unknown.

DISCUSSION

The present studies indicate that, in various mammalian systems, FEAU inhibits cell growth and natural nucleoside incorporation into cellular DNA substantially less than does FMAU. In addition, the incorporation of labeled FEAU into cellular DNA is far less than that of labeled FMAU. FEAU, however, is only 4- to 10-fold less effective in vitro than is FMAU against HSV-1 and HSV-2.

In cell extracts the K_is of FMAU and FEAU are similar for mammalian and viral enzymes. The difference in antiviral potency between FEAU and FMAU is probably due to their different affinity for virus encoded DNA polymerases. As indicated in Table 3, [2-^{14}C]FMAU incorporates sevenfold more radioactivity into HSV-1-infected Vero cells than does [2-^{14}C]FEAU. This difference in incorporation is comparable to the magnitude of the difference between their antiherpetic effects in vitro.

Despite the fact that FEAU is a somewhat weaker antiherpetic agent than FMAU, it is still one of the most potent agents currently available. The most important factor determining the therapeutic usefulness of FEAU and FMAU is the degree of toxicity exhibited toward the host. Although FMAU is more potent against HSV-1 and HSV-2 in vitro (ED_{50}s, 0.006 M and 0.023 μM, respectively) and against HSV-1 in mice (ED_{50}, 1 mg/kg [intraperitoneal dose]), FMAU produces encephalopathy in humans at 0.8 mg/kg per day (five intravenous doses), which severely limits its therapeutic usefulness (6). Encephalopathy does not occur in mice and rats at FEAU doses as high as 500 mg/kg per day (five intraperitoneal doses). Nor does encephalopathy occur in dogs at FEAU doses of 50 mg/kg per day (10 intravenous doses). These findings indicate that among different animal

TABLE 5. Toxic and lethal doses of FEAU and FMAU[a]

Animal species	Toxic dose (mg/kg per day) and effect of:		Lethal dose (mg/kg per day) and effect of:	
	FEAU	FMAU	FEAU	FMAU
Mouse	800, 4 i.p. doses; no weight loss	250, 5 i.p. doses; weight loss		1,000, 5 i.p. doses; 1 death/5 tested
Dog	50, 10 i.v. doses; 0 deaths/1 tested	1.25, 10 i.v. doses; 0 deaths/2 tested	100, 10 i.v. doses; 1 death/1 tested	5, 5 i.v. doses; 2 deaths/2 tested
Monkey	30, 6 i.v. doses[b]; normal appetite, normal hematological test results	10, 10 i.v. doses; slight weight loss; 20, 10 i.v. doses; weight loss, tremor		

[a] i.p., Intraperitoneal; i.v., intravenous.
[b] K. F. Soike, Tulane Delta Regional Primate Research Center, Covington, La., personal communication.

species there are great differences in toxicity between FEAU and FMAU. The negligible toxic effects of FEAU in various mammalian cell systems indicate its therapeutic potential. More detailed in vivo studies are necessary, however, for further development of this compound.

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