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Delayed Treatment with Combinations of Antiviral Drugs in Mice Infected with Herpes Simplex Virus and Application of the Median Effect Method of Analysis

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Mice were inoculated intracerebrally with a lethal dose of herpes simplex virus type 2. Three days later, the mice were treated intraperitoneally, twice daily for 4 days, with the following drugs alone or in combination: acyclovir (ACV), vidarabine (ara-A), 2′-fluoro-5-iodoaracytosine (FIAC), and 2′-fluoro-5-methylarauracil (FMAU). Despite delayed treatment, most of the animals receiving low doses of FMAU alone or in combination with ACV or ara-A survived. In contrast, significantly higher mortality rates were noted in mice receiving ara-A, ACV, or FIAC alone. The data were analyzed for quantitation of synergism, additivity, and antagonism of multiple drug effect by the median effect method. The median effective doses (in nanomoles per kilogram per day) calculated in this manner were: FMAU, 22.5; FIAC, 510; ara-A, 901; ACV, 7,587; ACV-ara-A (drug ratio, 1:1), 550; FIAC-ara-A (1:1), 376; FIAC-ACV (1:1), 133; FMAU-ACV (1:8), 60.3; and FMAU-ara-A (1:8), 65.2. Marked synergy was found throughout a wide range of effect levels with the five different combinations, with no increased toxicity over the single-drug treatments. Similar results were obtained when the data were analyzed by the isobologram method. Since many patients with severe herpetic infections, such as herpes encephalitis, have a poor prognosis despite single-drug therapy, the possible use of combinations including low doses of FMAU deserves further investigation.

Animal models have been useful in providing leads to antiviral drugs for the treatment of herpes simplex virus (HSV) infections. The finding that drugs like vidarabine (ara-A) and acyclovir (ACV) are effective in animal models for HSV encephalitis (14, 19) was later confirmed in humans with the same disease (26, 31–33). Nevertheless, beyond the neonatal stage, patients who are comatose when antiviral drugs are administered still do very poorly irrespective of the drug used (26, 31–33). Newer approaches are therefore necessary and may include combinations of drugs. We have studied several licensed and experimental drugs which could be candidates for this purpose. Based on animal studies with ara-A, ACV, several 2′-fluoroarabinosyl pyrimidine nucleosides, including 2′-fluoro-5-methylarabinosyluracil (FMAU) and 2′-fluoro-5-iodoarabinosylcytosine (FIAC) (10, 20, 22), and congeners of acyclovir, such as 9-[(2,3-dihydroxypropoxy)methyl]guanine (DHPG; also known as 2′-NDG, BIOLF-62, and BW759) (27) and buciclovir (9; unpublished data), we concluded that FMAU was the best candidate to be evaluated in humans with severe herpetic infection (20).

Several reports have demonstrated additive to synergistic effects with various combinations of ACV, ara-A, FIAC, and FMAU when treatment of HSV-infected mice was initiated within 48 h after virus inoculation (7, 13, 17, 22, 23; M. R. Karim, S. Bearney, D. E. Foster, C. Lopez, and K. A. Watanabe, Annu. Meet. Am. Soc. Microbiol. 1985, A39, p. 7). To mimic more closely the problem of severe human HSV encephalitis, it appeared to be necessary to evaluate these drugs alone and in combination by delaying treatment for several days after virus inoculation. With experimentally induced HSV encephalitis in mice, the combinations that were evaluated included FMAU-ACV, FMAU-ara-A, FIAC-ACV, FIAC-ara-A, and ACV-ara-A.

One of us (T.-C.C.) recently reported a new, simple, and computerized method—the median effect method—for determining synergy, additivity, or antagonism in combination studies of enzyme inhibitors, insecticides, and anticancer drugs (5, 6). It was therefore of interest to evaluate this method for the combinations of antiviral drugs used in the current studies and to compare the results obtained with data analyzed by isobolograms.

(Part of this work was presented at the Second Annual Meeting of the Inter-American Society for Chemotherapy, 8–11 December 1985, Tampa, Fla., p. 44.)

MATERIALS AND METHODS

Compounds. The 2′-fluoroarabinosyl pyrimidine nucleoside FIAC and FMAU were obtained from J. J. Fox and K. A. Watanabe, Memorial Sloan-Kettering Cancer Center, Rye, N.Y. ACV was a gift of G. Elion of Burroughs-Wellcome Co., Research Triangle Park, N.C. Ara-A was purchased from Sigma Chemical Co., St. Louis, Mo. All the nucleosides were the free bases. The compounds were dissolved in pH 7.4 phosphate-buffered saline (PBS) and were soluble at 37°C in PBS at most of the concentrations and doses reported. When this was not possible, as with ara-A and ACV, the solution was prepared by adjusting the pH to 8.0 with 1 M NaOH. High-pressure liquid chromatography of the resulting solution indicated that the nucleosides remained unchanged for more than 1 week at room temperature (unpublished results). The compounds were sterilized before use by passage through a membrane filter (0.22-μm pore size; Millipore Corp., Bedford, Mass.).

Cells and viruses. Vero cells were obtained from Flow

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Laboratories, McLean, Va. The plaque-purified G strain of HSV-2 was supplied by B. Roizman, University of Chicago (8); high-titered pools were prepared as described elsewhere (23).

Infection of mice. Random-bred Swiss ICR mice (female, 5 to 6 weeks old), obtained from Harlan-Sprague Co., Indianapolis, Ind., were acclimatized in the laboratory for 2 weeks. They then received inoculations into the right cerebral hemisphere under anesthesia (Metofane; Pitman-Moore Co., Washington Crossing, N.J.) with strain G of HSV-2. The virus (20 PFU, equivalent to about five 50% lethal doses [LD_{50}]) caused a mortality rate of over 95% in animals treated with PBS. The virus titer of the inoculum was determined in Vero cells by plaque assay (23). Control animals were inoculated intracerebrally with PBS (0.05 ml). The reasons for selecting this virus strain for the animal studies have been discussed previously (22, 23).

Drug treatment. Mice were inoculated intracerebrally at about 5 p.m. and treated with drug 72 h later. Recipients were randomized to receive drug or PBS 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, for a total of eight doses. For the combined treatments, the drugs were administered intraperitoneally with separate syringes, with minimum delay between the two doses. The dose ranged from 1.9 to 600 nmol/kg per day (see Table 1, footnote *b*, for conversion to milligram-per-kilogram equivalents), and was selected on the basis of delayed treatment results obtained with FMAU, FIAC, and ACV in a previous study (22). For most of the studies described in this report, a ratio of 1:1 was selected for combinations of ACV and ara-A, ACV and FIAC, and ara-A and FIAC. Because FMAU had been shown to be extremely effective when treatment was delayed, and since the levels of FMAU in the brain of mice are about 14-fold higher than the levels reported for acyclovir (22), a ratio of 1:8 was selected for combinations of FMAU and ACV or ara-A. Mice were weighed on days 0, 1, 3, 5, 7, 14, 21, and 30. The fluctuations in weight of the animals during treatment were not large enough to require adjustment of the dose. Only the data for the days of maximum average weight loss (usually days 7 and 14 after virus inoculation) are shown in Table 1. The cages were checked for dead mice at least twice daily for the 30-day duration of the study. The results from three different experiments are presented in Table 1. The data from these experiments were combined and analyzed together, since no significant difference was noted in the mortality rate and mean time to death of the PBS-treated mice.

ELISA. Infection was validated by testing the sera of surviving mice for the presence of HSV antibodies with an enzyme-linked immunosorbent assay (ELISA). On day 30 to 32 after virus inoculation, surviving mice were bled retroorbitally under anesthesia. The serum was screened for the presence of HSV antibodies as previously described (25); a peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Miles Laboratories, Inc., Naperville, Ill.) was used instead of anti-guinea pig immunoglobulin G.

Median effect method. Dose-effect relationships were analyzed by the median effect equation [(1–3, 5, 6; T.-C. Chou, I. Hirano, J. Chou, and P. Talalay, Fed. Proc., in press; T.-C. Chou and P. Talalay, *in* K. Harrap, ed., *New Avenues in Developmental Cancer Chemotherapy*, in press): $f_a/f_u = (D/D_m)^m$, or $\log (f_a/f_u) = m \log D - m \log D_m$ (equation 1), where f_a and f_u are the fractions of the system affected and unaffected, respectively, by dose D, D_m is the dose required to produce the median effect, and m is the slope of the median effect (Chou) plot. Consequently, a plot of $y = \log (f_a/f_u)$ with respect to $x = \log D$ will have a slope m. The magnitude of this slope will at once reveal whether the system behaves in accordance with hyperbolic ($m = 1$) or sigmoidal ($m \neq 1$) curves. Furthermore, the intercept on the x axis at $y = \log (f_a/f_u) = 0$ will give the median effect dose, because when $f_a + f_u = 1.0$, $f_a = f_u = 0.5$ and $f_a/f_u = 1$. Therefore, when $y = 0$, the y intercept occurs at $\log D_m$. Hence, D_m can be calculated from the antilog of ($-y$ intercept/m). When the m and D_m values are determined by the median effect plot, the dose that is required to produce any level of effect can be calculated from equation 1.

Synergy, summation (additivity), and antagonism of drug effects were quantitatively analyzed by the multiple-drug effect analysis developed by Chou and Talalay (5, 6). The summation effects of the two drugs can be described by $[(f_a)_{1,2}/(f_u)_{1,2}]^{1/m} = [(f_a)_1/(f_u)_1]^{1/m} + [(f_a)_2/(f_u)_2]^{1/m} + \alpha[(f_a)_1(f_a)_2/(f_u)_1(f_u)_2]^{1/m} = (D)_1/(D_m)_1 + (D)_2/(D_m)_2 + \alpha(D)_1(D)_2/(D_m)_1(D_m)_2$ (equation 2), where $(f_a)_{1,2}$ is the effect of the mixture of drug 1 and drug 2 in a specified dose ratio. When the median effect plots of each drug and their mixture are parallel ($m_1 = m_2 = m_{1,2}$), then a mutually exclusive drug effect is indicated (e.g., similar mode of action), and in this case $\alpha = 0$. When $m_1 = m_2$ but $m_{1,2}$ is greater and upwardly concave, then a mutually nonexclusive drug effect is indicated (e.g., independent mode of action), and in this case $\alpha = 1$. The interaction effect of two drugs was quantitatively determined by the combination index (C.I.), which is defined as C.I. $= (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2$ (equation 3), where D_x is the dose of each drug alone required to produce x% effect. The value of D_x at x% effect can be determined by substituting the m and D_m values into equation 1. The m and D_m values can be readily obtained from the median effect plot parameter or more conveniently by using a computer program (1). In equation 3, $(D)_1$ and $(D)_2$ are the components of the two drugs that in combination also produce x% effect (for an example, see Results). This analysis generates the combination effect as depicted below: when C.I. = 1, summation is indicated; when C.I. < 1, synergy is indicated; and when C.I. > 1, antagonism is indicated.

In equation 3, if the regression lines of the median effect plot for each drug alone are not parallel, exclusivity of drug effects cannot be clearly determined. Under these circumstances, the C.I. value should be determined by equation 3 with both mutually exclusive ($\alpha = 0$) and nonexclusive ($\alpha = 1$) assumptions.

Computer programs based on the above equations have been used in the present studies for automated analysis of dose-effect data with an Apple II microcomputer or an IBM-PC (1). The analysis quantifies synergy, summation, and antagonism effects at different effect levels. Relative potency, sigmoidicity of the dose-effect curves, regression coefficients, and graphics are also obtained from the computer analysis.

The application of this method is relatively simple. The experimental design requires that the ratio of the dose of the two drugs used in combination be kept constant and the dose of the mixture be varied. The experimental data obtained are logarithmically transformed, and the extreme points ($f_a = 1$ or 0) can be censored from the analysis since they cannot be transformed ($\log_{10} 0 = -\infty$). It is sometimes necessary to approximate mortality rates of 100% (e.g., 12 of 12 treated animals dead) to 96% (i.e., 11.5 of 12). With the program described above, the data are plotted as $y = \log [(f_a)^{-1} - 1]^{-1}$, $\log (f_a/f_u)$, or $\log [f_a/(1 - f_a)]$ versus $x = \log (D)$. The D_m

TABLE 1. Effect of delayed treatment with antiviral drugs alone and in combination in mice inoculated intracerebrally with HSV-2

Treatment[a] (drug ratio)	Dose[b] (nmol/kg per day)	Mortality (no. of mice dead/no. treated [%])[c]	Mean time to death ± SD[c,d] (days)	Avg % change in wt from day 1	
				Day 7	Day 14
Negative control (no virus)		0/28		5	14
PBS		48/50 (96)	8.1 ± 2.2	−21	−15
ACV	15.0	10/12 (83)[h]	5.9 ± 1.0[h]	−19	2
	37.5	10/12 (83)[h]	6.6 ± 1.6[h]	−19	−10
	150.0	19/25 (76)[f]	10.1 ± 3.2[f]	−13	−9
	300.0	17/25 (68)[e]	10.1 ± 3.1[f]	−14	3
	600.0	8/12 (67)[e]	13.4 ± 5.9[f]	−17	−10
Ara-A	15.0	11/12 (92)[h]	5.3 ± 1.2[h]	−19	−17
	37.5	12/12 (100)[h]	7.3 ± 3.1[h]	−22	
	150.0	24/25 (96)[h]	9.5 ± 3.4[h]	−12	−17
	300.0	22/25 (88)[h]	8.4 ± 2.1[h]	−18	−4
	600.0	8/12 (67)[g]	7.8 ± 2.8[h]	−20	−4
FIAC	100.0	9/12 (75)[f]	8.8 ± 3.6[h]	−15	−20
	150.0	7/13 (54)[g]	13.7 ± 3.9[g]	−14	−13
	300.0	8/13 (61)[e]	12.8 ± 2.6[g]	−10	−11
	500.0	6/12 (50)[g]	8.3 ± 5.9[h]	−11	−11
FMAU	1.9	12/12 (100)[h]	7.3 ± 2.1[h]	−21	
	4.7	12/12 (100)[h]	7.3 ± 2.4[h]	−25	
	10.0	9/12 (75)[f]	9.3 ± 4.2[h]	−27	−13
	18.8	3/12 (25)[g]	9.7 ± 2.3[h]	−10	−3
	37.5	4/12 (33)[g]	8.8 ± 1.3[h]	−5	0
	50.0	3/12 (25)[g]	13.0 ± 6.1[h]	−12	−12
	75.0	1/13 (8)[g]	12	0	8
	100.0	3/12 (25)[g]	10.7 ± 6.7[h]	−11	−6
	150.0	1/13 (8)[g]	12	−4	9
	300.0	0/13 (0)[g]		−3	5
FMAU-ACV (1:8)	1.9:15.0	12/12 (100)[h]	5.5 ± 1.7[h]	−17	
	4.7:37.5	4/12 (33)[g]	7.8 ± 3.0[h]	−8	−15
	10.0:80.0	5/12 (42)[g]	8.8 ± 6.1[h]	−8	−11
	18.8:150.0	1/12 (8)[g]	9	−3	7
	37.5:300.0	1/12 (8)[g]	12	−5	2
	75.0:600.0	1/12 (8)[g]	19	−7	2
FMAU-ara-A (1:8)	1.9:15.0	11/12 (92)[h]	5.6 ± 1.4[h]	−29	7
	4.7:37.5	9/12 (75)[f]	6.2 ± 1.7[h]	−13	−2
	10.0:80.0	4/12 (33)[g]	6.5 ± 1.3[h]	−8	−6
	18.8:150.0	1/12 (8)[g]	9	−4	2
	37.5:300.0	1/12 (8)[g]	6	−5	−4
	75.0:600.0	1/12 (8)[g]	13	−13	−16
FIAC-ACV (1:1)	100.0:100.0	4/12 (33)[g]	10.0 ± 4.1[h]	−12	−16
	150.0:150.0	3/13 (23)[g]	13.3 ± 1.2[f]	−3	−1
	300.0:300.0	1/13 (8)[g]	9	0	0
FIAC-ara-A (1:1)	100.0:100.0	9/12 (75)[f]	11.7 ± 5.1[f]	−13	−18
	300.0:300.0	3/13 (23)[g]	9.3 ± 4.5[h]	−4	9
	500.0:500.0	3/12 (25)[g]	6.7 ± 2.5[h]	−16	−21
ACV-ara-A (1:1)	100.0:100.0	8/11 (73)[g]	8.6 ± 3.8[h]	−16	4
	150.0:150.0	8/13 (61)[g]	12.6 ± 3.8[e]	−7	−18
	300.0:300.0	7/13 (54)[g]	12.9 ± 6.4[f]	−3	−1
	500.0:500.0	4/12 (33)[g]	15.3 ± 6.1[f]	−11	−23

[a] Swiss ICR (female, about 7 weeks old) mice were treated with drug 72 h after virus inoculation (HSV-2, strain G; about 5 LD_{50}). Doses were administered intraperitoneally twice a day for 4 days.

[b] The molecular weights of the compounds are: ACV, 225; ara-A, 267; FMAU, 260; and FIAC, 371. To convert nanomoles to milligrams divide the value by 4.44, 3.75, 3.85, and 2.70, respectively.

[c] Calculated on day 21. The probability that the observed increase in survivor number or increase in mean time to death was due to chance is noted as follows: *e*, $P \leq 0.01$; *f*, $P \leq 0.05$; *g*, $P \leq 0.001$; *h*, not significant ($P > 0.05$).

[d] Single numbers indicate death of single animal.

TABLE 2. Median effective doses and C.I. for antiviral drugs

Treatment (drug ratio)	Parameters[a]			Median effect dose ratio $[(D_m)_1/(D_m)_2]$	ED_{90} (nmol/kg per day)	C.I.[b] at f_a:		
	$m \pm$ SE	D_m (nmol/kg per day)	r			0.5	0.7	0.9
FIAC	0.50 ± 0.32	510	0.74		40,590			
FMAU	1.33 ± 0.16	22.5	0.95		117			
ACV	0.27 ± 0.05	7,587	0.96		>100,000			
Ara-A	1.78 ± 0.04	901	0.99		3,096			
FMAU-ACV (1:8)	1.79 ± 0.47	60.3	0.91	1/337	206	0.31	0.25	0.20
FMAU-ara-A (1:8)	1.81 ± 0.24	65.2	0.98	1/40	220	0.41	0.35	0.29
FIAC-ACV (1:1)	1.60 ± 0.14	133	0.99	1/15	525	0.14	0.04	0.007
FIAC-ara-A (1:1)	1.48 ± 0.55	376	0.94	1/1.8	1,659	0.65	0.46	0.29
ACV-ara-A (1:1)	0.96 ± 0.18	550	0.97	1/0.12	5,425	0.35	0.46	0.87

[a] m is the slope, D_m is the median effective dose, and r is the correlation coefficient determined from the median effect plot.
[b] C.I. values less than 1 indicate synergy. C.I. values were derived from equations 1 and 3, using $\alpha = 1$.

values and the other parameters shown in Table 2 for the single drug and the combination are calculated automatically.

Statistical analyses. The LD_{50} for the virus was determined in a pilot study and estimated by the median effect plot. Survival data from the mouse studies were assessed with nonparametric tests as previously described (23). The computation of P values took into account animals that died on or before day 21.

RESULTS

Single-drug treatment. Since PBS-treated mice inoculated with a low dose of virus first began to die 4 days later, we were obligated to start the various drug regimens after a 3-day delay (Table 1). The mean day of death of the PBS-treated mice was 8.1 ± 2.2 (standard deviation [SD]), and the overall mortality rate in this group was 96%. For the drug-treated groups, the mortality rate was significantly lower at doses ranging from 10 nmol/kg per day for FMAU to 600 nmol/kg per day for ara-A. Although administration of FIAC, ara-A, or ACV, even at high doses (600 nmol/kg per day), failed to reduce mortality by more than one-half, less than 20 nmol of FMAU per kg was able to reduce mortality by two-thirds to three-quarters.

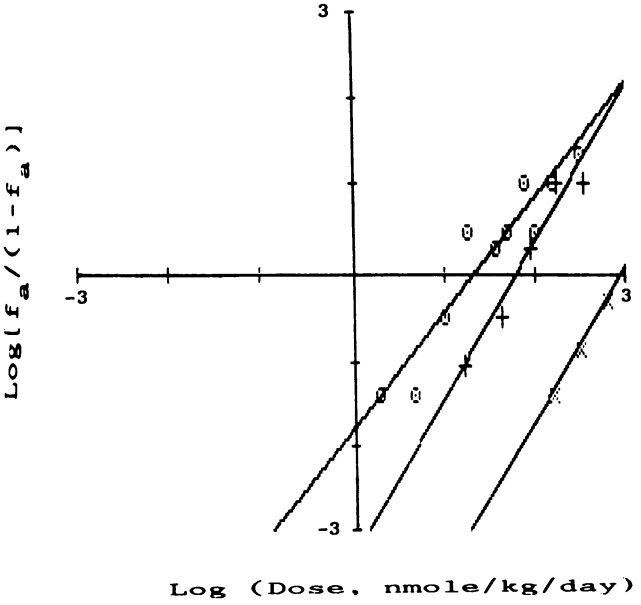

FIG. 1. The median effect plot for the dose-effect relationship for FMAU (○) and ara-A (×) alone and in combination (+) at a 1:8 ratio.

The estimated median effective dose was 22.5, 510, 901, and 7,587 nmol/kg per day for FMAU, FIAC, ara-A, and ACV, respectively (Table 2), with a median effect dose ratio relative to FMAU of 1:23, 1:40, and 1:337 for FIAC, ara-A, and ACV, respectively. It was not possible to test ACV, ara-A, and FIAC at doses above 600 nmol/kg without encountering drug-related toxicity. The toxicity of the four drugs alone (and in combination) in uninfected mice, with the same dose scheduling, has been reported previously (22, 23).

Combined treatment. Significant reduction in mortality compared with the controls and the single-drug treatment animals was noted for several doses of combinations (Table 1). Because the slopes of the regression curves for the drugs alone and in combination were not parallel, the combination index was determined by using mutually nonexclusive assumptions ($\alpha = 1$), an analysis that yields a more conservative estimate of synergy. The median effective dose for the combinations at a ratio of 1:1 was 133, 376, and 550 nmol/kg per day for FIAC-ACV, FIAC-ara-A, and ACV-ara-A, respectively (Table 2). For the combinations at a ratio of 1:8, the median effective dose was 60.3 and 65.2 nmol/kg per day for FMAU-ACV and FMAU-ara-A, respectively. In this type of analysis, the combination is treated as a third drug, and the contribution of FMAU to the median effective dose for the combination is only one-eighth that of ACV or ara-A.

When the survival data for the paired combinations of the four drugs were analyzed using the f_a versus C.I. plot (see Materials and Methods), a strong synergistic interaction (C.I. < 1) was noted at different f_a values (Table 2). As an example of how the survival analysis is performed, Fig. 1 and 2 show the dose-effect and f_a-C.I. plots for the combination of FMAU and ara-A (ratio, 1:8). The slope of the dose-effect curve (Fig. 1) for this combination was greater than for either drug alone, suggesting a mutually nonexclusive interaction. The f_a-C.I. plot (Fig. 2) indicated that the C.I. value was less than 1 over the entire range of f_a values, suggesting strong synergism at all effect levels.

As shown in Table 2, the slopes (m values) of the median effect plots were steeper for the combinations than for the single drugs, except for ara-A. This change in the slope of the dose-effect curve (sigmoidicity), in conjunction with the lower than expected median effect (D_m) values in combination, showed synergistic effects. For instance, in Table 2, the D_m for the combination of FMAU and ara-A (1:8) was 65.2 nmol/kg per day, which constitutes 7.25 nmol/kg per day for

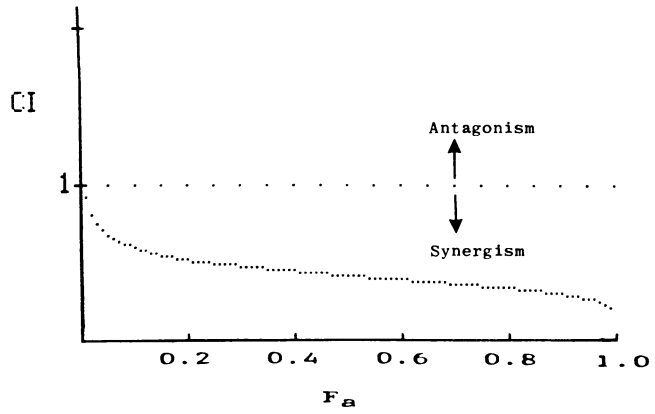

FIG. 2. Fraction affected (f_a)-C.I. plot for the combination of FMAU and ara-A at a 1:8 ratio. C.I. values were calculated from equations 1 and 3 ($\alpha = 1$).

FMAU plus 57.95 nmol/kg per day for ara-A. By contrast, the median effect dose for FMAU and ara-A alone, as shown in Table 2, was 22.5 and 901 nmol/kg per day, respectively. The C.I. for this pair of combinations at the 50% effective dose (ED_{50}) (at D_m or $f_a = 0.5$) can be calculated by equation 3 (see Materials and Methods). This yields a C.I. value of 0.41, well below 1, suggesting strong synergism. Computer-generated isobolograms based on the median effect method (5; Chou et al., in press) either in conventional form or in a conservative version (Fig. 3) showed strong synergism for FMAU and ara-A. These results confirm the analyses obtained in the f_a-C.I. plot (Fig. 2). When a drug ratio of 1:1 or 1:2 for the combination of FMAU and ACV or ara-A was used at doses ranging from 75 to 300 nmol/kg per day, less than 10% mortality was noted. The data (not shown) suggest that these combinations at high doses and different ratios are not antagonistic.

By day 7 after virus inoculation, significant reduction in body weight compared with the PBS-treated mice was apparent for all the single-drug treatment groups, except for the FMAU groups at doses greater than 10 nmol/kg. Most of the surviving mice gradually gained weight, and by day 30 after virus inoculation their weights had increased by more than 3% (range, 4 to 39%) compared with day 1 values (data not shown). A marked decrease in body weight was noted for the mice treated with ara-A or ACV at doses of 600 nmol/kg. However, when these drugs were combined with FMAU at the same dose, an increase in body weight was noted over that with each drug alone. The combination of FIAC and ACV was not only the most synergistic, but at all doses tested it was also the least toxic compared with the single drugs. Although the combination of ara-A and ACV at 500 nmol/kg produced significant survival, a marked decrease in body weight was noted compared with that of animals treated with 300 nmol/kg (Table 1). With the exception of combinations containing ara-A at the highest dose tested, the toxicity of the different combinations did not appear to be additive. The animals surviving the virus inoculation were bled on day 30 to 32. All the animals were shown to have seroconverted.

DISCUSSION

Since combination chemotherapy with antiviral drugs is currently being considered for the treatment of HSV encephalitis, it was important to determine the interaction of several drugs in a mouse model for severe HSV infections to provide some prediction of the potential clinical usefulness of this therapeutic modality. For these studies we selected an intracerebral rather than a peripheral route of virus inoculation, since in humans virus is already present in the brain at the time of treatment. To increase the severity of the test, and because this disease in humans is often diagnosed late, drug treatment was delayed by 72 h after virus inoculation. Significant reduction in mortality was achieved at very low doses of FMAU, but ACV, ara-A, and FIAC alone were only marginally active; a median effective dose for these drugs was obtained by extrapolation, since at high doses severe drug-related toxicity is observed (22). The results obtained with FMAU and FIAC were similar to those found in our previous study when treatment was delayed for 2 days; FMAU was more active than FIAC, but both of these 2′-fluoronucleosides were more potent than ACV (22).

One of the main problems in assessing the effectiveness of drug combinations has been the lack of agreement among investigators on how to analyze the experimental results in a quantitative manner that is simple and theoretically sound and does not require large numbers of measurements. The two most widely used methods of analysis for such systems are the isobologram introduced by Loewe (15), and the fractional product method, formalized by Webb (30). There are several major drawbacks to the conventional isobologram method and the fractional product method (5). Conventional isobolograms do not use data efficiently, since an isobol determines synergism-antagonism at one effect level and is not applicable to mutually nonexclusive drugs, whereas the method used in this report determines synergism-antagonism at all effect levels. The isobologram analysis requires the construction of dose-effect curves for each drug alone and for a series of mixtures of the drugs. The doses of each drug alone and in combination that are required to achieve an arbitrarily selected endpoint are then used to construct the isobologram as a secondary plot. The isobologram, which is a curve of equieffectiveness, reveals by inspection whether the drug mixture displays antagonism, summation (additivity), or synergism. An analysis based on the mutually nonexclusive equation should give a more conservative estimation of synergism than the conventional isobologram (Fig. 3), because the former has an additional term in calculating the value of C.I. (total, three terms). Therefore, the conventional isobolograms tend to overestimate synergism (C.I. has two terms). Figures 2 and 3 (broken lines) should give identical conclusions because these plots are based on equation 3 ($\alpha = 1$) and are derived from the same multiple-drug effect equation, except that the f_a-C.I. plot is effect-oriented and the isobologram is dose-oriented. It should be noted, however, that the f_a-C.I. plot is more convenient to use. From our experience with various combinations, isobolograms tend to jam the data points at the origin or at one axis. This causes difficulty in reading the points despite the fact that these isobolograms represent one side of the same theory (Chou and Talalay, in press). The conclusion that the summation of effects of two or more drugs can be calculated by multiplying the residual fraction of the activity unaffected by each drug individually is based on the view that two or more agents whose actions are totally independent of one another (and are therefore mutually nonexclusive) will each affect a constant fraction of a system. Although such a method of analysis has been widely used for combinations of anticancer drugs (29), its limitations have not been realized until recently (5). The main problem with the fractional product method (30) is that it

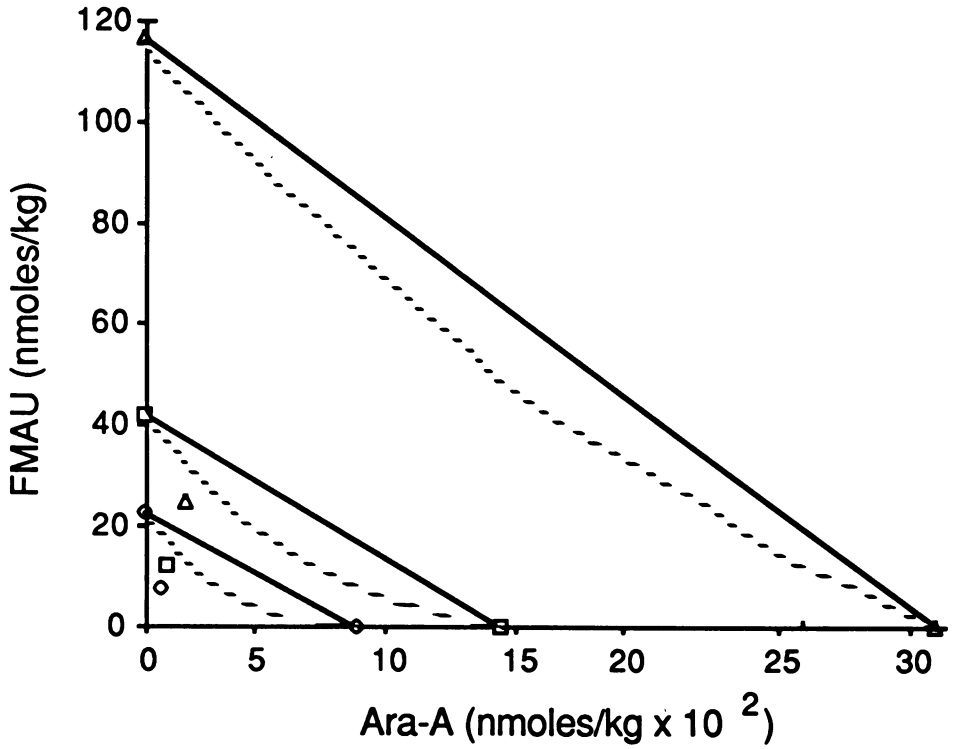

FIG. 3. Computer-generated isobolograms showing synergistic effect between FMAU and ara-A at ED_{50} (○), ED_{70} (□), and ED_{90} (△) levels. Solid lines, Conventional isobolograms based on mutually exclusive assumption (equation 3, $\alpha = 0$). Broken lines, Conservative isobolograms based on mutually nonexclusive assumption (equation 3, $\alpha = 1$).

does not take into account the shapes of the dose-effect curves. If the curve is hyperbolic for drug 1 and sigmoidal for drug 2, the fractional product method may yield erroneous conclusions. It has been shown from the derivation of equations that the fractional product method is valid if the following two conditions are satisfied: (i) each drug has a hyperbolic dose-effect curve ($m = 1$), and (ii) the effects of the two drugs are mutually nonexclusive (5).

As noted in Table 2, the slopes of the dose-effect curves for ACV (0.27) and FIAC (0.5) were very shallow, and for FMAU (1.33) and ara-A (1.78) they were sigmoidal. Since all the m values are different from 1, the applicability of the fractional product method to the present study is ruled out. For all the combinations tested, a strong synergism was noted at effect levels of 0.5 to 0.9 (Table 2). The results obtained with the combination of FMAU and either ACV or ara-A (1:8 ratio) were very similar (slope $\approx$ 1.8); however, the strongest synergy was noted for the combination of FIAC and ACV (1:1 ratio). For the combinations FMAU-ACV and FIAC-ACV, the slope of the dose-response curve was greater for the combination than for each drug alone (Table 2).

In the present study, the survival data were interpreted for synergy, summation, or antagonism by computer analysis, as described previously (1, 5, 6). This method of analysis is applicable to mutually exclusive and nonexclusive interactions. Usually the initial dose of each drug selected for a combination can be based on either the 50% effective dose (ED_{50}) for each drug or on the level of drug in serum or the target organ (22). The doses of the two drugs in combination are then varied upwards or downwards at a constant ratio. In more extensive studies, it may be necessary to test a different combination ratio (e.g., 1:2 or 2:1). This can be easily done, since it is not necessary to redetermine the median effective dose for each drug alone. Determination of the optimum drug ratio and schedule that will produce a synergistic effect over a wide range of affected fractions (f_a) without increased toxicity is important. It should be noted that for chemotherapeutic drugs, only the high-effect region ($f_a \geq 0.5$) is of potential clinical relevance.

The reasons for the synergy observed with the combinations reported here may be related to the greater blockade of the viral DNA polymerase. The metabolites of the nucleosides described here are known to interact with the viral DNA polymerase (12, 16, 20, 24). This enzyme, which is essential for HSV replication, is complex and contains various recognition sites. These include the 2′-deoxynucleoside 5′-triphosphate binding site, which recognizes the sugar and base moiety of normal and altered nucleosides, a pyrophosphate exchange-release site, and an aphidicolin-binding site (11; D. M. Coen, J. Antimicrob. Chemother., in press). Even if two inhibitors act on the same site, a more complete blockade can be achieved than with just one inhibitor (21). Ara-A 5′-triphosphate is known to compete at the DNA polymerase level with ATP, acyclovir 5′-triphosphate (ACVTP) competes with dGTP, and FMAU 5′-triphosphate competes with dTTP. With a combination of ACV and FMAU, therefore, a two-pronged attack may take place at the catalytic site: ACVTP will compete with dGTP, and at the same time FMAU 5′-triphosphate will compete with dTTP, resulting in more effective inhibition of this enzyme. Since ACVTP can also incorporate into the viral DNA as a chain terminator and irreversibly inactivate this enzyme (12), this step may be the rate-limiting one for

combinations of ACV with FMAU. FMAU 5'-triphosphate can also incorporate into DNA molecules that have not been inactivated by ACVTP. When ACV, FIAC, or FMAU is combined with ara-A, the rate-limiting step may be at the ribonucleotide diphosphate reductase step, since ara-A 5'-diphosphate is a potent inhibitor of this enzyme (16). The metabolism of FIAC is too complex to speculate at present on its interaction in combination with other antiviral agents at the DNA polymerse level (20). The findings of additional studies on the levels of nucleotide pools in virus-infected and uninfected cells (e.g., Spector et al. [28]) and the effect of dual inhibitors with purified viral DNA polymerase (e.g., Frank and Cheng [11]) may be relevant to the synergy observed in this animal model. The results of such studies may assist in the future in predicting the outcome of combinations in animal models.

Another factor that may influence the results obtained with combinations of antiviral drugs is that HSV may be selectively distributed in vivo at particular sites or sanctuaries, and that these agents may have different abilities to penetrate and accumulate in different tissues. 2'-Fluoroarabinosyl pyrimidine nucleosides, especially FMAU, have been shown to have remarkable absorption and distribution in tissues, including the brain, after intravenous, intraperitoneal, or oral administration (4, 18, 22). Therefore, these compounds may complement the therapeutic effect of other agents at nonbiochemical levels. Since antiviral nucleoside analogs require intracellular virus-specific enzymes for activation, the bioavailability of the analogs becomes an important determinant for therapeutic efficacy.

Of interest was the markedly higher body weight of mice treated with high doses of the combination of ACV with either FMAU or FIAC in comparison to those treated with single-drug therapy at the same dose. The combination of FMAU and ara-A also appeared to be less toxic at intermediate effective doses and as toxic as ara-A alone at high doses (Table 1). In general, the weights of the animals receiving the drug combinations were either higher than or similar to those of animals receiving single-drug therapy with the most toxic compound.

In conclusion, it appears from our analyses that all the combinations tested in this study are suitable for further investigation for clinical use. However, the toxicological profiles of these combinations and the optimum drug schedules will have to be ascertained before trials in humans with severe HSV infections are conducted.

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