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Combination Chemotherapy: Interaction of 5-Methoxymethyldeoxyuridine with Adenine Arabinoside, 5-Ethyldeoxyuridine, 5-Iododeoxyuridine, and Phosphonoacetic Acid Against Herpes Simplex Virus Types 1 and 2

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The antiviral activity of 5-methoxymethyl-2'-deoxyuridine (MMUdR) was compared with that of 5-iodo-2'-deoxyuridine (IUdR), 5-ethyl-2'-deoxyuridine (EtUdR), adenine arabinoside (Ara-A), and phosphonoacetic acid (PAA) against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). MMUdR was more potent than Ara-A and PAA but less active than EtUdR and IUdR against HSV-1 in rabbit kidney (RK-13) cells. In Vero cells, the antiviral activities of MMUdR, Ara-A, and PAA against HSV-1 were of the same order of magnitude. The antiviral potency against HSV-2 varied with the strain of virus used. All strains of HSV-2 were markedly inhibited by EtUdR and IUdR and to a lesser degree by PAA. However, considerable variation was noticed in the susceptibility of HSV-2 strains to Ara-A and MMUdR. Interaction of MMUdR with Ara-A, EtUdR, IUdR, and PAA was investigated by the method of response isobolograms. MMUdR showed synergistic activity in combination with Ara-A and PAA but antagonistic activity in combination with EtUdR and IUdR against herpesviruses. Minimum toxic dose (concentration required to produce definite evidence of microscopic cytoxicity in rapidly growing RK-13 cells) was determined for each compound and was found to be 512, 172, 64, 8, and <0.5 μg/ml for MMUdR, PAA, Ara-A, EtUdR, and IUdR, respectively. MMUdR was found to have the maximum antiviral index against HSV-1 (512) and HSV-2 strains X-265 (102) and ATCC (85). Antiviral index was defined as the minimum toxic dose divided by the dose that reduced plaque numbers by 50%.

Herpesviruses are responsible for a number of infectious diseases in humans which have not proven amenable to control by immunization. However, some measure of control has been achieved with antiviral chemotherapy. 5-Iodo-2'-deoxyuridine (IUdR) and 9-β-D-arabinofuranosyladenine (adenine arabinoside; Ara-A) have been used clinically (22, 23, 30), and several other compounds have been shown to inhibit herpes simplex virus replication in vitro (6, 7, 21, 25). IUdR and 5-ethyldeoxyuridine (EtUdR) have potent antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro and against herpes keratitis (18, 25). Phosphonoacetic acid (PAA) is also inhibitory against herpesviruses and has been shown to be effective in the treatment of skin or mucous membrane infections in animals (14, 21). IUdR and Ara-A exhibit varying degrees of toxicity toward host cells (12, 15, 20, 28). PAA has a tendency to accumulate in the bones, and use of this compound for the treatment of systemic infections is likely to be accompanied by side effects (B. A. Bopp, C. B. Estep, and D. J. Anderson, Fed. Proc. **36**:939, 1977). Thus, it is obvious that toxicity is one of the major problems of antiviral chemotherapy. Combination chemotherapy has proven to be an effective means of dealing with drug toxicity problems in antimicrobial and cancer chemotherapy (3, 8, 11, 13), but it has received scant attention in antiviral chemotherapy (1, 10, 17, 20). By selecting drugs whose mechanisms of action differ, it should be possible to find combinations that act synergistically, thus reducing the effective dose of each drug and diminishing the problem of toxicity.

We have developed a new nucleoside analog, 5-methoxymethyldeoxyuridine (MMUdR),

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which has been shown to have a higher therapeutic index than other drugs currently in use (1). It is devoid of toxicity to lymphoid functions in vitro (2), is non-immunosuppressive in vivo (26), and is effective in the treatment of keratitis in animals (20). It appears that MMUdR provides promise as an anti-herpes drug, especially if combined with other drugs which also have a high therapeutic index. In this paper, we compare the antiviral activity against HSV-1 and HSV-2, and cytotoxicity towards mammalian cells, of MMUdR, with those of Ara-A, EtUdR, IUdR, and PAA.

MATERIALS AND METHODS

Drugs. IUdR and Ara-A were obtained from Sigma Chemical Co., St. Louis, Mo., and Pfanstiehl Laboratories, Waukegan, Ill., respectively. PAA was kindly provided by L. R. Overby (Abbott Laboratories, Chicago, Ill.). EtUdR was a gift from Gert Streissle (Bayer, Wuppertal, W. Germany), and MMUdR was synthesized (4). All antiviral compounds were dissolved at the required concentration in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) and filter sterilized immediately before use.

Animals. New Zealand White female rabbits (2 to 3 kg) were obtained from the Animal Resources Centre, University of Saskatchewan. The animals were maintained in individual cages in special quarters designed for infectious studies.

Cell culture. RK-13 (rabbit kidney) and Vero (African green monkey kidney) cells were cultured as previously described (1). Confluent monolayers were prepared by seeding about 5×10^4 cells into each cup of microtiter tissue culture plates (no. 3040; Falcon Plastics, Oxnard, Calif.). The cultures were incubated at 37°C in a humidified CO_2 (5%) atmosphere, and the monolayers were confluent within 24 h.

Viruses. HSV-1 strain 76 was isolated from a human labial lesion in the Department of Microbiology. HSV-2 strains ATCC, MS, and X-265 were kindly provided by V. Pavilanis, Armand-Frappier Institute, Montreal, Canada. Stock viruses were prepared and titrated as previously described (1). Antibodies specific to each herpesvirus were prepared as follows: HSV-1 or HSV-2 ATCC (0.5 ml; virus titer, 10^7 plaque-forming units [PFU] per ml) was injected intravenously into rabbits. After two weeks, animals were challenged with a second dose of the same herpesvirus. Four weeks after the initial injection of virus, blood was collected, and serum was filter sterilized, heat inactivated, and titrated by virus neutralization. Antisera showed a neutralization titer of approximately 1/128 against the virus used for immunization.

Drug inhibition assays. For antiviral assays, confluent RK-13 or Vero cell cultures were infected with either 10 or 50 PFU of virus per well in a microtiter plate as was described previously (1, 2). Each antiviral compound, at the appropriate concentrations, was added to Eagle minimal essential medium containing 1 to 2 neutralizing units of specific antibody for each herpesvirus type. Plaques were allowed to develop for 72 h before fixation, staining, and enumeration (1, 2). In each experiment, toxicity controls (containing test compound and medium only), cell controls (containing medium only), and virus controls (containing virus and medium only) were also run simultaneously. The percentage of inhibition (calculated from the reduction in the number of plaques) at each concentration of the compound was determined. These data were used to draw dose-response curves for each antiviral compound. From these graphs, the concentrations of each compound required to reduce the number of plaques by 12.5%, 25%, 37.5%, 50%, 62.5%, and 75% was determined. For studying the type of interaction of two drugs, response isobolograms were drawn using the procedure described earlier (9, 29). Dose-response curves for single drugs and for each level of the first drug (e.g., MMUdR) in combination with various concentrations of the second drug (e.g., Ara-A) were drawn for each pair of antiviral drugs. From these curves, the amount of the second drug in combination required to give 50% reduction in PFU was determined. Fractional inhibitory concentrations were then calculated for each drug by dividing the concentration of each drug in the combination by the amount of drug that would be required to give the same degree of inhibition by itself.

Fractional inhibitory concentrations of each pair of drugs were then plotted to determine the nature of interaction. When the effects of two compounds are additive, the points fall on a straight line, connecting unity on the ordinate axis with unity on the abscissa axis. Deviations to the left of this theoretical line indicate synergism; deviations to the right represent interference or antagonism between the two drugs.

Inhibition of cell growth. For determination of cytotoxic activity, RK-13 cells (6×10^5) were seeded into tissue culture plates containing 24 cups (Falcon Plastics). Solutions of drugs at appropriate concentrations were added within 5 to 10 min. Normal controls (cells + medium + 10% fetal calf serum) were also run simultaneously. After 72 h, cells were washed with Hanks solution, trypsinized, diluted with maintenance medium, and counted using a model FN Coulter Counter (Coulter Electronics Inc., Hialeah, Florida). Some cell samples were also counted using a hemacytometer. A paired t test showed there was no significant difference between the two methods of counting (data not shown). The toxicity of drugs in combination was also studied. The drugs were added together in the proportion that resulted in either maximum synergism or antagonism. These concentrations were determined from isobolograms.

RESULTS

Comparison of antiviral potency of MMUdR, EtUdR, IUdR, Ara-A, and PAA against HSV-1 and HSV-2. Dose-response curves for antiviral drugs against HSV-1 and HSV-2 in RK-13 cells are shown in Fig. 1. The antiviral activity against HSV-1 decreased in the following order: IUdR > EtUdR > MMUdR > Ara-A $\approx$ PAA in RK-13 cells. In Vero cells, MMUdR, Ara-A, and PAA were almost equipotent against HSV-1. All strains of HSV-2 were

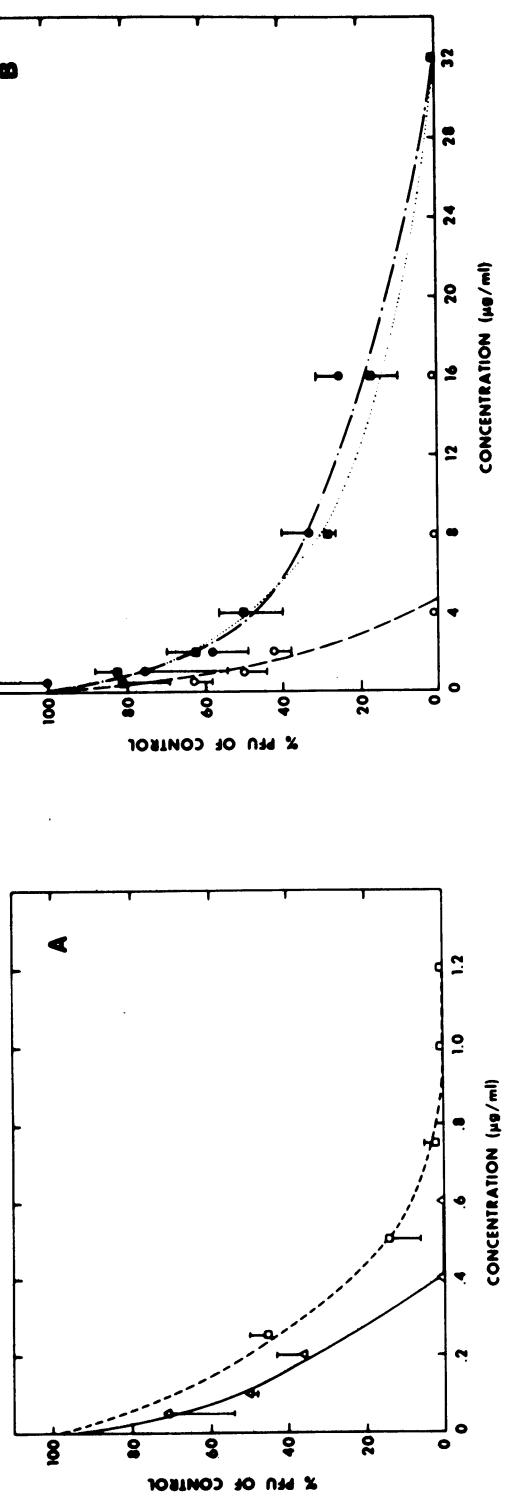

FIG. 1. *Dose-response curves for antiviral drugs against HSV-1 76 (A and B) and HSV-2 ATCC (C and D) in RK-13 cells. Amount of virus used was 10 PFU. (A) EtUdR (□), IUdR (△); (B) MMUdR (○), Ara-A (●), and PAA (■). Each point represents an average of eight determinations. Vertical bar indicates standard error.*

markedly inhibited by EtUdR and IUdR. However, considerable variation was noticed in susceptibility of different strains of HSV-2 to Ara-A, MMUdR, and PAA. In general, the antiviral potency of PAA against HSV-2 was greater than those of Ara-A and MMUdR. MMUdR and Ara-A were almost equipotent against HSV-2 X-265. However, MMUdR was less active than Ara-A against HSV-2 strains ATCC and MS.

Inhibition of HSV-1 and HSV-2 plaque formation by simultaneous treatment with combinations of two antiviral drugs. The amount of each drug alone and in combination required to cause 50% reduction in PFU of HSV-1 and HSV-2 was determined. Dose-response curves for two typical experiments are shown in Fig. 2. MMUdR in combination with Ara-A resulted in marked inhibition of plaque formation (Fig. 2A). In marked contrast, the effect of MMUdR and EtUdR combination was less than for the individual drugs (Fig. 2B). To study the type of interaction of two drugs, isobolograms were drawn (Fig. 3). MMUdR in combination with either Ara-A or PAA showed synergistic activity (Fig. 3A and B), whereas MMUdR showed an antagonistic interaction when combined with either EtUdR or IUdR (Fig. 3C and D). The amount of each drug required for the inhibition of viral growth was significantly reduced when used in combination (Table 1).

Effect of antiviral drugs on uninfected rapidly growing RK-13 cells. The relationship between the dose of IUdR, EtUdR, Ara-A, PAA, and MMUdR and the decline in the number of RK-13 cells is shown in Fig. 4A. MMUdR was the least toxic of the compounds used in this study. Minimum toxic dose (concentration required to produce definite evidence of microscopic cytotoxicity) was 512, 172, 64, 8, and <0.5 for MMUdR, PAA, Ara-A, EtUdR, and IUdR, respectively. In combination chemotherapy experiments, MMUdR gave significant protection to cells from EtUdR toxicity (Fig. 4B). MMUdR in combination with IUdR showed a variable response. At concentrations between 1.5 and 6 μg of IUdR per ml, MMUdR gave significant protection to cells from IUdR toxicity. However, at higher concentrations MMUdR slightly enhanced the toxicity of IUdR. In contrast, MMUdR at high concentrations afforded cells some protection against the toxicity of Ara-A and PAA. MMUdR was nontoxic at concentrations of up to 1,024 μg/ml, against confluent monolayers of RK-13 cells.

Antiviral indexes of MMUdR, EtUdR, PAA, Ara-A, and IUdR. Results of antiviral indices of these compounds against HSV-1 and HSV-2 are shown in Table 2. MMUdR had the highest antiviral index against HSV-1 and against HSV-2 strains ATCC and X-265. Against HSV-2 MS, the antiviral index was PAA > EtUdR > MMUdR > Ara-A > IUdR. The antiviral index was defined as the minimum dose at which toxicity was observed microscopically, divided by the dose that reduced the plaque numbers by 50%.

DISCUSSION

It is now well established that a number of viruses induce their own enzymes for the synthesis of viral nucleic acid and that some of these virus-coded enzymes differ from host cell enzymes (5, 16, 19). One way of reducing toxicity is to develop antiviral drugs which act by specifically inhibiting virus-coded functions during the viral replication cycle. Such drugs might thus be expected to have minimum effects on host cell metabolism. The results reported earlier (1, 2, 20, 26) and in this communication indicate that MMUdR is a unique antimetabolite. This drug is highly efficacious against HSV-1 and has moderate activity against HSV-2. In contrast to most other nucleosides (12, 28), MMUdR is nontoxic to rapidly growing cells at concentrations far greater than those required for inhibition of herpes simplex virus. Computation of the antiviral index revealed that MMUdR was much safer to use than any other drug tested (Ara-A, EtUdR, IUdR, or PAA) against HSV-1 and against HSV-2 strains X-265 and ATCC.

Another way of improving the therapeutic index of antiviral drugs is to use them in combination. By selecting pairs of drugs which inhibit viral replication by acting at different enzymatic steps, enhancement or synergism may result due to a sequential blockade, concurrent inhibition, or complementary inhibition (29). We have shown that combination of MMUdR with Ara-A or PAA resulted in synergistic inhibition of herpes simplex virus replication. As a result, the total amount of each drug required for inhibition of virus replication was significantly reduced. Of particular interest was the finding that synergistic activity was achieved without significant concurrent increase in toxicity to rapidly growing cells. These results indicate that combination chemotherapy may indeed represent a very effective method of combating herpes simplex infections.

Several other benefits may also accrue by combination chemotherapy of viral diseases. First, in vivo, where disease occurs as a result of extensive virus replication, combination chemotherapy might be effective in reducing the virus load such that severity of the clinical disease would be reduced. Second, combination chemo-

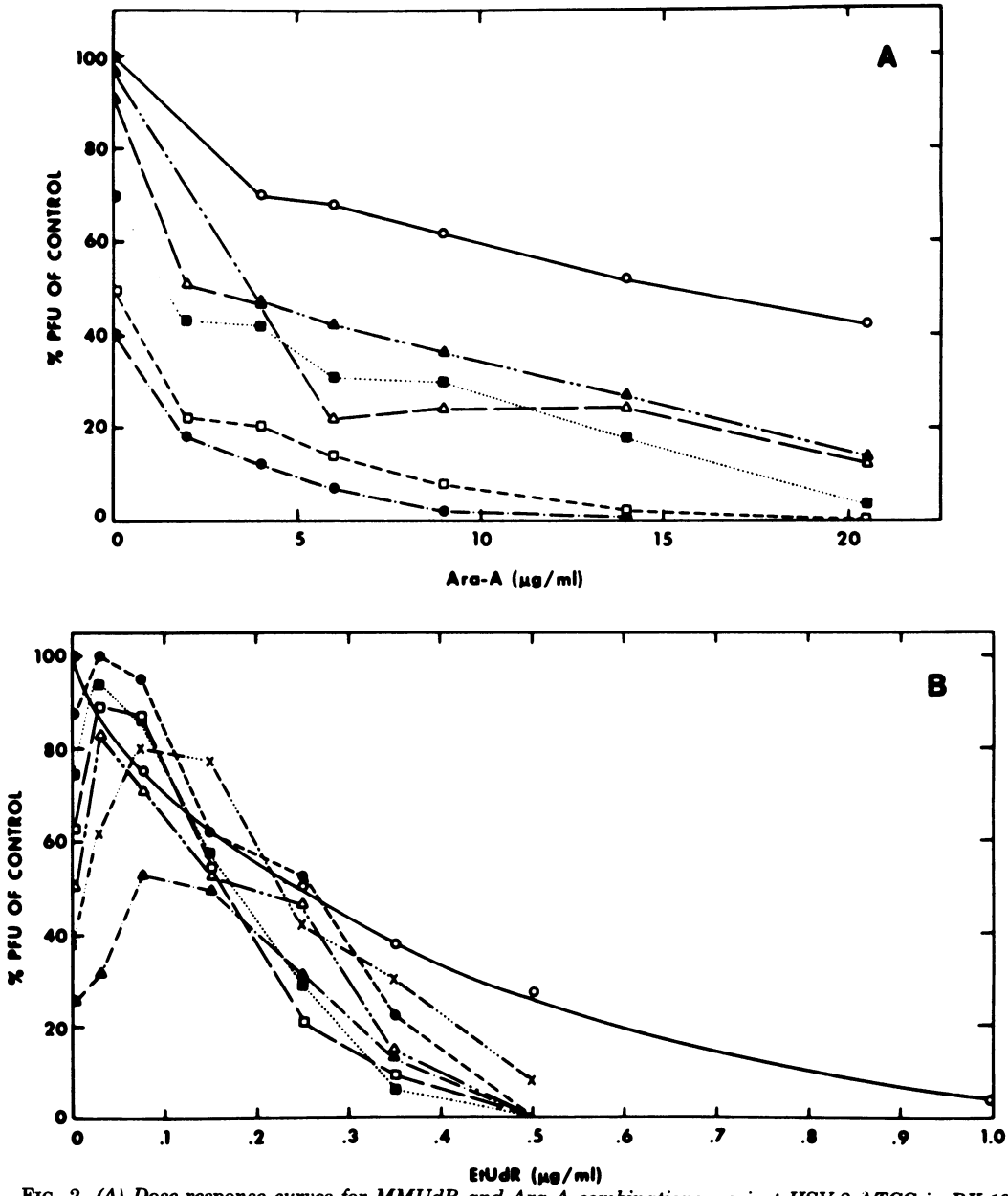

FIG. 2. *(A) Dose-response curves for MMUdR and Ara-A combinations against HSV-2 ATCC in RK-13 cells. MMUdR concentrations in combination with Ara-A: zero (○), 1 μg/ml (▲), 3 μg/ml (△), 16 μg/ml (■), 34 μg/ml (□), and 76 μg/ml (●). The points lying on the response axis represent the percent PFU of control produced by these MMUdR concentrations in single-drug experiments performed concurrently with the combination experiments. Amount of virus used was 50 PFU. (B) Dose-response curves for MMUdR and EtUdR combinations against HSV-1 76 in RK-13 cells. MMUdR concentrations in combinations: zero (○), 0.25 μg/ml (●), 0.63 μg/ml (■), 1.25 μg/ml (□), 2 μg/ml (△), 3 μg/ml (×), and 5 μg/ml (▲). The points lying on the response axis represent the percent PFU of control produced by these MMUdR concentrations in single-drug experiments performed concurrently with the combination experiment. Amount of virus used was 50 PFU.*

therapy is likely to be of considerable value in the treatment of infections due to virus strains which may be only marginally susceptible to individual drugs. A very significant finding in these studies was the synergistic activity of MMUdR and Ara-A combinations against HSV-2 MS. These results indicate that chances of success for the treatment of infections due to

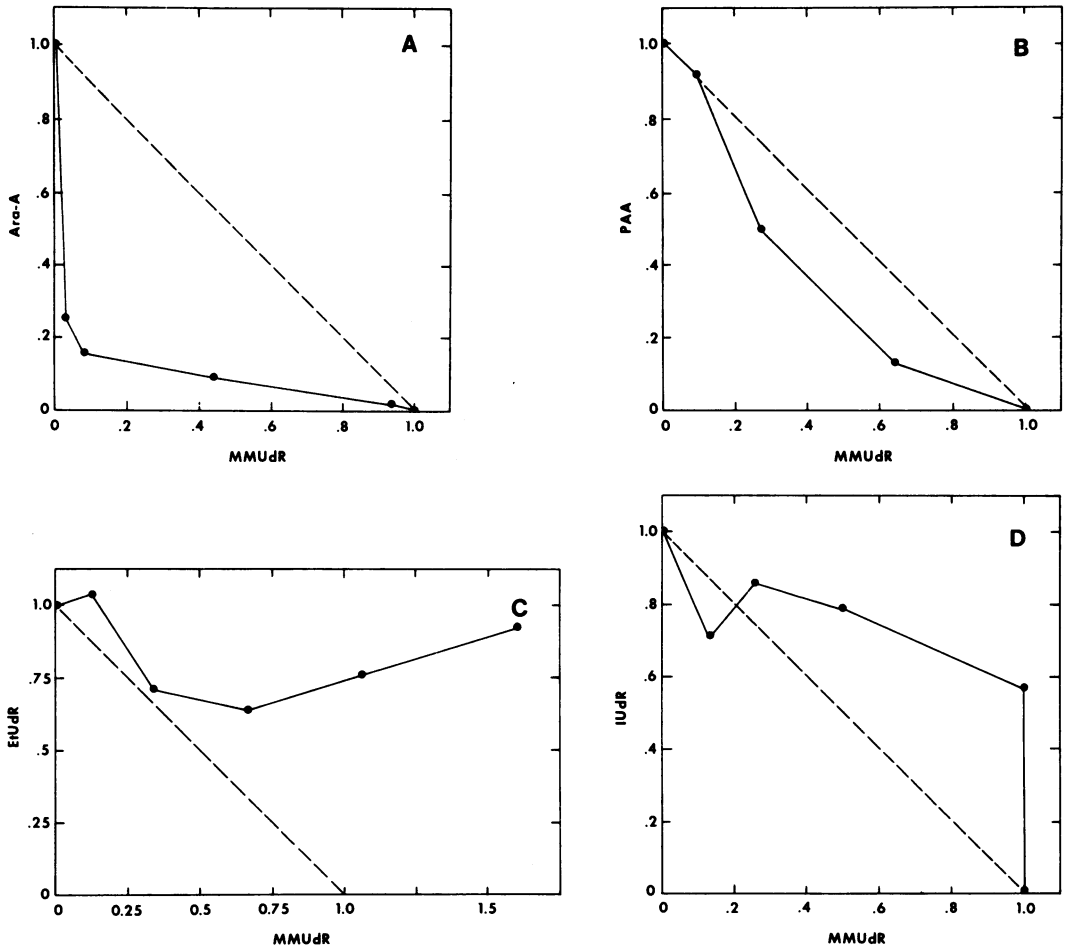

FIG. 3. *Isobolograms for combinations of antiviral drugs (A) MMUdR and Ara-A and (B) MMUdR and PAA against HSV-2 ATCC in RK-13 cells, and (C) MMUdR and EtUdR and (D) MMUdR and IUdR against HSV-1 76 in RK-13 cells. Amount of virus used was 50 PFU. Experimental details and interpretation of the plots are given in the text.*

TABLE 1. *Effectiveness of combination of MMUdR with Ara-A and PAA against HSV-1 and HSV-2*

Virus[a]	Drug combination	Sum of FIC[b]	Amt of each drug required	
			In combination (μg/ml)	Alone (μg/ml)
HSV-1	MMUdR		0.60	1.88
	+	0.42	0.80	8.0
	Ara-A			
	MMUdR		0.32	1.88
	+	0.30	1.28	9.88
	PAA			
HSV-2 ATCC	MMUdR		2.56	36
	+	0.20	1.44	16
	Ara-A			
	MMUdR		14.4	36
	+	0.60	3.8	19
	PAA			
HSV-2 MS	MMUdR		16.0[c]	64
	+	0.50	11.5[c]	46
	Ara-A			

[a] Amount of virus used, 50 PFU.

[b] FIC, Fractional inhibitory concentrations, calculated from isobologram figures according to the procedure described previously (9).

[c] Antiviral assays were carried out using Vero cells.

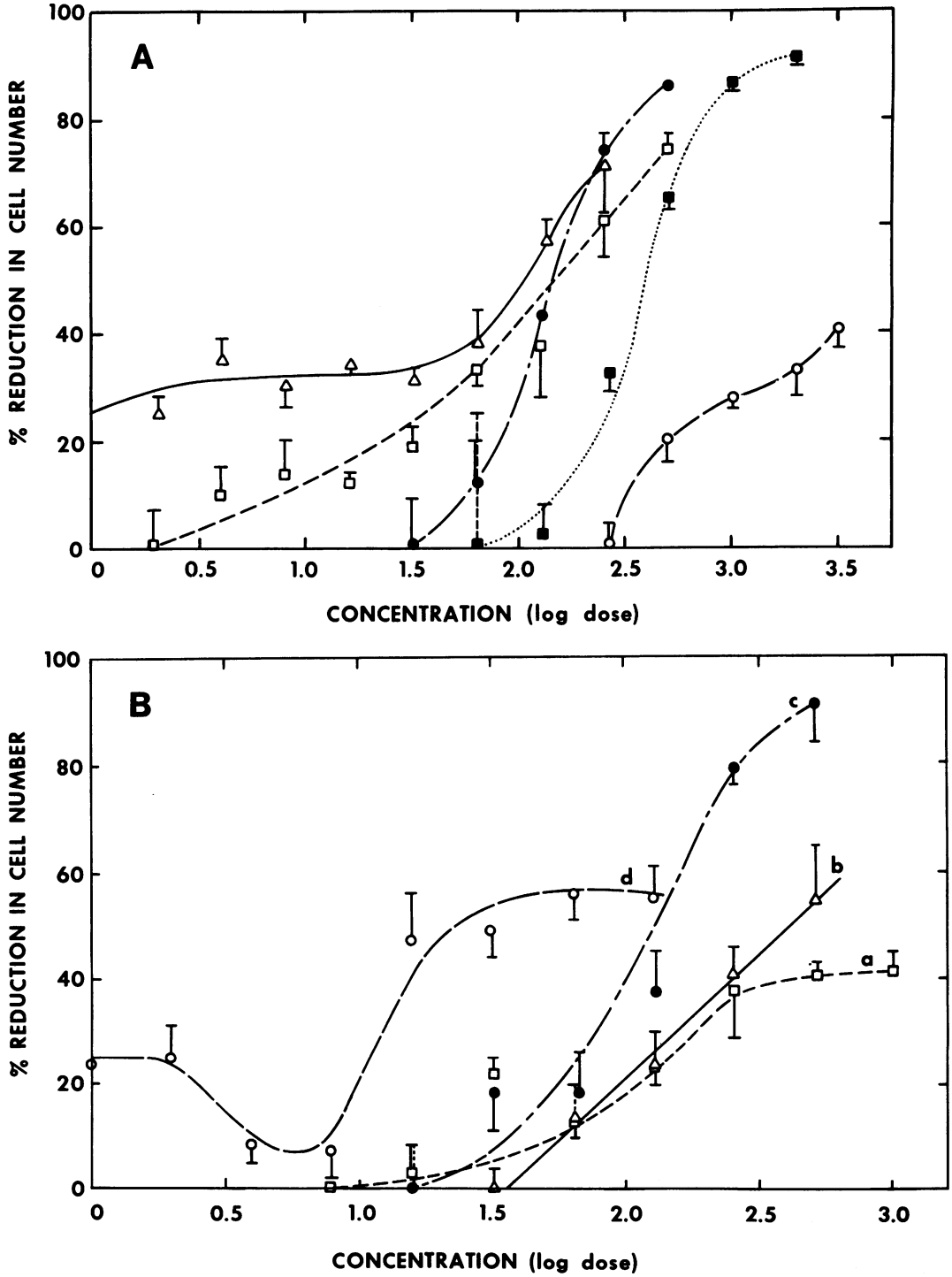

FIG. 4. *(A) Effect of individual drugs on growth of rapidly growing RK-13 cells. MMUdR (○); PAA (■), Ara-A (●), EtUdR (□), and IUdR (△). (B) Effect of combination of antiviral drugs on growth of RK-13 cells. (a) MMUdR + PAA (2:1); (b) MMUdR + EtUdR (64:1); (c) MMUdR + Ara-A (1:2); and (d) MMUdR + IUdR (2:1). Ratios for each pair of drugs were calculated from isobolograms.*

TABLE 2. *Antiviral indices of MMUdR, EtUdR, PAA, Ara-A, and IUdR*

Compound	Minimum toxic dose[a] (μg/ml)	Antiviral index[b]			
		HSV-1 76	HSV-2		
			X-265	ATCC	MS
MMUdR	512	512[c] (256)[d]	102 (64)	85 (14)	8 (4)
EtUdR	8	80 (32)	23 (16)	35 (20)	11 (8)
PAA	172	43 (17)	51 (43)	43 (9)	57 (25)
Ara-A	64	17 (8)	11 (6)	13 (4)	1 (1)
IUdR	<0.5	3.6 (<2.0)	2 (<1.2)	2.4 (<1.4)	0.7 (<0.3)

[a] Concentrations required to produce definite evidence of microscopic cytotoxicity.
[b] Determined by dividing the toxic dose by the minimal effective antiviral dose.
[c] Antiviral index for virus input of 10 PFU.
[d] Parentheses indicate antiviral index for virus input of 50 PFU.

marginally susceptible virus strains would be better by the use of combination chemotherapy. Finally, the chance of a drug-resistant mutant arising would be markedly reduced because even if a mutant does arise that is resistant to one particular drug, that mutant would still be prevented from replication in the presence of another drug whose mechanism of action is different. Thus combination chemotherapy can also be expected to cause a reduction in the overall problem of mutant development. Experiments are in progress to evaluate the comparative efficacy of these drugs in the treatment of HSV-2 infections and to evaluate whether combination chemotherapy can alleviate the problem of development of drug-resistant mutants.

ACKNOWLEDGMENTS

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