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Comparison of the antiviral effects of 5-methoxymethyldeoxyuridine-5′-monophosphate with adenine arabinoside-5′-monophosphate

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Methoxymethyldeoxyuridine-5′-monophosphate (MMUdR-MP) and arabinofuranosyladenine-5′-monophosphate (Ara-AMP) had significant antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in RK-13 and Vero cells. MMUdR-MP and Ara-AMP were more potent than methoxymethyldeoxyuridine (MMUdR) and arabinofuranosyladenine (Ara-A) against the MS strain of HSV-2. MMUdR-MP inhibited replication of HSV-1^r (mutant resistant to MMUdR). MMUdR in combination with Ara-AMP showed additive activity; whereas the MMUdR-MP and Ara-AMP combination was antagonistic against herpes viruses. MMUdR in combination with Ara-A was synergistic in reducing the log virus yield. Cytotoxicity (microscopic lesions) was observed on exposure to MMUdR-MP and Ara-AMP at 450 and 90 μM, respectively. Rapidly proliferating RK-13 cells exposed to Ara-AMP (64 μM) were killed. In the same system, the cells surviving after incubation with MMUdR-MP (650 μM), multiplied at an almost normal rate.

methoxymethyldeoxyuridine monophosphate; adenine arabinoside monophosphate; combination chemotherapy; antiviral activity; cytotoxicity

Introduction

The spectrum of antiviral substances is wide. However, toxicity mitigates the therapeutic usefulness of a large number of antiviral substances. In recent years, several 5-substituted-2′-deoxyuridine analogs with selective antiherpes activity have been described [5,8,18,19]. In this laboratory, we have been studying the antiviral activity and biological properties of one of these novel nucleoside analogs, 5-methoxymethyl-2′-deoxyuridine (MMUdR) [10 and refs. cited therein]. The selective antiviral activity of MMUdR results from its phosphorylation by herpes virus-induced pyrimi-

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dine deoxyribonucleoside kinase [26]. The nucleotide, after conversion to its corresponding triphosphate, is a potent competitive inhibitor of DNA-dependent DNA polymerase of herpes simplex virus (Gupta et al., unpubl. results). In earlier studies, it was shown that the antiviral potency of MMUdR against herpes simplex type 2 (HSV-2) varied with the strain of virus used [2]. One possible reason for limited activity against naturally occurring HSV-2 strains is that they lack the ability to phosphorylate MMUdR. If this premise is correct, then one can expect that the 5'-phosphorylated derivative of MMUdR should have antiviral activity against these strains, provided it can enter the cell intact. In order to test this hypothesis, methoxymethyl-2'-deoxyuridine-5'-monophosphate (MMUdR-MP) was synthesized. In this communication, we report the antiviral activity of MMUdR-MP against herpes viruses, and vaccinia virus and compare its antiviral activity and lethality towards mammalian cells with arabinofuranosyladenine-5'-monophosphate (Ara-AMP), arabinofuranosyladenine (Ara-A) and MMUdR.

Materials and methods

Cell culture

Rabbit kidney (RK-13) and Vero cells were cultured as previously described [2,3]. Cells (5×10^4) were seeded into each well of microtitre tissue culture plates (No. 3040, Falcon Plastics, Oxnard, CA, U.S.A) and incubated at 37° in a humidified CO_2 (5%) atmosphere. The monolayers were confluent within 24 h.

Viruses

The viruses used were: HSV-1, herpes simplex virus type 1 MacIntyre (MAC), Nahmais (NAH) and WCVM (76) strains; HSV-2, herpes simplex virus type 2 (X-265, ATCC and MS strains); EHV-1, equine herpes virus type 1, strain KYD and vaccinia virus. HSV-1 (strains NAH and MAC), and HSV-2 strains X-265, ATCC and MS were kindly provided by Dr. V. Pavilanis, Armand-Frappier Institute, Montreal, Canada. EHV-1 (KYD) was procured from the Animal Diseases Research Institute, Ottawa, Ontario and vaccinia virus was acquired from the Department of Microbiology, University of Saskatchewan. HSV-1 strain 76 was isolated from a human labial lesion by Dr. Babiuk, Department of Microbiology, University of Saskatchewan. Stock viruses were prepared and titrated as previously described [2,4]. Antibodies specific to each herpes virus were prepared according to the procedures discussed earlier [4]. Resistant virus (HSV-1^r) was obtained by growing HSV-1 strain 76 in the presence of increasing concentration of MMUdR. The virus was grown two times in drug-free medium prior to use in antiviral assays.

Chemicals and drugs

Cyanoethyl phosphate (barium salt), dicyclohexylcarbodiimide (DDC) and Ara-A

were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Ara-AMP (sodium salt) was a generous gift from Dr. A.E. LePage, Cancer Research Unit, University of Alberta, Edmonton. MMUdR was synthesized by methylation of 5-hydroxymethyldeoxyuridine and purified by chromatography on silica gel [10]. DEAE-cellulose (Schleicher and Schull, Inc.) was purified using the method of Mathews and Huennekens [11] and stored as an aqueous suspension. Ara-A stock solution was prepared as follows: the drug (1024 μg/ml) was dissolved in phosphate-buffered saline (0.05 M, pH 7.0) at 50°, filter sterilized and diluted with assay media (MEM containing 4% FCS) to give a final concentration of 512 μg/ml. All other drugs were dissolved in the assay media.

Methoxymethyldeoxyuridine-5'-monophosphate (MMUdR-MP)

This compound was synthesized using one of the following procedures. (i) *Cyanoethyl-PO_4 method* [24] phosphorylation of MMUdR using a limited quantity of phosphorylating reagent (β-cyanoethyl-PO_4-MMUdR ratio 1:1) at 25° for 20 h in the presence of DCC gave a mixture of MMUdR-MP and MMUdR-3',5'-diphosphate approximately in the ratio of 3:1. The purification of MMUdR-MP was accomplished by chromatography on DEAE-cellulose and a typical elution profile is shown in Fig. 1. (ii) *Phosphorous oxychloride method* [22] 3'acetyl-MMUdR was phosphorylated with $POCl_3$. After deblocking with alkali, MMUdR-MP was isolated as the barium salt. The barium salt was converted to the ammonium salt prior to use.

Drug inhibition assay

For antiviral assays, confluent monolayers of RK-13 cell cultures were infected with either 10, 50 or 100 plaque forming units (PFU) of virus per well. Each antiviral compound, at the appropriate concentrations, was added. For plaque assays, 1 or 2 U of neutralizing antibody specific for each herpes virus was included in the culture fluids. Plaques were allowed to develop for 72 h prior to fixation, staining and enumeration [2,3]. In each experiment, toxicity controls (containing test compound and medium only) and cell controls (containing medium only) were also run simultaneously. From dose–response curves, the concentration of each compound required to cause 50% reduction in plaque numbers was determined [2]. For studying the type of interaction between drugs, the method of dose isobologram was used [2,23]. Dose–response curves for single drugs and for each level of the first drug (e.g., MMUdR-MP) in combination with various concentrations of the second drug (e.g., Ara-AMP) were drawn for each pair of antiviral drugs. The concentrations of various drugs in combination were multiples (0.25, 0.50, 1, 2, 4 times) of amounts required to cause 50% inhibition of PFU when used alone. From these curves, the amount of the second drug in combination required to give 50% reduction in PFU was determined. Fractional inhibitory concentrations (FIC) 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. FIC of each pair of drugs were then plotted to determine the nature of interaction. When the effects of two

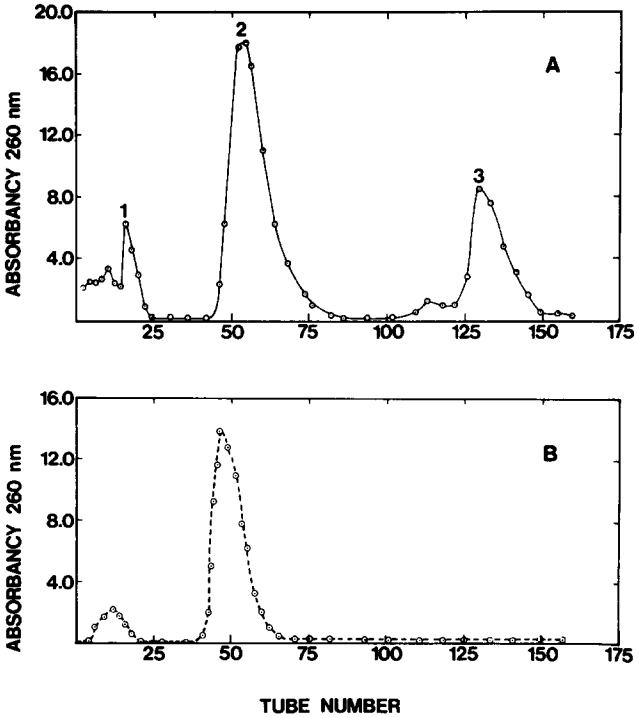


Fig. 1. Elution profile of a DEAE cellulose column. A. Phosphorylated reaction mixture was diluted with water, pH was adjusted to 7 and the solution (250 ml) was percolated through a column (3 × 29 cm) previously equilibrated with 0.05 M ammonium acetate. Elution was with the following ammonium acetate solutions: 250 ml of 0.05 M (tubes 1-26), 250 ml of 0.1 M (tubes 27-60) and 800 ml of 0.1-0.4 M (tubes 61-160). Fractions (9.6 ml) were collected and every fourth tube was monitored for absorbancy at 260 nm. Peak 1 (unreacted MMUdR plus side products); peak 2 (MMUdR-MP) and peak 3 (MMUdR-3', 5'-diphosphate). B. Rechromatography of peak 2. The contents of peak 2 (of A) were pooled together, lyophilized, reconstituted with water to a final volume of 250 ml and chromatographed on a DEAE-cellulose column using conditions listed under A.

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 right of this theoretical line suggest interference or antagonism; deviations to the left indicate synergism between two drugs.

Virus yield studies

Confluent monolayers in microtitre plates were infected with 10 PFU of HSV-1, strain 76. After 1 h of incubation at 37°, unadsorbed virus was aspirated, monolayers were washed once with Hank's medium and drugs were added. Antiserum was not included in the overlay. Earlier studies have shown that MMUdR in combination with Ara-A shows maximum synergistic activity in the ratio of 3:4 [2]. Therefore, these two

drugs were used in this ratio. In contrast, combination of MMUdR-MP and Ara-AMP was antagonistic, therefore the concentration of these drugs in combination experiments were arbitrarily fixed in the ratio of 1:1. In all experiments, virus control (virus and medium) and drug control (drug and medium) were also run simultaneously. After 72 h of incubation at 37°, the microtitre plates were kept at -70° for 2 h, then allowed to thaw at 22° and the medium from samples which had received similar treatment were pooled and titrated to determine the amount of virus present [2,3].

Inhibition of cell growth

The cytotoxic activity of the compounds was determined using RK-13 cells [2]. Experiments were also caried out to determine whether inhibition of cell growth was permanent or reversible. Normal controls (cells + medium + 10% FCS) and cells containing drugs at appropriate concentrations were incubated for 72 h at 37°. At the end of the incubation period, the medium was aspirated and the cells were washed twice with Hank's solution. Fresh growth medium was added and the tissue culture plates were incubated for 3 days. The cells were trypsinized, diluted with medium and counted using a model FN Coulter counter (Coulter Electronics Inc., Hialeah, FL, U.S.A.).

Results

Comparison of antiviral potency of MMUdR-MP and Ara-AMP

The antiviral activity against HSV-1 and HSV-2 was determined using RK-13 and Vero cells. Both compounds had significant antiviral activity against HSV-1 and HSV-2 (Table 1). However, the magnitude of antiviral activity was dependent on the cell lines used and differed for each compound. Dose–response curves for antiviral drugs against HSV-2 in Vero cells are shown in Figs. 2 and 3. MMUdR and MMUdR-MP inhibited replication of HSV-1 at lower concentrations than Ara-A and Ara-AMP in RK-13 cells. The antiviral activity of nucleotides was two- or three-fold greater than nucleosides against HSV-2, MS. On the other hand, higher concentration of phosphorylated compounds were required to inhibit HSV-2, X-265, particularly at higher multiplicities of infection. Ara-AMP was also more potent than Ara-A against HSV-1 strains NAH and MAC. The results of antiviral activity of Ara-A and Ara-AMP are in agreement with other workers [7,9,16,20]. MMUdR-MP showed moderate activity against EHV-1; whereas MMUdR was inactive. HSV-1^r (virus resistant to MMUdR) was susceptible to MMUdR-MP.

Inhibition of HSV-2 plaque formation by simultaneous treatment with two antiviral compounds

The interaction of MMUdR-MP and Ara-AMP and MMUdR and Ara-AMP was investigated by determining the amount of each drug alone and in combination

TABLE 1

Relative antiviral activity of MMUdR, MMUdR-MP, Ara-A and Ara-AMP against herpes viruses and vaccinia virus[a]

Virus	Amount of virus PFU[b]	Minimum inhibitory concentration[b] (μM)			
		MMUdR[c]	MMUdR-MP[c]	Ara-A[c]	Ara-AMP[b]
HSV-1 (76)	10	4	20	14	12
	50	7	40	30	14
HSV-1 (NAH)	10	30	16	120	46
	50	60	38	140	92
HSV-1 (MAC)	10	7	32	240	46
	50	15	32	240	92
HSV-1[r]	50	1176	90	12	–[d]
HSV-2 (X-265)	10	18	17	23	12
	50	30	57	38	87
HSV-2 (ATCC)	10	22	30	15	20
	50	132	90	70	65
HSV-2 (MS)	10	235	105	240	43
	50	470	150	240	80
EHV-1 (KYD)	10	>940[e]	90	15	24
	100	>940[e]	366	30	46
VV	10	>470[e]	>730[e]	15	6
	100	>940[e]	>730[e]	30	12

[a] Antiviral assays were carried out using RK-13 cells. All compounds were added immediately after virus infection.

[b] Concentration required to cause 50% reduction in plaque numbers.

[c] Minimum toxic dose (concentration required to produce definite evidence of microscopic toxicity) against confluent monolayers of RK-13 cells were: MMUdR, 3800 μM; MMUdR-MP, 800 μM; Ara-A, >960 μM and Ara-AMP, >740 μM.

[d] Not tested.

[e] Highest concentration tested.

required to cause 50% reduction in PFU of HSV-2 and the results are shown in Fig. 3. The effect of MMUdR-MP and Ara-AMP combination was less than for the individual compounds (Fig. 3A,B). In contrast MMUdR in combination with Ara-AMP resulted in greater inhibition of plaque formation (Fig. 3C). In order to study the nature of interaction, the dose isobolograms were drawn and the results are shown in Fig. 4. MMUdR-MP showed an antagonistic interaction in combination with Ara-AMP (Fig. 4A,B); whereas MMUdR in combination with Ara-AMP showed additive activity against HSV-2, strain MS (Fig. 4C).

Effect of antiviral drugs on HSV-1 replication (virus yield)

The concentration of each compound required to cause 50% reduction in log virus yield was: MMUdR (8.3 μM), Ara-A (75 μM), MMUdR-MP (79 μM) and Ara-AMP

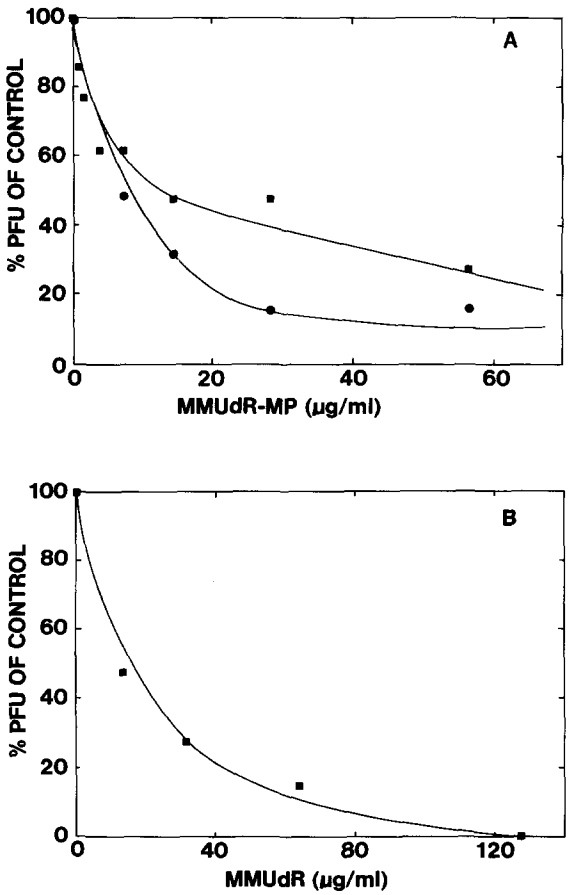
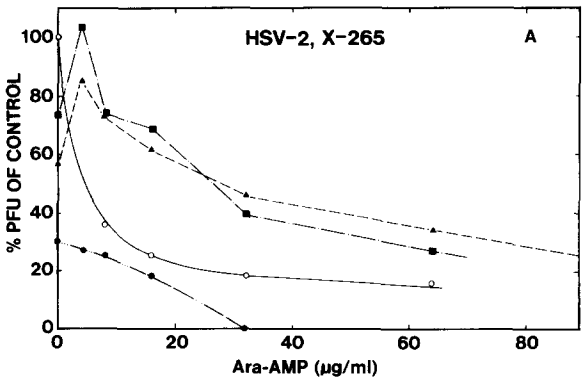


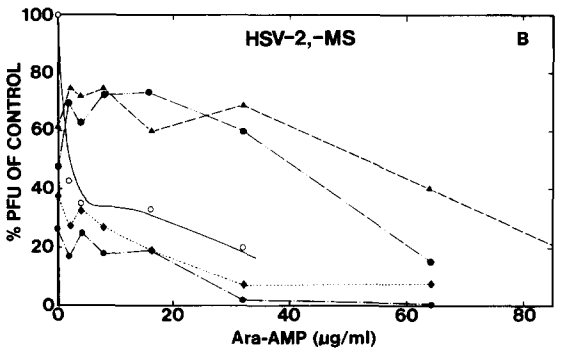
Fig. 2. Dose–response curves for antiviral drugs against HSV-2 in Vero cells. Amount of virus used was 10 PFU. A. MMUdR-MP against HSV-2 strain X-265 (●—●—●) and HSV-2 strain MS (■—■—■). B. MMUdR against HSV-2 strain MS.

(99 μM). In combination, MMUdR (4.9 μM) plus Ara-A (6 μM) and MMUdR-MP (52 μM) plus Ara-AMP (57 μM) caused a 50% reduction in log virus yield. These results show that a considerably enhanced antiviral effect resulted when MMUdR was used in combination with Ara-A. Similarly, a lower concentration of MMUdR-MP in combination with Ara-AMP was required to affect the same degree of virus yield reduction over the activity of these compounds when used individually. The relationship beteeen the dose of drugs and virus yield is shown in Fig. 5.

Effect of MMUdR, MMUdR-MP, Ara-A and Ara-AMP on rapidly growing RK-13 cells

Definite microscopic lesions were observed on exposure to MMUdR-MP and Ara-AMP at 450 and 90 μM, respectively. Previous studies have shown that the





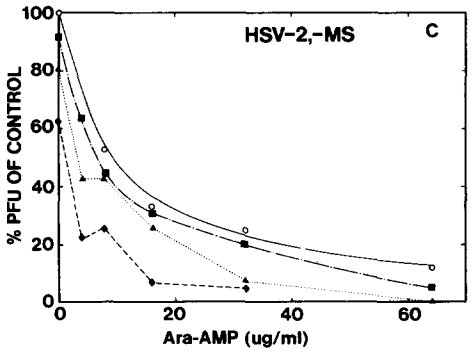


Fig. 3. Dose–response curves for MMUdR-MP and Ara-AMP combinations A. HSV-2 strain X-265 and B. HSV-2 strain MS. MMUdR-MP concentrations in combination with Ara-AMP were: zero ○——○, 3.5 μg/ml (■– · –■), 7.1 μg/ml (▲– –▲), 14.1 μg/ml (●– · – · –●), 28.3 μg/ml (◆ · · · ◆) and 56.1 μg/ml (●– · –●). The points lying on the response axis represent the percent PFU of control produced by the above MMUdR-MP concentrations in single-drug experiments performed concurrently with the combination experiments. C. Dose–response curves for MMUdR and Ara-AMP combinations against HSV-2 strain MS. MMUdR concentrations in combination with Ara-AMP were: zero (○——○), 8 μg/ml (■–·–■), 16 μg/ml (△ · · · △) and 32

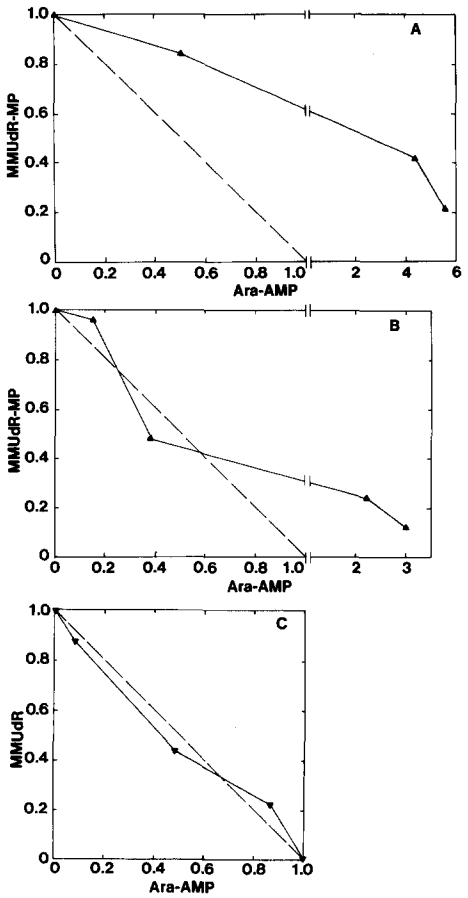


Fig. 4. Isobolograms for combinations of antiviral drugs. A. MMUdR-MP and Ara-AMP against HSV-2 strain X-265. B. MMUdR-MP and Ara-AMP against HSV-2 strain MS. C. MMUdR and Ara-AMP against HSV-2 strain MS. Antiviral assays were carried out using Vero cells. Amount of virus used was 10 PFU.

minimum toxic dose (concentration required to produce definite evidence of microscopic cytotoxicity) was 1020 and 240 μM for MMUdR and Ara-A, respectively [2]. These results indicate that the nucleotide analogs are considerably more toxic than the corresponding nucleosides. In order to determine whether toxic lesions induced by each drug were reversible or permanent, rapidly growing RK-13 cells were exposed to MMUdR (2500 μM), Ara-A (240 μM), MMUdR-MP (650 μM) and Ara-AMP (64 μM).

μg/ml (◆ · · · ◆). The points lying on the response axis represent the percent PFU of control produced by the above MMUdR concentrations in single-drug experiments performed concurrently with the combination experiments. Amount of virus used in all experiments was 10 PFU and antiviral assays were carried out in Vero cells.

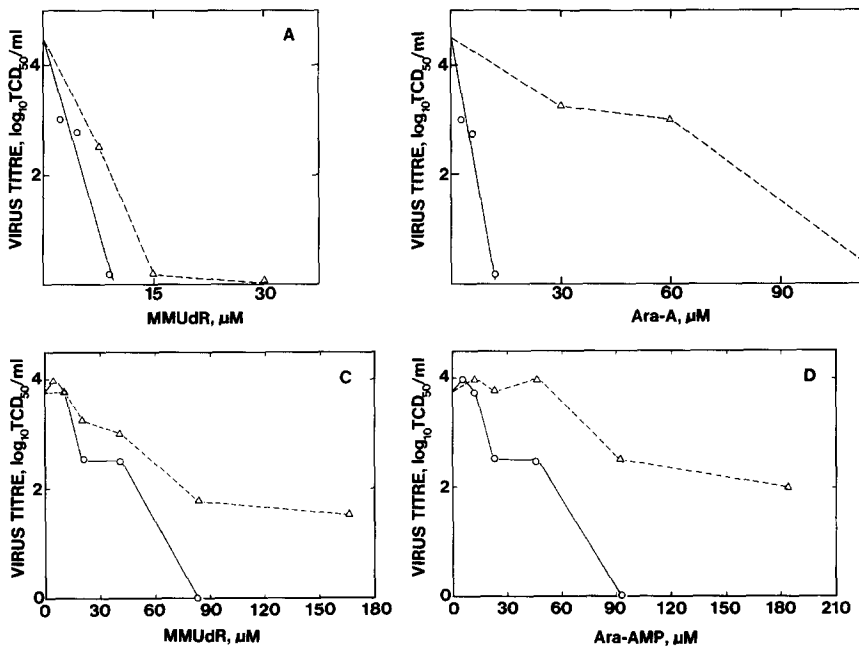


Fig. 5. Effect of antiviral drugs on HSV-1 strain 76 replication (virus yield). A. MMUdR △ · · · △); MMUdR + Ara-A ○——○. B. Ara-A △ · · · △; MMUdR + Ara-A ○——○. C. MMUdR-MP △ · · · △; MMUdR-MP + Ara-AMP ○——○. D. Ara-AMP △ · · · △; Ara-AMP + MMUdR-MP ○——○. In combination experiments, MMUdR and Ara-A were used in the ratio of 3:4; whereas MMUdR-MP and Ara-AMP were combined in the ratio of 1:1.1. The antiviral assays were carried out using RK-13 cells.

Cells incubated with MMUdR remained viable, and resumed almost normal growth after removal of this drug. Under similar conditions, surviving cells exposed to Ara-A and MMUdR-MP resumed partial growth; whereas cells treated with Ara-AMP failed to resume growth. These results suggested that MMUdR, Ara-A and MMUdR-MP toxicity was essentially reversible (Fig. 6).

Discussion

The most interesting observation was that the nucleotide analogs were potent inhibitors of HSV-2, MS. This virus strain is only marginally* susceptible to MMUdR and Ara-A. Another interesting finding was that HSV-1, strains NAH and MAC which are essentially resistant to Ara-A, were susceptible to Ara-AMP. The marginal activity of MMUdR against these herpes virus strains could be due to one of the

*Concentration required to cause 50% reduction in plaque number was > 200 μM.

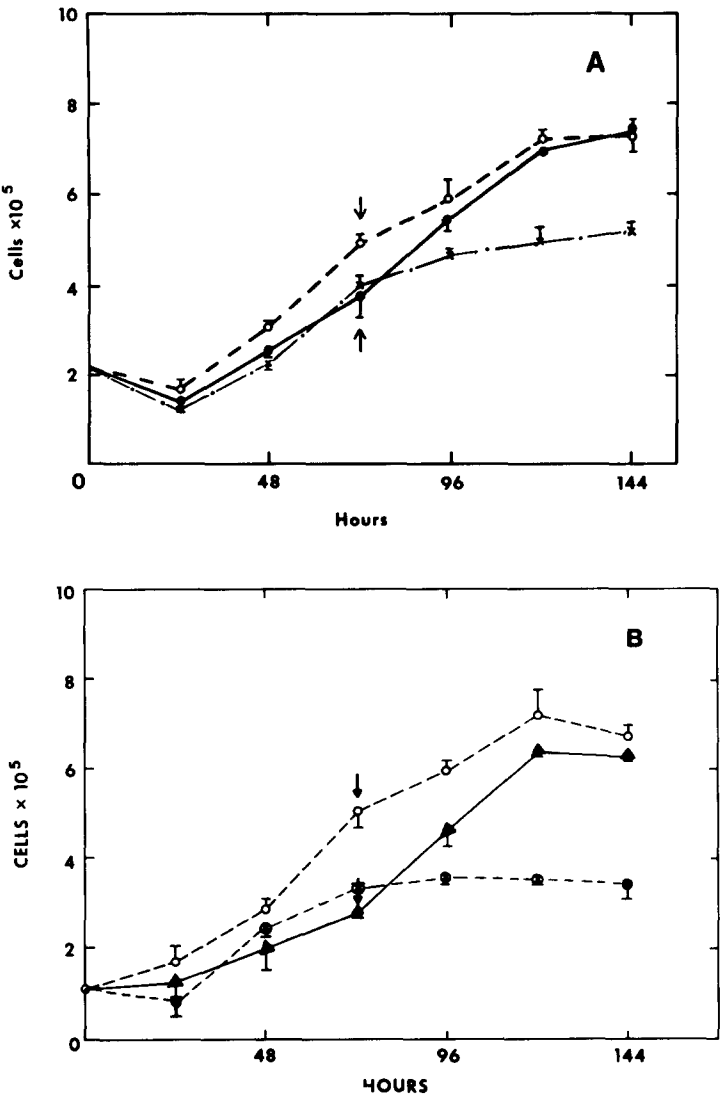


Fig. 6. Viability of rapidly growing RK-13 cells after incubation with drugs. A. Control ○ – – ○; MMUdR, 250 μM ●——●; MMUdR-MP, 650 μM, x – · – x. B. Control ○ – – ○; Ara-A, 240 μM, ▲——▲; and Ara-AMP, 65 μM, x – – x. Arrows indicate when cells were washed with Hank's medium for removal of drugs.

following reasons: (i) considerable decrease in the concentration or inability to induce viral kinase capable of phosphorylating MMUdR and (ii) further phosphorylation of the nucleotide to its active form, the 5'-triphosphate, does not occur and hence it fails to inhibit viral DNA synthesis. Since MMUdR-MP is active against these strains, it is logical to assume that lack of formation of triphosphate of MMUdR is

likely not responsible for its refractoriness. Studies are under way to elucidate mechanism(s) responsible for natural resistance to MMUdR. Ara-A has been characterized as having a 'multi faceted' mechanism of action [21]. Ara-A is incorporated into the DNA of cells and herpes viruses [13,16]. The compound, in its di- and tri-phosphate forms, is an inhibitor of ribonucleotide phosphate reductase [13] and of terminal deoxynucleotidyl transferase [15]. Ara-ATP has been shown to preferentially inhibit viral DNA-dependent DNA polymerase and this is generally believed to be responsible for its antiviral effect [13]. It is difficult to explain the low activity of Ara-A against these virus strains (HSV-1, NAH and MAC) because earlier studies have shown that adenosine or deoxyadenosine kinase activity (enzyme responsible for the phosphorylation of Ara-A) is not affected in primary rabbit kidney cells after infection with herpes simplex viruses [7].

Antagonistic interaction observed when MMUdR-MP was used in combination with Ara-AMP suggests that these nucleotides may be competing for the same transport mechanism for entry into the cell. This suggestion is further supported by the finding that when MMUdR was combined with Ara-AMP, results of interaction were additive. Further studies using labelled MMUdR-MP are necessary to determine if MMUdR-MP entered RK-13 cells intact and its metabolic fate after entry into the cell.

Earlier studies have shown that Ara-AMP enters cells slowly and its toxicity is expressed at a slower rate as compared to Ara-A. However, its toxic effects are considerably more lethal compared to the parent nucleoside [17]. Comparison of the toxicity of Ara-AMP and MMUdR-MP on RK-13 cells revealed that both these nucleotides inhibited growth. However, damage to cell metabolism incurred in the presence of Ara-AMP was irreversible. In the same system, surviving cells multiplied almost at the normal rate after removal of MMUdR-MP. Results presented on the effects of Ara-A and Ara-AMP are in agreement with other workers [13,17].

MMUdR-mutants can be isolated from infected cell cultures treated with this drug [1,25]. We have previously reported that MMUdR-resistant virus is susceptible to several other antiviral drugs [1]. The observation that MMUdR-MP inhibited replication of MMUdR-resistant virus is significant and suggests that its nucleotide may be useful for the treatment of infections due to MMUdR-resistant virus. Studies are presently in progress to determine the efficacy of MMUdR-MP in the treatment of herpes simplex infections.

We have earlier reported that combination of MMUdR with Ara-A resulted in synergistic inhibition of herpes simplex virus replication [2]. Simultaneous application of MMUdR and Ara-A in combination has been also found to be more effective than individual drugs for the treatment of keratitis [12] and herpes vaginitis (Ayisi et al., in preparation). The results of virus yield experiments further substantiate these results, and support the hypothesis that by selecting drugs whose mechanism of action differs, it is possible to achieve inhibition of viral replication at concentrations considerably lower than when these drugs are used alone.

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