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Activities of 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine and Its Metabolites against Herpes Simplex Virus Types 1 and 2 in Cell Culture and in Mice Infected Intracerebrally with Herpes Simplex Virus Type 2†

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As measured by plaque and yield reduction assays, several metabolites of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) were highly active against herpes simplex virus types 1 and 2. These metabolites included the 2′-deoxy-2′-fluoroarabinosyl derivatives of 5-iodouracil (FIAU), cytosine (FAC), uracil (FAU), and thymine (FMAU). In mice inoculated intracerebrally with herpes simplex virus type 2, the relative order of potency of these compounds and licensed antiviral drugs was as follows: FMAU >> FIAC ≈ FIAU > acyclovir ≈ vidarabine >> FAC ≈ FAU. One of the main metabolites of FMAU, 2′-fluoro-5-hydroxymethyl-arabinosyluracil, was essentially inactive in vivo. FIAC-, FIAU-, FMAU-, FAC-, and FAU-resistant herpes simplex virus variants prepared in cell culture were found to be (i) devoid of viral thymidine kinase, (ii) cross-resistant to one another and resistant to drugs requiring viral thymidine kinase for activation, and (iii) sensitive to vidarabine or phosphonoformate. These results indicate that FIAC, FIAU, and FMAU require the virally encoded thymidine kinase for activation and suggest that the antiviral activity of FAU and FAC in cell cultures is also mediated by this enzyme. The interaction of the fluoroarabinosyl pyrimidine nucleosides with herpes simplex virus thymidine kinase in a cell-free system is also described.

Recently, the activities of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (2′-fluoro-5-iodoarabinosylcytosine; FIAC) and its thymine analog, 2′-fluoro-5-methyl-arabinosyluracil (FMAU), were compared with those of vidarabine (ara-A) and acyclovir (ACV) in mice infected intracerebrally with herpes simplex virus type 2 (HSV-2). FIAC and FMAU were more effective in this model than either ACV or ara-A when the drugs were administered intraperitoneally, and FMAU was the most potent nucleoside antiviral agent, with a therapeutic index of more than 3,000 (30).

Several metabolites of FIAC that are produced in mammalian systems by chemical or enzymatic deamination, deiodination, and methylation reactions have recently been identified (7–9, 15, 19, 23, 26; T.-C. Chou, C. Lopez, J. M. Colacino, J. Grant, A. Feinberg, T.-L. Su, K. A. Watanabe, J. J. Fox, and F. S. Philips, Proc. Am. Assoc. Cancer Res. **24**:205, 1983). The metabolites found in plasma and urine include 2′-fluoro-5-iodoarabinosyluracil (FIAU), 2′-fluoro-arabinosylcytosine (FAC), 2′-fluoroarabinosyluracil (FAU), and FMAU (Fig. 1). In addition, there are at least two metabolites of FMAU that have been isolated from the urine of mice (15). These metabolites include 2′-fluoro-5-hydroxymethyl-arabinosyluracil (FHMAU) and a glucuronide of FMAU. The glucuronide conjugates of FIAC, FIAU, FAU, and FAC have also been isolated from the urine of humans receiving FIAC (14).

In this study we compared the activities of the parent drug and its metabolites in cell cultures by using plaque and yield reduction assays and in mice inoculated intracerebrally with HSV-2. The patterns of resistance and cross-resistance of FIAC-, FIAU-, FMAU-, FAU-, and FAC-resistant herpes simplex virus (HSV) variants were also examined, as they provided an insight into the mechanism of action of these drugs and an appreciation of the potential problems that may be encountered clinically.

(Part of this work was presented at the Eighth International Herpesvirus Workshop, 31 July to 5 August 1983, Oxford, England, p. 235.)

MATERIALS AND METHODS

Compounds. The 2′-fluoronucleosides FIAC, FMAU, FIAU, FAC, FAU, and FHMAU were synthesized as previously described (15, 16, 17, 34, 35). ACV was a gift from G. Elion, Burroughs-Wellcome Co., Research Triangle Park, N.C. Other compounds were obtained as follows: ara-A, from Sigma Chemical Co., St. Louis, Mo.; trisodium phosphonoformate hexahydrate, from Astra Läkemedel AB, Södertälje, Sweden; 5-*E*-(2-bromovinyl)-2′-deoxyuridine, from E. de Clercq, Rega Institute for Medical Research, Leuven, Belgium; and 5-ethyl-2′-deoxyuridine, from Ortho Pharmaceutical Ltd., Toronto, Canada. All of the nucleosides except FAC were free bases; FAC was obtained as the HCl salt. The compounds were dissolved in pH 7.4 phosphate-buffered saline (PBS) and were soluble at 37°C in medium and in PBS at all of the concentrations and doses described below. The compounds were sterilized just before use by passage

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† This paper is dedicated to the memory of Frederick S. Philips, a pioneer in the development of 2′-fluoro-arabinosyl-pyrimidine nucleosides.

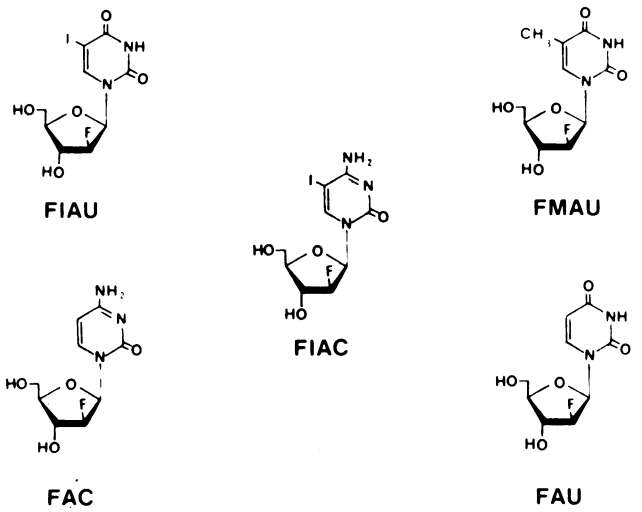

FIG. 1. Chemical structures of FMAU, FIAC and its metabolites.

through a membrane filter (0.22 μm; Millipore Corp., Bedford, Mass.).

Cells and viruses. Vero and HEp-2 cells were obtained from Flow Laboratories, McLean, Va. Plaque-purified HSV-1 strain F and HSV-2 strain G were supplied by B. Roizman, University of Chicago, Chicago, Ill. (12); high-titer pools were prepared as described elsewhere (31).

Antiviral and cytotoxicity assays. The compounds examined in this study were screened for antiviral activity against HSV-1 and HSV-2 by using plaque and yield reduction assays with Vero cells and the methods described previously (29–31). In the yield reduction assays, residual drug was removed by washing the cells three times with PBS after virus adsorption (31).

The cytotoxic activities of the drugs were measured for 3 days in rapidly dividing Vero cells, as described previously (31). The trypsinized cells were counted with a hemacytometer in the presence of 3% trypan blue.

Isolation of resistant variants. Drug-resistant HSV variants were prepared by single passage of the virus in Vero cells at a multiplicity of infection of 10^{-3} in the presence of a drug (10 μM) dissolved in a 1% agarose overlay. Individual plaques were picked, and a high-titer pool was prepared in HEp-2 cells. The different viruses were then tested for increased resistance to that drug and related antiviral drugs. The 50 and 90% effective doses (ED_{50}s and ED_{90}s, respectively) for the viruses were estimated from a liner regression analysis of plots of percentages of plaque reduction versus the logarithm of the drug concentration. Only the linear portion of the plot of the raw data titration curve was used in the analysis.

TK assay. The enzyme extract preparations used in the thymidine kinase (TK) assays were obtained from HeLa Bu-25 cells (deficient in cytocellular TK) which had been infected with HSV-1 strain F and HSV-2 strain G. The methods used for preparation of the enzymes and for the assay have been described previously (6). The amount of enzyme used was 0.1 U. The controls had no nucleoside analogs in the assay mixture. The final concentrations of the thymidine and nucleoside analogs were 100 and 200 μM, respectively.

Infection of mice. Acclimatized random-bred Swiss ICR mice (female; 5 to 6 weeks old; Harlan-Sprague Co., Indianapolis, Ind.) were inoculated in the right cerebral hemisphere under anesthesia (Metofane; Pitman-Moore Co., Washington Crossing, N.J.) with HSV-2 strain G (12). The virus (35 PFU; equivalent to about 7 50% lethal doses) caused a mortality rate of 92% in PBS-treated animals. The reasons for selecting this virus strain for the animal studies have been discussed previously (30, 31). The virus titer of the inoculum was determined in Vero cells by a plaque assay. Control animals were inoculated intracerebrally with PBS (0.05 ml).

Drug treatment. Mice were inoculated intracerebrally at noon (day 0) and were treated with a drug 5 h later. The recipients of the drug or PBS were randomized after virus inoculation. The drug solutions were injected in 0.5-ml doses intraperitoneally twice daily at 9 a.m. and 5 p.m. for 4 days (total of eight doses). The dosage ranges, 0.5 to 200 mg/kg per day, were based on results obtained with FMAU, FIAC, and ACV in previous studies (30, 31). Mice were weighed on days 0, 1, 3, 5, 7, 14, 21, and 30. The fluctuations in the weights of the animals during treatment were not large enough to necessitate adjustment of the dose. Only the data for the maximum weight loss are shown in Table 4. The cages were checked for dead mice at least twice daily throughout the duration of the study.

Drug toxicity in mice. Uninfected mice were divided so that each group had the same mean body weight, and the weight of each animal was within 1 g of the mean group weight at the beginning of the experiment. The drugs dissolved in PBS were administered intraperitoneally to ear-marked mice. The animals were weighed individually on days 0, 1, 3, 5, 7, 10, 14, 21, 28, and 30.

UV absorption and solubility characteristics. The UV spectra of the drugs were obtained in acid or base solutions by using a model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The solubilities of the drugs were determined by measuring the levels of absorbance at the λ_{max}s of the compounds in saturated aqueous solutions that had been kept at 37°C for 48 h.

Statistical analyses. The 50% lethal doses for the viruses were determined from a pilot study and were estimated by the method of Reed and Muench (27). The survival data from the mouse studies were assessed by using a survival analysis computer program (BMDP1L), which was obtained from the University of California Health Services Computing Facility, Los Angeles; *P* values derived by the Breslow (Savage) test are reported below. Animals that survived past day 21 were included as censored data. Differences in the mortality rates among the various groups were assessed by Fischer's exact tests. Probit analysis was used to estimate the ED_{50}s for each drug in the mouse studies, as described previously (30). A linear regression analysis of a plot of percentage of plaque reduction versus logarithm of drug concentration was used to determine the ED_{50}, ED_{90}, and 50% inhibitory dose (ID_{50}) for each drug. A program obtained from Basic Business Software, Las Vegas, Nev., assisted in these determinations.

RESULTS

Antiviral and cytotoxicity assays. FIAC, FMAU, FIAU, FAC, and FAU inhibited HSV-1 and HSV-2 plaque formation by 50% in Vero cells at concentrations ranging from 0.009 to 0.07 μM. FIAC, FMAU, FIAU, and FAU were more active than FAC. These findings were even more apparent at the ED_{90} level (Table 1).

The results were different when the drugs were tested by

TABLE 1. Antiviral and cytotoxic activities and effects on viral TK of FIAC and its metabolites and other licensed drugs

Compound	Antiviral activity				Cytotoxicity (ID_{50}) (μM)[b]	Therapeutic index (ID_{50}/ED_{90}) for:		TK activity (of control value)[c]	
	HSV-1 strain F		HSV-2 strain G						
	ED_{50} (μM)[a]	ED_{90} (μM)[a]	ED_{50} (μM)	ED_{90} (μM)		HSV-1 strain F	HSV-2 strain G	HSV-1 strain F	HSV-2 strain G
FIAC	0.023	0.048	0.030	0.084	21.7	452	258	40.6	96.5
FMAU	0.028	0.061	0.033	0.11	7.9	130	72	36.0	76.2
FIAU	0.014	0.042	0.009	0.040	10.3	245	258	21.8	61.0
FAC	0.07	0.35	0.070	0.87	15.1	43	17	72.0	106.4
FAU	0.014	0.065	0.020	0.33	2.9	45	9	83.4	105.6
FHMAU	0.48	8.1	2.6	24.5	297	37	12	109.9	116.5
ACV	0.071	0.31	0.043	0.36	1,600	5,161	4,444	97.6	93.4
Ara-A	30.3	59.8	50.0	94.7	60.0	1	0.6	ND[d]	ND

[a] ED_{50} and ED_{90}, Concentrations of compound that reduced the number of plaques by 50 and 90%, respectively, compared with the virus control cultures containing no compound.
[b] ID_{50}, Concentration of compound that inhibited 50% of cell growth on day 3 in rapidly dividing Vero cells.
[c] ^{14}C-labeled thymidine phosphorylation activity. The final drug concentration was 200 μM.
[d] ND, Not determined.

the yield reduction assay. FIAC and FIAU were about 1 order of magnitude more active than FMAU, FAC, or FAU, but were less effective against HSV-2 than against HSV-1 (Table 2). FAU appeared to be more effective against HSV-2 than against HSV-1 (Table 2), and FMAU and FAC were equally effective against both viruses. Whereas the activity of FIAU against HSV-2 (but not HSV-1) plateaued at a concentration of about 0.5 μM, the dose-response curves for FMAU were almost identical for the two viruses (Table 2).

The ranking of the compounds with respect to cytoxicity was as follows: FAU > FMAU > FIAU > FAC > FIAC. This ranking suggested that the cytosine analogs were less toxic to Vero cells than the uracil derivatives (Table 1). However, the rankings with respect to therapeutic index (ID_{50}/ED_{90}) were as follows: FIAC > FIAU > FMAU > FAC = FAU for HSV-1 and FIAC = FIAU > FMAU > FAC > FAU for HSV-2 (Table 1). Although the 5-hydroxymethyl analog (FHMAU) exhibited significant antiviral activity, it was about 1 order of magnitude less active and less toxic than FMAU, FIAC, or any of its metabolites (Table 1). The antiviral activity of FHMAU was considered to be specific since it was essentially nontoxic to host Vero cells. ACV was the least toxic of the compounds tested, with a therapeutic index of more than 4,000 for both viruses. For comparison, ara-A, which was also included in these cell culture studies (Table 1), was markedly less toxic and less active than the 2'-fluoroarabinosyl nucleosides.

Enzyme studies. The phosphorylating behaviors of HSV-1- and HSV-2-induced TKs in the presence of the nucleoside analogs at concentrations of 200 μM are also shown in Table 1. Most of the compounds competed effectively with thymidine for the viral TK extracted from HSV-1-infected HeLa Bu-25 (TK^-) cells. However, with the exception of FMAU and FIAU, the fluorinated compounds tested did not affect the phosphorylation of thymidine by the HSV-2 TK.

Resistance studies. Drug-resistant clones were isolated from single passages of HSV-1 and HSV-2 in the presence of the fluorinated nucleosides at concentrations of 10 μM, and high-titer pools were prepared in the absence of drugs. FIAC-, FMAU-, FAU-, and FAC-resistant HSV-1 and HSV-2 variants were readily produced in cell cultures, and these variants were cross-resistant to one another in Vero cells (Table 3). Compared with the parent virus cloned in the absence of drugs, the variants tested were essentially refractory to FIAC, FMAU, ACV, 5-*E*-(2-bromovinyl)-2'-deoxyuridine, and 5-ethyl-2'-deoxyuridine treatment, but responded to ara-A or phosphonoformate. For comparison, an ACV-resistant clone was included. All of the drug-resistant clones were found to be TK^- since they induced less than 0.2% of the TK activity of the parent clone.

Mouse studies. When mice were inoculated with about 7 50% lethal doses of HSV-2, the mortality rate was 92% in the PBS treated group (Table 4). However, administration of FIAC and FIAU (10 mg/kg per day) or ACV (60 mg/kg per day) produced significant increases in both numbers of

TABLE 2. Effects of FIAC, FMAU, FIAU, FAC, and FAU against HSV-1 and HSV-2 as determined by yield reduction assays in Vero cells

Compound	Concn (μM)	Yield (% of control value)[a]	
		HSV-1 strain F	HSV-2 strain G
FIAC	0.01	39	50
	0.05	22	41
	0.1	6.3	29
	0.5	0.92	2.5
	1.0	0.05	0.36
FMAU	0.1	55	49
	0.5	32	29
	1.0	21	18
	2.5	7.5	10
	5.0	1.5	2.0
	10.0	0.35	0.41
FIAU	0.1	14	40
	0.5	0.5	12
	1.0	0.3	9.9
FAU	0.1	26	39
	0.5	18	13
	1.0	9.2	8.5
	2.5	6.2	2.1
	5.0	2.8	1.1
	10.0	0.8	0.18
FAC	0.1	90	98
	0.5	60	89
	1.0	33	42
	5.0	4.0	8.9
	10.0	2.2	3.0

[a] The virus yields in the absence of drug were as follows: strain F, 1.14×10^7 PFU/ml; strain G, 1.09×10^7 PFU/ml. The multiplicity of infection was 0.1.

TABLE 3. Cross-resistance studies with FMAU-, FIAC-, FIAU-, FAU-, FAC-, and ACV-resistant HSV-1 and HSV-2 variants derived from strains F and G

Virus	ED_{50} (μM) of:									
	FIAC	FIAU	FMAU	FAU	FAC	ACV	BVDU[a]	EDU[b]	ara-A	PFA[c]
F-FMAU-C2[d]	8.0 (400)[e]	ND[f]	11 (550)	ND	ND	19 (146)	280 (9,333)	>200 (>20)	60 (1.7)	30 (1.1)
G-FMAU-C1	18 (545)	13 (1,444)	8.5 (283)	>40 (>2,000)	3.4 (49)	20 (33)	ND	773 (94)	73 (1.6)	47 (1.6)
F-FIAC-C3	51 (2,550)	ND	10 (500)	ND	ND	14 (108)	210 (7,000)	>200 (>20)	60 (1.7)	55 (2.0)
G-FIAC-C1	21 (636)	9.0 (1,000)	11 (367)	3.5 (175)	3.3 (47)	35 (58)	ND	257 (31)	85 (1.9)	66 (2.2)
F-FIAU-C1	11 (560)	8.5 (607)	6.9 (345)	>200 (>10^4)	6.1 (87)	13 (97)	ND	ND	34 (1.0)	32 (1.2)
G-FIAU-C1	29 (879)	15 (1,667)	9.2 (307)	>200 (>10^4)	2.8 (40)	>10 (>17)	ND	ND	39 (0.87)	19 (0.63)
F-FAU-C2	3.8 (190)	7.8 (557)	3.3 (165)	49 (3,500)	8.1 (116)	34 (262)	ND	ND	54 (1.5)	28 (1.0)
G-FAU-C2	3.6 (109)	29 (3,222)	12 (400)	>200 (>10^4)	1.4 (20)	>5 (>8)	ND	ND	24 (0.53)	26 (0.87)
G-FAC-C1	15 (455)	17 (1,922)	8.6 (287)	140 (7,000)	7.3 (104)	17 (28)	ND	ND	48 (1.1)	18 (0.60)
F-ACV-C4	22 (1,100)	ND	12 (600)	ND	ND	34 (262)	>300 (>10^4)	>400 (>40)	56 (1.6)	29 (1.0)

[a] BVDU, 5-*E*-(2-Bromovinyl)-2′-deoxyuridine.
[b] EDU, 5-Ethyl-2′-deoxyuridine.
[c] PFA, Phosphonoformate.
[d] The viruses are coded according to the parent strain from which they were derived, the drug in which they were prepared, and the clone number.
[e] The numbers in parentheses are fold increases in resistance, which were calculated as follows: ED_{50} of resistant variant/ED_{50} of the corresponding parent cloned strain.
[f] ND, Not determined.

survivors and survival time. The results obtained with FMAU and FIAC were consistent with our previous observations; FMAU was at least 1 order of magnitude more active than FIAC (30). The median effective daily doses were 3.23 mg/kg (95% confidence interval, 0 to 12.7 mg/kg) and 6.4 mg/kg (95% confidence interval, 0.21 to 18.0 mg/kg) for FIAC and FIAU, respectively. Although FIAC appeared to be more active than FIAU, when the slopes and equality of the dose-response lines were compared, this difference was not significant (analysis of variance, $P \geq 0.14$). FAU and

TABLE 4. Effects of FIAC and its metabolites on mice inoculated intracerebrally with HSV-2 strain G

Treatment[a]	Dose (mg/kg per day)	Mortality (no. of mice dead/no. of mice treated)[b]	Mean day of death[c]	Maximum wt change		% of surviving animals with cataracts[d]	ED_{50}	
				%	Day		mg/kg per day	μmol/kg per day
PBS		12/13 (92)	8.3	−20	14	100		
FMAU	0.5	8/23 (35)[e]	11.1[f]	0	7	25	0.12[g]	0.6[g]
FIAC	5	6/13 (46)[h]	14.9[i]	−9	14	83	3.23–9.3[g]	8.7–25.1[g]
	10	2/13 (15)[f]	15.0[f]	−6	5	40		
	20	1/12 (8)[i]	(8)	2	3	18		
	40	1/12 (8)[i]	(16)	4	3	40		
	80	0/12 (0)[i]		2	5	0		
FIAU	5	9/13 (69)[j]	13.2[e]	−9	7	50	6.4	17.2
	10	5/13 (38)[f]	12.2[e]	−6	7	25		
	20	2/12 (17)[f]	13.5[i]	−6	5	25		
	40	2/11 (18)[f]	11.5[f]	1	3	0		
	80	1/12 (8)[i]	(13)	0	5	18		
FAU	30	13/14 (93)[j]	7.0[j]	−15	7	0	>160	>650
	80	12/12 (100)[j]	11.7[j]	−28	14			
	160	10/12 (83)[j]	7.3[j]	−12	7	50		
FAC	30	11/14 (79)[j]	8.4[j]	−26	14	0	>200	>710
	80	10/12 (83)[j]	10.9[j]	−20	14	100		
	100	8/12 (67)[j]	9.6[j]	−12	7	50		
	200	11/15 (73)[j]	12.9[j]	−18	14	50		
ACV	20	7/12 (58)[j]	12.5[j]	−6	7	75	33.1–60.9[g]	147–271[g]
	40	8/12 (67)[j]	8.0[j]	−11	7	100		
	60	4/12 (33)[f]	13.5[f]	6	7	14		
Control (no virus)		0/12 (0)		−4	7	0		

[a] Mice were treated 5 h after intracerebral inoculation. Doses were administered intraperitoneally twice a day for 4 days.
[b] Calculated on day 21. The numbers in parentheses are percentages.
[c] Calculated on day 21. The numbers in parentheses indicate that a single animal died.
[d] Mice were examined on day 31.
[e] Probability that the observed increase in survivor number or increase in mean day of death was due to chance, <0.01.
[f] $P < 0.001$.
[g] Data from reference 30. For comparison, the ED_{50} for ara-A was 25.1 mg/kg per day or 94 μmol/kg per day.
[h] $P < 0.05$.
[i] $P < 0.0001$.
[j] Not significant.

TABLE 5. UV and solubility characteristics of selected antiviral nucleosides

Compound	0.01 MHCl[a]		0.01 MNaOH[a]		Solubility (mg/ml)[b]
	λ_{max} (nm)	λ_{min} (nm)	λ_{max} (nm)	λ_{min} (nm)	
FMAU	268.5 (14,690)[c]	238 (4,420)[c]	271 (11,440)[c]	249 (7,930)[c]	>20
FAU	262 (12,300)	232 (3,813)	263 (10,186)	245 (8,056)	>20
FAC	281 (16,609)	243.5 (3,378)	273 (11,682)	252 (8,867)	>20
FIAC	311 (9,646)	266 (2,690)	298 (8,255)	268 (5,287)	7.50
FIAU	288 (11,346)	252 (4,464)	281 (8,928)	256 (6,138)	4.85
ACV	258 (13,838)	230 (4,050)	265 (12,825)	236 (6,413)	1.36
Ara-A	261 (15,086)	235 (4,272)	264 (15,486)	233 (4,539)	1.25

[a] Determined with a Beckman model 25 spectrophotometer.
[b] Determined by UV spectrophotometry at 37°C in PBS (pH 7.2).
[c] The numbers in parentheses are ϵ values.

FAC were essentially inactive when they were administered in daily doses of up to 200 mg/kg.

In a separate experiment designed to determine whether FHMAU, the main metabolite of FMAU, had activity comparable to that of FMAU, groups of 10 mice were inoculated intracerebrally with a lethal dose of HSV-2 strain G. After 5 h the mice were treated intraperitoneally with one drug or the other or with PBS twice a day for 4 days. The mortality rates for animals treated with PBS, FHMAU (4 mg/kg per day), and FMAU (1 mg/kg per day) were 90% (mean day of death, 5.8 ± 1.4), 100% (mean day of death, 5.6 ± 1.5), and 55% (mean day of death, 14.0 ± 3.7; $P < 0.01$), respectively.

Weight loss was usually greater in infected mice that received the lower dose of a drug (Table 4). The mice that initially lost weight or failed to gain weight gradually gained weight after day 14. There were no signs of toxicity, such as decrease in weight, failure to gain weight, or death, in uninfected mice given FIAU in half daily doses of 100 mg/kg per day for 4 days, FAU in doses of 200 mg/kg or FAC in doses of 400 mg/kg (data not shown). The toxicities of FMAU, FIAC, ACV, and ara-A when the same dose schedule was used have been reported previously (30, 31).

A significant number of surviving mice developed unilateral or bilateral cataracts by day 31 after virus inoculation. The proportion of mice with cataracts was generally lower when a higher dose of an antiviral drug was used (Table 4). Even when an effective dose of FIAC was used (survival rate, 92%), almost one-half the animals developed cataracts.

Drug solubility. The aqueous solubilities at 37°C of the 2'-fluoroarabinosyl nucleosides were compared with those of ACV and ara-A by using UV spectroscopy. The order of solubility of the free nucleosides was as follows: FMAU and FAU > FIAC > FIAU > ACV > ara-A (Table 5).

DISCUSSION

The findings that FIAC has a high level of activity against experimentally induced HSV encephalitis in mice (30) and a complex metabolism (Fig. 2) prompted us to determine whether any of the metabolites of this compound had comparable activity in vitro and in vivo. These studies are particularly relevant to clinical trials with FIAC and FMAU in patients with herpesvirus infections (13, 14, 36; M. Fanucchi, B. Leyland-Jones, J. Burchenal, C. Young, B. Clarkson, C. Lopez, et al., Proc. Am. Assoc. Cancer. Res. **24**:150, 1983; B. Leyland-Jones, H. Donnelly, P. Myskowski, A. Donner, S. Goshen, C. Lopez, D. Armstrong, C. W. Young, and J. J Fox, Clin. Res. **31**:369A, 1983).

Using chromatographic analyses of plasma and urine obtained from mice or rats given FIAC, Philips et al. (26) showed that this substance is deaminated to FIAU as a major metabolite. Deiodinated metabolites (FAC and FAU) and their glucuronides have also been detected; only trace amounts of FMAU (<0.5% of the administered dose) were found in urine (26). In dogs, the main metabolites are FAC and FAU, and only trace amounts of FIAU are found (26). In humans, FIAC is rapidly transformed to FIAU and to FAC, FAU, and the glucuronide conjugates of FIAC, FIAU, FAC, and FAU; FMAU could not be detected in plasma or urine (8, 14). Chou et al. (8) reported that FIAU, FAU, FAC, and FMAU appeared in mouse blood and livers and in DNAs of mouse small intestines or leukemic cells that had been treated with FIAC (Fig. 2). The deamination of FIAC to FIAU in vivo is probably mediated by a pyrimidine nucleoside deaminase (8). However, the deiodination of the 2'-fluoro-5-iodoarabinosylcytosine 5'-monophosphate (FIACMP) appeared to be mediated by thymidylate

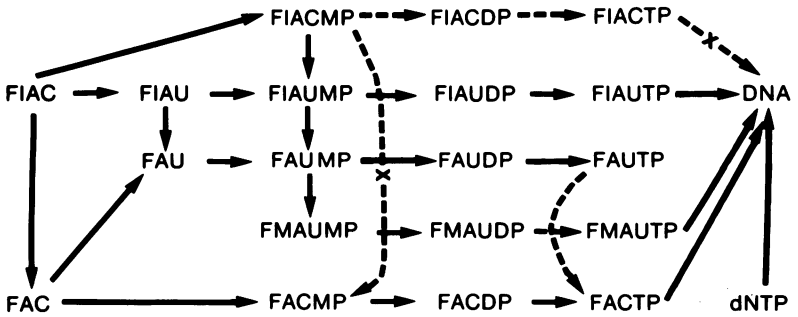

FIG. 2. Probable metabolites of FIAC in mammalian systems. The dashed lines indicate pathways which may occur but for which there is at present no evidence. The crosses indicate that the pathways do not occur. dNTP, 2'-Deoxynucleoside 5'-triphosphates.

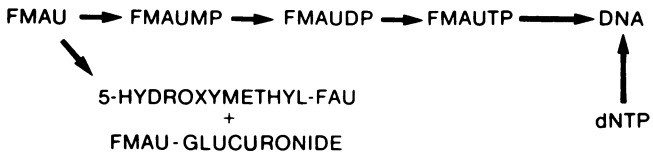

FIG. 3. Probable metabolites of FMAU in mammalian systems. dNTP, 2'-Deoxynucleoside 5'-triphosphates.

synthase (T. J Braun, A. Feinberg, T.-C. Chou, K. A. Watanabe, J. J. Fox, and F. S. Philips, Proc. Am. Assoc. Cancer Res. **24**:213, 1982). 2'-Fluoro-5-iodoarabinosyluracil 5'-monophosphate (FIAUMP) can be further biotransformed in vitro to the nucleotide of FMAU (Fig. 2) (Braun et al., Proc. Am. Assoc. Cancer Res. **24**:213, 1982). Neither FIAU, FIAC, nor FIAC serves as a substrate for thymidylate synthase (Braun et al., Proc. Am. Assoc. Cancer Res. **24**:213, 1982). However, it is chemically possible to deiodinate FIAC to FAU via FAC (i.e., without first deaminating FIAC) (Fig. 2) (19). The enzyme responsible for this bioconversion is not known. The radioactivity of ^{14}C-labeled FIAC is incorporated in vivo into the DNAs of normal proliferating tissues and of leukemic cells of mice, where it appears as FAC, FIAU, and FMAU, but not as FIAC (19). In contrast, ^{14}C-labeled FMAU is incorporated into viral DNA (Fig. 3). FMAU is incorporated into termini and internucleotide linkages of viral DNA at a 250-fold higher level than it is incorporated into the DNA of uninfected cells, resulting in short viral DNA strands (Chou, et al., Proc. Am. Assoc. Cancer Res. **24**:205, 1983). After administration of FMAU to rodents, FHMAU and the glucuronide of FMAU can also be detected in urine (15). Allaudeen et al. (1) and Ruth and Cheng (28) previously reported that 2'-fluoro-5-iodoarabinosylcytosine 5'-triphosphate (FIACTP) was more inhibitory to the DNA polymerases of HSV-1 and HSV-2 than to the cellular DNA polymerases. However, there was no appreciable difference in the K_m-K_i ratios for the viral and host polymerases. FIAU and FMAU appear to be more potent inhibitors of DNA synthesis in P815 leukemic cells than FIAC is (7). In HSV-infected cells, FIAC and FIAU are rapidly taken up and phosphorylated only to the 5'-monophosphate (23); presumably, the monophosphates are further phosphorylated by cellular enzymes to the corresponding triphosphates (Fig. 2). Of particular importance was the finding that the metabolites of FIAC retain their antiviral activity in cell culture (16, 17, 24) (Table 1). Since little is known about the fate of FIAC and its metabolites in HSV-infected cells and animals, it was important to determine the antiviral activities of these compounds in mice inoculated intracerebrally with HSV, a model that appears to be a good prognosticator of drugs which can be effective in humans (31).

First, we confirmed that all of the main metabolites of FIAC had potent antiviral activity in cell cultures against HSV-1 and HSV-2 (Table 1) and that the antiviral activities of the drugs were not secondary to their toxic effects. The order of the therapeutic index (ID_{50}/ED_{90}) values of the drugs was as follows (in increasing order): ACV > FIAC ≈ FIAU > FMAU > FAC ≈ FAU ≈ FHMAU >> ara-A. The relative potencies of the 2'-fluoronucleosides in cell cultures are also consistent with findings reported previously (16, 17, 24, 34, 35), with the exception of FAU and FHMAU, which previously were found to have poor antiviral activity compared with the other fluorinated compounds (15–17, 24). Technical differences must account for these disparities. These differences include virus type, virus and cell passage history, the composition and concentration of natural nucleosides in the medium, multiplicity of infection, delay in adding the overlay after virus adsorption, and the duration of incubation of the drugs. It is interesting that FHMAU was essentially nontoxic to the host Vero cells (ID_{50}, ≈300 μM), whereas 5-hydroxymethyl-2'-deoxyuridine (which differs structurally from FHMAU only by the absence of a 2'-fluoro group) was cytotoxic to a variety of normal and tumor cell lines (32). The activities of the fluorinated nucleosides were also determined by yield reduction assays. Whereas by the plaque reduction assay all of the drugs inhibited HSV plaque formation by 50% at concentrations ranging from 0.009 to 0.07 μM, in yield reduction assays FIAC and FIAU were 1 order of magnitude more active than FMAU, FAU, or FAC. The dose-effect plots for FIAC and FIAU against HSV-2 were different, suggesting that these two drugs may not be equivalent in this cell culture system.

In mice inoculated intracerebrally with HSV-2, FMAU was the most potent compound. Based on this finding and a previous report (30), the relative potencies of the agents tested were as follows: FMAU >> FIAC ≈ FIAU > ara-A ≈ ACV >> FAC ≈ FAU (Table 4). Although at the doses tested FAC, FAU, and FHMAU were potent inhibitors of HSV-2 in cell culture, these compounds were essentially devoid of antiviral activity in the encephalitis model. This dichotomy between in vitro activity and in vivo activity suggests that these agents do not cross the blood-brain barrier in sufficient concentrations to exert activity or that they are rapidly metabolized, detoxified, or excreted. In contrast to the in vivo conditions, concentrations of the drugs remained almost constant in vitro throughout the test period. These results also suggest that FIAU is probably the main metabolite of FIAC in mice, since on an equal dose schedule, FIAC was essentially equivalent to FIAU (Table 4) and much less potent than FMAU (30).

It might be argued that direct brain inoculation is not a "natural" route of infection and that the mechanical trauma to brain tissue at the injection site may alter the blood-brain barrier and allow drug leakage. In other experimental infections in mice, including infections produced by intranasal, intravaginal, or intradermal inoculation of virus (10, 11, 21, 22), an antiviral drug may inhibit virus replication and spread before the virus reaches the brain. Therefore, unless a delayed treatment schedule is used, these infections may be of little value for predicting the efficacy of drugs used for treatment, rather than prophylaxis, of clinical neonatal and adult herpes encephalitis. In addition, immunological factors, such as macrophages, can play a role in limiting virus spread when virus is inoculated by other routes (e.g., intraperitoneally) (20, 25). We and other workers have shown that a localized injury to a brain does not appear to increase drug transport to the brain (2, 30). In intracerebral infections agents must rapidly cross the blood-brain barrier or choroid plexus (33) and accumulate in the target organ in effective amounts in order to inhibit virus replication or limit the spread of virus to other critical organs or both. All of this must happen before the drug is metabolized and excreted. In addition, it is important to ascertain that the antiviral drug is effective on any virus that is already in the brain. Thus, experimentally induced mouse encephalitis is one of the most severe tests for potential antiviral drugs.

By using experimentally induced HSV encephalitis in mice, it is possible to determine the capacities of antiviral agents to prevent the secondary ocular effects of the virus, such as cataract formation or retinitis (31a; R. F. Schinazi,

J. A. Gammon, R. D. Stulting, J. R. F. Kuck, J. D. Wright, and A. J. Nahmias, Invest. Ophthalmol. Vis. Sci. **25**:23, 1984). As shown in Table 4, the percentage of surviving animals developing unilateral or bilateral cataracts 1 month after virus inoculation generally increased with decreasing dose of the antiviral drug. This trend was more apparent when the number of survivors was greatest and when an effective therapeutic agent was used.

FIAC-, FIAU-, FMAU-, FAC-, and FAU-resistant HSV-1 and HSV-2 variants were readily prepared in cell cultures. These clones were cross-resistant to compounds that require a viral TK for activation, such as ACV and 5-*E*-(2-bromovinyl)-2'-deoxyuridine, but were sensitive to drugs that interact with the viral DNA polymerase, such as ara-A and phosphonoformate. These results not only confirm that FIAC, FIAU, and FMAU require the virally encoded TK for activation (5, 16, 17) but also strongly suggest that FAU and FAC operate by similar mechanisms, at least with regard to the initial phosphorylation to the 5'-monophosphate. Subsequent conversion to the diphosphate analogs may require a viral enzyme or a cellular enzyme or both (4).

In a cell-free system, ACV, FMAU, FIAC, FIAU, FAU, and FAC inhibited phosphorylation of thymidine by the HSV-1-encoded TK to a greater degree than they inhibited phosphorylation of thymidine by the HSV-2-encoded TK, indicating that the degree of competition between thymidine and the nucleoside analogs was greater for HSV-1 TK than for HSV-2 TK. These results are consistent with the inhibitory constants (K_i) obtained by Cheng et al. (5) with FIAC, FIAU, and FMAU. Despite the potent activities of the analogs against HSV-2 in cell cultures, FMAU and FIAU were the only agents that inhibited HSV-2 phosphorylation of thymidine by more than 20%, suggesting that these two drugs compete for the HSV-2 TK in a cell-free system. The phosphorylating activity of FIAC was not altered in the presence of 200 μM tetrahydrouridine or 2'-deoxytetrahydrouridine, which are potent inhibitors of 2'-deoxycytidine and cytidylate deaminase, respectively (18; data not shown). These results suggest that there is a poor correlation between the affinity of the compounds for HSV-2 TK and the findings in cell culture or animal studies. The low affinity for HSV-2 TK compared with HSV-1 TK has been noted previously for several nucleoside analogs (6).

In conclusion, no metabolite of FIAC was more effective than FMAU in mice inoculated intracerebrally with HSV-2. The results obtained with infected mice treated with FMAU and FIAC are consistent with our previous findings (16, 17, 30). Other workers have also confirmed our finding related to the high efficacy and potency of FMAU in mice infected with HSV (E. R. Kern, J. P. Richards, and P. E. Vogt, Abstr. Inter-Am. Soc. Chemother. 1984, 5, p. 2; M. R. Karim, S. Bearney, D. E. Foster, C. Lopez, and K. A. Watanabe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, A39, p. 7). Because FMAU has greater aqueous solubility (Table 5) and in vivo antiviral activity than either FIAC or FIAU, it should be considered first for evaluation in the treatment of HSV infections.

In preliminary studies in patients with advanced cancer, FMAU appeared to produce irreversible neurologic damage at doses greater than 32 mg/m^2 per day. Other side effects at these doses included diarrhea, nausea, and blood count depression (13; Fanucchi et al., Proc. Am. Assoc. Cancer Res. **24**:150, 1983). If we can extrapolate from mice to human studies, this dose is about 100 times higher than the median effective dose of FMAU in our mouse encephalitis model. A therapeutic and pharmacological study of oral and intravenous FMAU treatments by using a dose of 2 mg/m^2 per day for 5 days in immunosuppressed patients with herpes zoster virus and HSV infections is planned at Memorial Sloan-Kettering Cancer Center.

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