Therapeutic Activities of 1-(2-Fluoro-2-Deoxy- β -D-Arabinofuranosyl)-5-Iodocytosine and -Thymine Alone and in Combination with Acyclovir and Vidarabine in Mice Infected Intracerebrally with Herpes Simplex Virus

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The therapeutic effectiveness of two new antiviral agents, 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine and 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)thymine, was compared with that of acyclovir and vidarabine. In mice inoculated intracerebrally with high 50% lethal doses of herpes simplex virus type 2, nontoxic intraperitoneal or oral treatments with the two new fluorinated antiviral agents were highly effective in reducing mortality. The two drugs were also effective when treatment was begun as late as 48 h after virus inoculation. The relative order of potencies of the drugs when compared on a molar basis or in terms of therapeutic index was 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)thymine $\gg$ 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine $>$ vidarabine $\approx$ acyclovir. The new pyrimidine analogs were also found to lack immunosuppressive activity in mice. The combination of 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine and vidarabine was the most effective; significantly greater reduction in mortality was achieved with this combination than with either drug alone. Thirty minutes after intraperitoneal treatment with the fluorinated analogs, the drugs (or their metabolites) were transported to the brains of virus-inoculated and normal mice at levels about one-third to two-thirds those in the blood. The levels of 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)thymine in the blood or brain were consistently higher than those found with equivalent intraperitoneal doses of the 5-iodocytosine analog.

The mouse encephalitis model has been a useful indicator of drug efficacy in the treatment of human herpes encephalitis. This was demonstrated when the results of studies on the effect of vidarabine (ara-A) or 5-iodo-2'-deoxyuridine in mice infected intracerebrally with herpes simplex virus (HSV) (10, 12) were compared with those for humans with HSV encephalitis (2, 26). Whereas ara-A was effective in mice and in humans, 5-iodo-2'-deoxyuridine was shown to be inactive. Pharmacokinetic studies in mice and humans (at autopsy) later revealed that 5-iodo-2'-deoxyuridine does not cross the blood-brain barrier (10, 13). Two new antiviral agents, 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine (2'-fluoro-5-iodoaracytosine; FIAC) and its thymine analog (2'-fluoro-5-methylarauracil; FMAU), were previously shown to have potent antiviral activities in cell culture and in mice inoculated intraperitoneally with HSV type 1 (HSV-1) (8, 9, 14, 24). It was therefore of interest to determine whether these drugs are also effective when virus is inoculated intracere-

brally. Other than the route of infection, the efficacy of antiviral agents in the mouse encephalitis model depends on the (i) viral strain; (ii) infectious dose; (iii) time of initiation of treatment; (iv) route of treatment; (v) drug dose and schedule; and (vi) animal species, weight, and age. Several of these variables were examined in this report. In addition, since any evaluation of new antiviral agents in the treatment of human encephalitis within the United States will probably necessitate a comparative double-blind study with ara-A and possibly acyclovir (ACV), we incorporated these two drugs in parallel with FIAC and FMAU in most of our mouse encephalitis studies.

Drug resistance development is already a serious problem in the treatment of herpes keratitis, and resistance to the newer drugs, like ACV, has already been confirmed in clinical situations (see reference 21). Since the rapid emergence of mutants resistant to antiviral agents or infection with an already resistant virus would impair the chances for cure, several combinations of these

promising antiviral agents were also evaluated in the mouse encephalitis model. This allowed us to predict the most effective combinations for use in humans.

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MATERIALS AND METHODS

Drugs. FIAC and FMAU were generously provided by K. A. Watanabe, C. Lopez, and J. Fox of the Sloan-Kettering Institute for Cancer Research (New York, N.Y.). ACV was a gift of G. Elion of Burroughs-Wellcome Co. (Research Triangle Park, N.C.). Ara-A was obtained from Sigma Chemical Co. (St. Louis, Mo.). The micronized drugs, dissolved in pH 7.4 phosphate-buffered saline (PBS) at 36°C, were sterilized just before use by passage through a membrane filter (0.22 μ m; Millipore Corp., Bedford, Mass.). For oral treatment, FIAC or FMAU was dissolved in tap water at a concentration of 0.5 mg/ml or lower. For intraperitoneal treatment, when the size of a dose of ara-A, ACV, or FIAC precluded solution in PBS, the compound was suspended in sterile PBS containing 0.5% carboxymethyl cellulose, as indicated in the tables. FMAU was soluble at all the doses used in this report.

Infection of mice. The details related to the acclimatization and grouping of the mice before infection have been described previously (21). Random-bred Swiss ICR mice (female, 6 to 8 weeks old) obtained from Harlan-Sprague Co. (Indianapolis, Ind.) were inoculated in the right cerebral hemisphere under anesthesia (Metofane; Pitman-Moore Co., Washington Crossing, N.J.) with strain G (6) of HSV-2. For experiments with HSV-1, the KOS strain, obtained from D. Coen (Sidney Farber Cancer Institute, Boston, Mass.), was used. The virus titer was determined before and immediately after inoculation of all the animals by a plaque assay in Vero cells as previously described (21). Control animals were inoculated intracerebrally with PBS (0.05 ml).

Drug treatment. Mice were inoculated intracerebrally at noon (day 0) and treated with drug 5 h later unless otherwise stated. The drug solutions were injected in 0.5-ml doses intraperitoneally twice daily at 9 a.m. and 5 p.m. for 4 days. The fluctuations in weights of the animals during treatment were not large enough to necessitate adjustment of the dose. Water bottles for the mice receiving oral treatments were removed at the time of virus inoculation and replaced 5 h later with bottles containing the drug solutions. Water consumption was measured daily, and the average daily water intake (3.5 ml) over the 4 days of treatment was calculated for each group of mice. Mice were weighed on days 0 to 4, 7, 14, 21, and 30. Only the latter four weighings are shown in the tables, since these usually do not reflect the weight loss due to the trauma of the intracerebral inoculation and intraperitoneal treatment. Mice surviving past day 21 were observed for 2 months. The mouse cages were checked for dead animals at least three times daily.

Drug toxicity. The mice were grouped when 6 to 8 weeks old. 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. Uninfected mice were treated with either the same or larger doses than those administered to virus-inoculated groups. Mice were weighed individually on days 0, 1, 3, 5, 7, 10, 13, 16, 21, and 30.

Definition of therapeutic index. The therapeutic index for each drug was calculated as the maximum tolerated dose divided by the 50% effective dose (ED_{50}). The maximum tolerated dose was defined as the dose of antiviral agent that caused significant loss in weight compared with PBS-treated animals for 3 or more days posttreatment. The ED_{50} was defined as the dose of antiviral agent which prevented half the infected mice from dying (see below).

Determination of virus in brains. The brains of dead mice were removed aseptically and frozen at -70°C in 2 ml of Hanks minimum essential medium containing 2% newborn calf serum and penicillin (100 U/ml) and streptomycin (100 μ g/ml). The brains were thawed, homogenized, and titrated by plaque assay as described previously (21).

Drug levels in blood and brain. FIAC or FMAU was administered intraperitoneally to mice 5 h after intracerebral inoculation with 200 PFU of HSV-2 (strain G) in 50 μ l of PBS or to uninoculated mice (four mice per group). The mice were bled retroorbitally 30 min after drug administration and immediately sacrificed, at which time brains were removed, weighed, and homogenized in minimum essential medium (2 ml) containing antibiotics. After two cycles of freeze-thawing, the brain homogenate was centrifuged ($3,000 \times g$ for 20 min). The supernatant was placed in a petri dish (60 by 15 mm) and exposed to a General Electric G8T5 UV bulb for 5 min under constant swirling at a dose of $45 \text{ ergs s}^{-1} \text{ mm}^{-2}$, as measured by a Blak-Ray UV dosimeter (model J-225; Ultraviolet Products, Inc., San Gabriel, Calif.), and then frozen at -70°C . The brain extracts treated in this way had no detectable infectious virus by plaque assay. The blood was sonicated at 4°C (half power for 10 s, microprobe; sonicator model W140; Ultrasonics, Inc., Plainview, N.Y.), filtered (0.45 μ m), and immediately frozen at -70°C . Twofold dilutions of the brain and blood extracts (up to 6.1×10^{-5}) in 2% newborn calf serum-minimum essential medium containing penicillin, streptomycin, sodium bicarbonate (25 mM), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 25 mM) (maintenance medium), and 0.1% pooled human globulin (Travenol Laboratories, Glendale, Calif.) were added to six-well plates (35-mm diameter; Costar, Cambridge, Mass.). In the wells were near-confluent Vero cells that had been infected for 1 h with 150 to 200 PFU of HSV-1 strain F (6). Previous studies have shown that the concentration range of activity for FIAC and FMAU in plaque reduction assays in Vero cells is similar irrespective of virus strain (Fig. 1); strain F was generally more susceptible to the drugs than strain G. Various concentrations of FIAC and FMAU (0.005 to 0.1 μ M) were added in triplicate to similarly infected cells for each experiment. After 48 h of incubation at 37°C in 5% CO_2 -95% air, the plates were fixed in sodium acetate-buffered Formalin (10%), stained with crystal violet (0.5%) in ethanol-water (20%), and dried, and the number of plaques formed was enumerated. The amount of FIAC and FMAU in the samples was determined from linear regression plots of log drug concentrations (micromolar) for the

two drugs versus the percent reduction in plaque number.

Neutralization test. Pooled heat-inactivated (56°C for 30 min) sera from three mice per group were obtained 2 months after virus inoculation. Twofold dilutions of these sera were made in replicate glass tubes (1:2 to 1:256) with maintenance medium. A 0.2-ml sample dilution was mixed with 0.2 ml of standardized HSV (diluted in maintenance medium to contain 240 PFU per 0.2 ml), and the virus-serum mixtures were incubated at 37°C for 1 h. After incubation, 0.1 ml of each virus-serum mixture was inoculated in triplicate onto confluent Vero cells in six-well plates. After viral adsorption for 1 h, maintenance medium containing 0.1% pooled human globulin was added. After 48 h of incubation at 37°C in 5% CO₂-95% air, the residual PFU were enumerated as previously described (21). Titrations to determine the virus concentration after incubation at 37°C for 1 h with mouse serum from uninfected animals were included in every assay. The neutralizing titer was taken to be the last dilution of serum which showed less than 50% plaque reduction.

Statistical analyses. The survival data from drug-treated animals and control groups were assessed by using a survival analysis computer program (BMDP1L) obtained from the University of California Health Sciences Computing Facility, Los Angeles. *P* values derived by the Breslow (Savage) test are reported. Animals that survived past day 21 were included as censored data. Differences in the mortality among the various groups were assessed by Fischer exact tests. The 50% lethal dose (LD₅₀) for the viruses was predetermined from pilot experiments and calculated by the method of Reed and Muench (19). A point estimate of the ED₅₀ for each drug was obtained by using the probit transformation method (7). The estimated sigmoidal dose-response curve (where response was measured as the proportion of mice alive after 21 days) was linearized by transforming the estimated proportion alive at each dose to probits (*y*) and dose to log₁₀ dose (*x*). Maximum likelihood estimates of the intercept (α) and slope (β) of the probit equation ($y = \alpha + \beta x$) were obtained by using the BMD Biological Assay Subprogram (5) with an estimated natural response rate of 1/24. The subprogram incorporates Newton's iterative scheme. The estimate of log₁₀ ED₅₀ was calculated from the equation: $(5 - \alpha)/\beta$.

RESULTS

Intraperitoneal treatment. When mice were inoculated intracerebrally with 200 PFU (equivalent to 55 LD₅₀) of HSV-2 (strain G), 80% of the PBS-treated group died with a mean day of death (MDD) of 9.6 (Table 1, experiment 1). Administration of FMAU intraperitoneally 5 h after infection at doses of 15 to 80 mg/kg per day (twice a day for 4 days) resulted in only 1 death of 60 infected mice. Whereas FIAC treatments of 30 mg/kg per day resulted in 27% mortality (MDD = 15.8), treatments with ACV or ara-A alone, at the same dosage schedule, resulted in 47% (MDD = 12.1) and 40% (MDD = 10.2) mortality, respectively (Table 1, experiment 1).

A second experiment (Table 1) with lower

doses of the drugs clearly indicated that FMAU was the most effective compound, with significant activity at a dose of 0.1 mg/kg per day. The ED₅₀ was found to be 0.12 mg/kg per day with a 95% confidence interval of 0.076 to 0.23. Antiviral activities of the same order for FIAC, ara-A, and ACV were noted at doses at least 50- to 300-fold greater.

Groups of 10 mice were also inoculated with 10 times the dose of virus (equivalent to 550 LD₅₀) and treated 5 h after intracerebral inoculation with the drugs (40 mg/kg per day) twice a day for 4 days (data not shown). Compared to the 100% mortality (MDD = 3.3) for the PBS-treated control mice, treatment with FMAU, FIAC, and ACV resulted in final mortalities of 0, 50 (MDD = 14.0), and 90% (MDD = 11.0), respectively. There was also no mortality in mice treated with FMAU at a dose of 10 mg/kg per day.

Additional preliminary studies with mice inoculated intracerebrally with 3.0×10^4 PFU of HSV-1 (strain KOS) showed that FIAC (5 mg/kg per day) or FMAU (1 mg/kg per day) reduced mortality from 50% in PBS-treated mice to 20 and 0%, respectively (10 mice per group).

Combined treatment. Combination studies with FIAC or FMAU given with ACV or ara-A are also shown in Table 1. Five hours after inoculation with 55 LD₅₀s of HSV-2, the mice received the drugs intraperitoneally, either alone or in combination, at a dose of 30 mg/kg per day (experiment 1). Because of the low mortality observed with high doses of FIAC or FMAU alone, it was not possible to determine whether these drugs in combination with ara-A or ACV were more effective than FIAC or FMAU alone.

A second experiment, with lower doses of the individual drugs, indicated that the combination of FIAC and ara-A (2 and 10 or 5 and 10 mg/kg per day) was the most effective; this combination was the only one that significantly decreased mortality when compared to treatment with either of the two drugs alone (Table 1, experiment 2).

Delayed treatment. Presented in Table 2 are the effects of delayed treatment with FIAC or FMAU (40 mg intraperitoneally per kg per day) on the survival of mice inoculated intracerebrally with 220 PFU of HSV-2 (equivalent to 61 LD₅₀s). When treatment was delayed for 12, 24, or 48 h after virus inoculation, significant antiviral effect and increased MDD were noted. At the same dose, FMAU was again found to be more effective than FIAC in that no death occurred when treatment was initiated as late as 48 h after virus inoculation. Treatments with FIAC under the same conditions resulted in 27% mortality (MDD = 15.8), whereas the mortality in the PBS-treated controls was 93% (MDD = 7.2).

TABLE 1. Effect of FMAU, FIAC, ACV, and ara-A alone and in combination in mice inoculated with HSV-2 (strain G)

Treatment	Dose (mg/kg per day) ^a	MDD ^b	No. of mice dead/no. treated (%) ^c	% Wt change on day ^d :			
				7	14	21	30
Expt 1 (200 PFU)							
PBS		9.6	12/15 (80)	-14	-28	-16	19
FMAU	15		0/15 (0) ^e	-4	5	12	20
	30	(9.0)	1/15 (7)	-3	-7	12	16
	50		0/15 (0) ^e	-3	5	10	14
	80		0/15 (0) ^e	-5	2	6	9
FIAC	15	14.8 ^f	4/12 (33) ^g	-2	-7	0	9
	30	15.8 ^f	4/15 (27) ^h	-2	-4	1	8
	50		0/15 (0) ^e	-2	2	5	11
	80		0/15 (0) ^e	2	3	8	15
ACV	30	12.1 ⁱ	7/15 (47) ^j	-5	-10	-6	8
Ara-A	30	10.2 ⁱ	6/15 (40) ^j	-10	-23	-11	-3
FMAU/ACV	30/30	(16.0)	1/15 (7) ^e	-3	1	5	9
FMAU/ara-A	30/30	(14.0)	1/15 (7) ^e	-2	3	10	12
FIAC/ACV	30/30	(16.0)	1/15 (7) ^e	-1	-2	3	7
FIAC/ara-A	30/30		0/15 (0) ^e	-5	-2	1	10
Control (no virus)			0/10 (0)	5	7	10	15
Expt 2 (220 PFU)							
PBS		7.3	23/24 (96)	-22	-23	-2	6
FMAU	0.01	6.7 ⁱ	11/12 (92) ^j	-13	-17	-7	5
	0.05	7.3 ⁱ	10/12 (83) ^j	-23	-31	-11	-3
	0.1	9.0 ⁱ	6/12 (50) ^h	-12	-36	8	5
	0.5	(9.0)	1/12 (8) ^e	2	8	9	14
	1.0		0/12 (0) ^e	4	8	9	17
	5.0		0/12 (0) ^e	6	13	18	23
FIAC	1.0	7.3 ⁱ	12/12 (100) ^j	-27	-41		
	2.0	10.4 ^g	9/11 (82) ^j	-10	-5	8	23
	5.0	10.0 ^g	7/12 (58) ^h	-9	0	3	9
Ara-A	10.0	9.6 ⁱ	10/12 (83) ^j	-18	-25	1	13
	15.0	13.3 ^g	6/12 (50) ^h	-13	-16	-9	2
	30.0	8.7 ⁱ	6/12 (50) ^h	-9	-9	8	17
ACV	10.0	9.2 ⁱ	10/12 (83) ^j	-13	-20	-7	15
	15.0	8.0 ⁱ	11/12 (92) ^j	-21	-6	-10	3
	30.0	13.3 ^g	7/12 (58) ^h	-7	0	9	17
FMAU/ACV	0.5/15		0/12 (0) ^e	-1	5	9	14
	0.05/10	10.3 ^g	7/12 (58) ^h	-5	-3	10	17
FMAU/ara-A	5/30		0/10 (0) ^e	5	15	19	20
	0.5/15	7.5 ⁱ	2/11 (18) ^e	1	3	5	14
	0.05/10	9.8 ^g	11/12 (92) ^j	-9	-15	-11	1
FIAC/ACV	5/10	10.5 ^g	6/12 (50) ^h	-8	-9	-2	5
FIAC/ara-A	5/10	(9.0)	1/12 (8) ^e	1	1	11	21
	2/10	7.7 ⁱ	3/12 (25) ^e	0	2	8	14
Control (no virus)			0/20 (0)	3	7	4	14

^a Given 5 h after intracerebral inoculation. Dose schedule, twice a day for 4 days.^b Calculated on day 21; numbers in parentheses indicate day of death of a single animal.^c Calculated on day 21.^d Average percent weight change compared to day 1.^e Probability that the observed increase in survivor number or increase in MDD was due to chance, $P < 0.0001$.^f $P < 0.001$.^g $P < 0.05$.^h $P < 0.01$.ⁱ Not significant.

Oral treatment. FMAU was found to be more effective than either FIAC or ACV when given in drinking water 5 h after intracerebral virus inoculation (Table 2). Whereas ACV was inef-

fective at a dose of 7.5 mg/kg per day, a similar dose of either FIAC or FMAU significantly reduced mortality from 93% in PBS-treated mice to 55 and 0%, respectively.

TABLE 2. Effect of delayed treatment or oral therapy with FMAU and FIAC in mice inoculated intracerebrally with HSV-2

Treatment	Time (h) ^a	Dose (mg/kg per day) ^b	MDD ^c	No. of mice dead/no. treated (%) ^d	% Wt change on day ^e :		
					7	14	21
Intraperitoneal							
PBS	12		7.2	14/15 (93)	−22	−26	15
FIAC	12	40	(14.0)	1/15 (7) ^f	−6	−5	13
	24	40	11.0 ^g	2/15 (14) ^f	−7	−1	5
	48	40	15.8 ^f	4/15 (27) ^f	−12	−7	6
FMAU	12	40		0/15 (0) ^f	−13	5	11
	24	40	(6.0)	1/15 (7) ^f	−9	6	11
	48	40		0/15 (0) ^f	−14	5	10
Oral							
FIAC	5	75	(14.0)	1/15 (7) ^f	4	7	17
	5	7.5	9.0 ^h	6/11 (55) ^h	5	15	24
	5	0.75	7.4 ^j	12/12 (100) ^j	−14		
FMAU	5	75	(16.0)	1/15 (7) ^f	−3	10	17
	5	7.5		0/11 (0) ^f	15	21	36
	5	0.75	17.0 ^g	5/12 (42) ⁱ	13	0	15
ACV	5	75	9.0 ^h	9/12 (75) ^j	−3	−2	−13
	5	7.5	6.6 ^j	12/12 (100) ^j	−18		
Control (no virus)				0/14 (0)	−2	8	9

^a Time of treatment after intracerebral inoculation of HSV-2 (220 PFU of strain G).^b Dose schedule was twice a day for 4 days.^c Calculated on day 21; numbers in parentheses indicate day of death of a single animal.^d Calculated on day 21.^e Average percent weight change compared to day 1.^f Probability that the observed increase in survivor number or increase in MDD was due to chance, $P < 0.0001$.^g $P < 0.001$.^h $P < 0.05$.ⁱ $P < 0.01$.^j Not significant.

Virus in brains. HSV was recovered from all the mice that died up to 9 days after intracerebral inoculation with virus; the titer of virus ranged from 2.4×10^2 to 6×10^5 PFU/g of brain. The five mice treated with high doses of FIAC or FMAU (30 mg/kg per day) that died did not have

detectable virus ($<10^2$ PFU/0.5 g of tissue) in their brains. Similarly, 10 of 13 of the mice receiving ara-A or ACV (30 mg/kg per day) that died after day 9 also had no infectious virus in their brains.

Toxicity studies. In the uninfected animals, no

TABLE 3. Effect of high doses of nucleoside antivirals on body weight

Treatment	Dose (mg/kg per day) ^a	Mean wt change (g) on posttreatment day ^b :								
		1	3	5	7	10	13	16	21	30
PBS		0.2 (6)	-0.1 (6)	-0.4 (6)	0.3 (6)	1.5 (6)	2.8 (6)	2.5 (6)	2.5 (6)	4.1 (6)
FIAC	800 ^c	-0.8 (6)	-1.4 ^d (6)	-2.0 ^d (6)	-1.9 ^d (6)	0.5 ^d (6)	2.2 (6)	1.8 (6)	1.5 (6)	3.0 (6)
	1,200 ^c	1.0 (4)	-0.5 (4)	-1.2 ^d (3)	-1.4 ^d (3)	0.3 ^d (3)	-1.5 ^d (3)	-0.3 ^d (3)	-0.1 ^d (3)	1.6 ^d (3)
FMAU	400	0.5 (6)	-0.7 (6)	-3.7 ^d (6)	-4.3 ^d (6)	-1.3 ^d (6)	-0.8 ^d (6)	0.4 ^d (6)	1.3 ^d (6)	1.1 ^d (6)
	800	-0.1 (6)	-1.4 ^d (6)	-4.8 ^d (6)	-7.6 ^d (6)	-6.5 ^d (4)	-4.2 ^d (4)	-2.3 ^d (4)	-1.5 ^d (4)	2.0 ^d (4)
ACV	160	-1.1 (6)	-1.7 ^d (5)	-1.0 (5)	-0.2 (5)	1.3 (5)	2.7 (5)	2.8 (5)	3.8 (5)	5.2 (5)
	400 ^c	-0.8 (6)	-1.9 ^d (6)	-2.0 ^d (6)	-1.1 ^d (6)	0 ^d (6)	0.5 ^d (6)	1.0 ^d (6)	1.1 ^d (6)	2.9 ^d (6)
Ara-A	400 ^c	-0.6 (6)	-1.0 (6)	-2.1 ^d (6)	-1.5 ^d (6)	-0.3 ^d (6)	-1.4 ^d (6)	-4.5 ^d (6)	0 ^d (6)	0.4 ^d (6)
	600 ^c	-0.8 (6)	-4.1 ^d (5)	-5.8 ^d (3)	-7.3 ^d (3)					

^a Given intraperitoneally twice a day for 4 days to 6- to 8-week-old outbred Swiss female mice.^b Numbers in parentheses indicate the number of mice weighed.^c Drug suspended in PBS containing 0.5% carboxymethyl cellulose.^d Significantly different from PBS-treated mice, $P \leq 0.05$.

TABLE 4. Concentrations of FIAC and FMAU or their antiviral metabolites in blood and brain of mice 30 min after administration

Treatment	Dose (mg/kg)	Mean concn (nmol/ml) $\pm$ SD		
		Blood	Brain	Brain/blood drug ratio (%)
FMAU	1 ^a	20 $\pm$ 2	13 $\pm$ 2	65
	1 ^b	25 $\pm$ 2	15 $\pm$ 1	60
	15 ^a	52 $\pm$ 15	37 $\pm$ 2	71
	15 ^b	70 $\pm$ 8	38 $\pm$ 1	54
	80 ^b	305 $\pm$ 22	210 $\pm$ 16	69
FIAC	15 ^a	37 $\pm$ 8	15 $\pm$ 1	41
	15 ^b	29 $\pm$ 3	9 $\pm$ 4	31
	80 ^b	208 $\pm$ 18	90 $\pm$ 12	43

^a Dose given intraperitoneally to mice inoculated intracerebrally with virus (220 PFU of strain G).

^b Dose given intraperitoneally to uninoculated mice.

apparent toxicity—as measured by deaths, decrease in weight, or failure to gain weight—was noted with FIAC or FMAU at doses of 15 to 80 mg/kg per day, given twice a day for 4 days, when tested alone or in combination with ara-A or ACV (data not shown). By day 7 posttreatment, significant reduction of body weights, compared to PBS-treated mice, was apparent only at very high doses of FIAC (800 mg/kg), FMAU (400 mg/kg), ACV (400 mg/kg), or ara-A (400 mg/kg) (Table 3). Severe weight loss or death or both was noted at higher doses of the drugs. Most of the surviving mice gradually gained weight after day 13, and no apparent toxicity or further death occurred up to 2 months posttreatment.

Treatment of virus-inoculated mice with FMAU or FIAC (5 to 80 mg/kg) resulted in only slight reductions of body weight relative to sham-infected control mice (Table 1). Weight loss was usually greater in virus-inoculated animals receiving ara-A or ACV alone than in mice given the drugs in combination with FMAU or FIAC. Virus-inoculated mice receiving oral FIAC or FMAU rapidly gained weight after the initial trauma of the intracerebral inoculation (Table 2).

Serology and resistance to reinfection. Survivors of the intracerebral inoculation in the delayed treatment experiment (Table 2) were bled retroorbitally 2 months post-inoculation. The HSV-antibody titers in sera from virus-inoculated untreated and drug-treated mice were $>1:256$, whereas the titers of PBS-inoculated mice were $<1:4$, as determined by plaque neutralization assays (data not shown). Lower titers were found (1:16 to 1:64) in the 15 mice treated early (<24 h) with FMAU. Thirty-nine other mice and six uninfected control mice were randomly selected from FIAC- or FMAU-treated

groups (Table 1, experiment 2) and rechallenged 42 days after the initial infection with 130 LD₅₀s of the homologous virus. Whereas all of the control mice died (MDD = 8.0), all but one of the previously infected drug-treated mice survived for more than 30 days after virus reinoculation.

Drug levels in blood and brain. The levels of FIAC and FMAU in the blood and brains of virus-inoculated and uninoculated animals 30 min after receiving a single relatively low or high dose of the drugs are shown in Table 4. These were calculated from dose-response linear regression plots determined at the same time as the plaque assays were done (Fig. 1). For each dose tested, similar drug levels were found in mice inoculated intracerebrally as in uninoculated animals. The levels of FMAU in the brain were about two- to fourfold greater than those found for FIAC for each treatment. The drugs were transported to the brain at levels about one-third to two-thirds those in the blood.

DISCUSSION

We have confirmed the earlier observation by Fox et al. (8) that FMAU and FIAC are equally effective on HSV-1 in cell culture by plaque reduction assay and extended this observation to HSV-2 (Fig. 1). An HSV-2 strain was chosen for most of the studies in mice inoculated intracerebrally since this virus type is isolated in about three-fourths of neonates with disseminated herpes or herpes encephalitis (A. Nahmias,

FIG. 1. Dose-response plots obtained by plaque reduction assay in Vero cells (21) for FIAC (open symbols) or FMAU (closed symbols) with HSV-1 strain F (○, ●) and HSV-2 strain G (□, ■). The coefficient of correlation for each drug was $\geq 97\%$.

H. Keyserling, and G. Kerrick, in J. Remington and J. Klein, ed., *Infectious Diseases of the Fetus and Newborn Infant*, in press). In addition, almost all the antiviral agents currently in use or being tested for the treatment of HSV infections are more effective in cell culture against HSV-1 than against HSV-2 strains (4). For example, in cell culture *E*-5-(2-bromovinyl)-2'-deoxyuridine is much less active against HSV-2 strains than HSV-1 strains (15, 20). We and others have shown that this also appears to be the case in mice inoculated intracerebrally or intraperitoneally (R. Schinazi, unpublished results; E. R. Kern, J. T. Richards, J. C. Overall, and E. De Clercq, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 433, 1982). Hence, it is likely that if a drug is effective with HSV-2 strains it would also be effective with HSV-1 strains.

Similar to previous studies with mice inoculated intraperitoneally with HSV-1 (8, 9), we also found that, on an equal drug dose and schedule, FMAU was significantly more effective than FIAC in reducing mortality in mice infected intracerebrally with HSV-2. This difference in activity may be partly due to the longer half-life of FMAU in mouse plasma than FIAC (9). In this encephalitis model, FMAU was not only more effective than FIAC, but was also more active than ACV or ara-A. FMAU was at least 70 times more effective than the other drugs in mice inoculated with 61 LD₅₀s (220 PFU) of HSV-2, showing significant activity at a dose of 0.1 mg/kg per day, equivalent to 2.3 µg or 8.85 nmol of drug per mouse per day (Table 1).

The maximum tolerated intraperitoneal doses of FIAC and FMAU in uninfected mice were determined at the same time as those of ara-A and ACV (Table 3). The significant weight loss noted with ACV or ara-A at a dose of 400 mg/kg was consistent with that reported previously (18, 21). Weight loss was also noted when FIAC or FMAU was given twice a day for 4 days at doses equal to or greater than 800 and 400 mg/kg per day, respectively. F. S. Philips (Sloan-Kettering Institute for Cancer Research, personal communication) has found that FIAC and FMAU were also toxic to 5-week-old Swiss outbred male mice when administered intraperitoneally daily for 5 days at doses of 1,000 mg/kg per day. The greater weight loss noted with FMAU at 400 mg/kg per day than with the other drugs at the same dose (Table 3) may be related to higher circulating levels of FMAU since this drug was not given in suspension.

From our data, the therapeutic indices (see above for definition) for these drugs were found to be, in increasing order: FMAU >> FIAC > ara-A ≈ ACV. When compared on a molar

basis, the order of activity was the same (Table 5). Hence, FMAU was not only the most effective compound on a molar basis but also was the drug with the highest therapeutic index.

Treatments with FMAU were much more effective than with either FIAC or ACV when given to mice infected with as much as 550 LD₅₀ equivalents of HSV-2 (see above). Even when therapy was withheld for 48 h after virus inoculation, treatments with FMAU were clearly superior to FIAC therapy (Table 2). An important advantage of FMAU or FIAC is that oral treatments of these drugs were more effective than equivalent doses of ACV (Table 2), suggesting that oral therapy with FMAU or FIAC should also be considered in humans. In similar studies with mice infected intracerebrally with HSV, oral ACV or ara-A was also effective but at much higher drug doses and longer treatments (11, 22, 23).

Preliminary results with intraperitoneal treatments of low doses of FIAC or FMAU indicated that FMAU was more effective than FIAC in mice inoculated intracerebrally with 1 LD₅₀ of the KOS strain of HSV-1 (see above). The new drugs may, therefore, also be effective in treating herpes encephalitis in adults since HSV-1 is usually recovered from these patients (25, 26).

Of importance was the fact that none of the combinations tested significantly increased mortality relative to the least active drug alone, even when relatively high doses of the drugs were used. The combination of FIAC and ara-A was found to be the most effective (Table 1). At the present time, any hypothesis concerning the mechanisms involved for these antiviral agents in combination would be speculative. In unpublished work we have completed, the combinations of FIAC or FMAU and ACV or ara-A in cell cultures infected with HSV-2 (strain G) resulted only in additive interactions. Further-

TABLE 5. Therapeutic indices for antiviral nucleosides in mice^a

Compound	Mol wt	Maximum tolerated dose (mg/kg per day)	ED ₅₀ (mg/kg per day) ^b	Therapeutic index
FMAU	260	400	0.12 (0.46)	3,333
FIAC	371	800	9.3 (25.1)	86
Ara-A	267	400	25.1 (94.0)	16
ACV	225	400	33.1 (147.1)	12

^a Antiviral agents were given intraperitoneally twice a day for 4 days to 6- to 8-week-old outbred Swiss female mice. See the text for definitions of the parameters listed.

^b In mice treated 5 h after intracerebral inoculation with 220 PFU of HSV-2 strain G. Numbers in parentheses are ED₅₀s in micromoles per kilogram per day.

more, there was no cross-resistance between FIAC-resistant (thymidine kinase-deficient) and ara-A-resistant (DNA polymerase-altered) HSV variants; the titer of FIAC-resistant virus in the presence of ara-A and that of ara-A-resistant virus in the presence of FIAC were similar to that shown by the parent strain (manuscript in preparation). However, these cell culture studies suggest that cross-resistance between FIAC- or FMAU-resistant variants and ACV-resistant variants is more likely to occur than with ara-A-resistant virus. The dosage schedules of FIAC, FMAU, ACV, and ara-A, alone or in combination, were also not toxic to infected or uninfected mice. The apparent lack of toxicity for the combination of FIAC and ara-A, coupled with the increased antiviral effect relative to the drugs alone and lack of cross-resistance, make this combination particularly attractive for future studies in humans.

The finding of appreciable amounts of HSV antibodies 2 months after infection of mice treated with FMAU or FIAC suggests that these drugs do not suppress the humoral immune response to HSV infection. Early effective therapy (<24 h after virus inoculation) with high doses of FMAU did not always allow the virus to replicate sufficiently to induce a strong immune response. However, of 39 infected mice which were treated early with relatively low doses of FIAC or FMAU and were rechallenged with a lethal dose of homologous virus, all but one of the mice lived for more than 30 days after virus reinoculation. This correlates with the finding in the serum neutralization assays of HSV antibodies in virus-inoculated mice that had been treated with the antiviral agents and confirms that these mice had been infected.

Preliminary single-dose drug transport studies (Table 4) indicated that both FIAC and FMAU or their active metabolites were transported to the brain at levels about one-third to two-thirds those in the blood. The levels of the drugs in the brain were considerably higher, at least 200-fold greater, than the mean effective dose required for 50% plaque reduction for FIAC or FMAU in Vero cells (Table 4 and Fig. 1). The level of FMAU in the blood and brain for each dose administered was consistently greater than those found for FIAC and may explain the higher activity of FMAU in this mouse model. Using 2-¹⁴C-labeled FIAC and FMAU, Fox et al. (9) recently found that both of these nucleosides are also well absorbed after intragastric administration in mice and rats, and diffused into the brain with great facility. In addition, on a molar basis, ACV brain levels in mice 30 min after a single intraperitoneal dose of 100 mg/kg were recently shown to be 6- and 14-fold lower than the levels reported here for FIAC and FMAU, respective-

ly, after a dose of 80 mg/kg (1). These results may also explain the consistently higher order of efficacy, FMAU >> FIAC > ACV, observed in our experiments.

It is likely that similar brain drug levels would be obtained in infected mice since the blood brain barrier is disrupted late in the course of HSV infection (R. R. McKendall, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, S5, 285). The localized injury to the blood-brain barrier during intracerebral virus inoculation did not appear to increase drug transport to the brain (Table 4). This is corroborated by the work of Biron et al. (1), who showed that the ability of ACV to penetrate this barrier was not increased in intracerebrally infected and uninfected mice.

Infectious virus could not be isolated from the brains of most of the drug-treated mice that died after day 9, an observation consistent with our previous findings in this model (21). This phenomenon has also been described in patients with falsely negative biopsy who die after the 18th day of illness (17). Death may be attributed to dissemination of the virus from the brain to other organs, to severe brain edema which accompanies the infection, or to both. In addition, the presence of local HSV antibody on day 9 may interfere with the isolation of infectious virus, since neutralizing antibodies are usually detected in sera of mice 5 to 7 days after virus infection (3, 16).

In conclusion, these observations in the mouse herpes encephalitis model indicate that at effective doses FIAC or FMAU are at least as safe and as effective as ara-A or ACV. FMAU was shown to be the most potent and selective antiviral agent discovered to date for the treatment of mouse encephalitis caused by HSV-2, with a therapeutic index greater than 3,000. In addition, the combination of FIAC and ara-A can be used safely to achieve much greater effectiveness than either drug alone. This modality could prove useful in individuals unresponsive to conventional therapy with ara-A or ACV for severe HSV infections. It is hoped that the results of phase 1 and 2 clinical studies will permit a controlled evaluation of FMAU, and possibly FIAC, for the treatment of HSV encephalitis in neonates and adults. The remarkably high efficacy in mice of low doses of FMAU and the ease of transport to the brain of this drug suggest that human trials should be initiated with considerably lower dosages of the drug than those shown to be effective for ara-A.

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