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Efficacy of 5-methoxymethyl-2′-deoxyuridine in combination with arabinosyladenine for the treatment of primary herpes simplex genital infection of mice and guinea pigs

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Summary

The relative efficacy of 5-methoxymethyl-2′-deoxyuridine (MMdUrd), arabinosyladenine (ara-A) and the combination of MMdUrd and ara-A in the treatment of experimental genital herpes (GH) was investigated using mouse and guinea pig models. The infection was initiated by intravaginal inoculation using either HSV-2, strain X-265 or HSV-2, strain MS. Treatment was initiated 3 h post virus inoculation. The parameters used to evaluate efficacy were: (i) percent mortality; (ii) mean day of death; (iii) virus yield from the vaginal secretions; and (iv) mean lesion score. The simultaneous application of 5% MMdUrd and 5% ara-A was an effective treatment for controlling primary GH in both animal models. Combination chemotherapy was also effective in preventing recurrence of infection as well as the emergence of drug resistant virus. At 20% concentration, ara-A was effective in providing protection against GH. However, lesions due to recurrent GH appeared after cessation of treatment and the virus isolated from vaginal secretions of ara-A treated animals required higher concentration of drug for inhibition of virus replication in cell culture. 20% MMdUrd was only partially effective in controlling GH.

The production of infectious virus particles (virus yield) in cell culture after exposure to either ara-A of MMdUrd alone or in combination was determined. When MMdUrd and ara-A were used together, a substantially lower amount of each drug

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was needed to inhibit virus production completely and removal of drugs did not result in an increase in virus yield.

genital herpes; methoxymethyl-2'-deoxyuridine; ara-A; combination chemotherapy

Introduction

A wide variety of drugs have been tested for their antiviral activity against herpesvirus and a few have been licenced for use in humans (for reviews see Refs. 6,11,14,34,36,37). Although these drugs have brought some relief from genital herpes [6,11,14], keratitis [14,34] and other herpes virus infections [14,36,37], the search for selective antiviral drugs is being actively pursued [7,8]. An alternative to the development of new drugs is the possibility of using existing drugs in combination. The use of combination chemotherapy in the treatment of cancer and bacterial infections is well established. This approach, however, has only received moderate attention in antiviral chemotherapy [1,3–5,9,21,26,28,29,32,38].

In our laboratory, we have been studying the antiviral activity and biological properties of an antimetabolite, 5-methoxymethyl-2'-deoxyuridine (MMdUrd, [1–4,12,21]). These studies have shown that MMdUrd in combination with arabinosyladenine (ara-A) shows synergistic activity against herpes simplex viruses in cell culture [1] and is considerably more effective in reducing the production of infectious virus particles of HSV-1 as compared to individual drugs [2]. Interestingly, synergistic activity was achieved without concurrent increase in cytotoxicity. The simultaneous application of 2% MMdUrd and 2% ara-A was found to be effective for the treatment of keratitis [21]. Of particular significance was the finding that there were no therapeutic failures when drugs were used in combination; whereas 5–10% treatment failures were recorded with the use of 5% MMdUrd or 5% ara-A. Thus potential benefits expected from use of antiviral drugs in combination which show synergistic interaction are: (i) better therapeutic response for the treatment of herpes simplex infections; (ii) decreased chances for the emergence of drug resistant mutants during treatment; and (iii) possibly reduced chance of toxicity to host. This communication describes results of efficacy trials of MMdUrd and ara-A alone and in combination for the treatment of primary genital herpes infection of mice and guinea pigs.

Materials and Methods

Drugs

Trifluorothymidine (F_3dThd), arabinosyl adenine (ara-A), and phosphonoformate (PFA) were purchased from Sigma Chemicals Co., St. Louis, MO. Acyclovir (ACV) was obtained from Burroughs Wellcome, Research Triangle, NC. 5-Methoxymethyl-2'-deoxyuridine-5'-monophosphate (MMdUrd-MP) and MMdUrd were synthesized [3,12]. Molecular weights of these drugs are: ACV, 225; ara-A, 267; F_3dThd, 296; MMdUrd, 273; MMdUrd-MP, 353; and PFA, 192. For antiviral assay in cell culture,

all compounds were dissolved at the required concentration in Eagle's minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, NY) and filter-sterilized immediately before use. Derma base was purchased from Borden Company, Don Mills, Ontario, Canada. For in vivo studies, MMdUrd dissolved in water and ara-A in suspension were incorporated in derma base to provide the desired concentrations.

Animals

Swiss Webster mice (6–8 weeks old) and female albino guinea pigs (Hartley strain, weighing about 200 g) were obtained from the Animal Resources Centre, University of Saskatchewan. 10-day pregnant mice were randomly selected and five animals were transferred to each cage. The guinea pigs were randomly divided and placed two per cage. The infected animals were housed in a special cabinet (HEPAIRE, Model No. DAE-18, Canadian Cabinets, Ottawa, Canada) designed for infectious disease studies. All animals were allowed free access to standard commercial pelleted diets and tap water in bottles (Vitamin C was added for the guinea pigs) with stainless steel sipper tubes.

Cell culture

RK-13 (rabbit kidney) cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) as previously described [2,4]. Confluent monolayers were prepared by seeding 5×10^4 cells into each well of a microtitre tissue culture plate (No. 3040; Falcon Plastics, Oxnard, CA). The cultures were incubated at 37°C in a humidified CO_2 (5%) atmosphere, and the monolayers were confluent within 24 h.

Viruses

HSV-2, strain X-265 and strain MS were kindly provided by Dr. V. Pavilanis, Armand-Frappier Institute, Montreal, Canada. The conditions for the preparation of virus stocks and antibodies have been described [1].

Drug inhibition assay

(i) Plaque reduction assay. For antiviral assays, confluent monolayers were infected with 10 or 50 plaque forming units (PFU) of virus per well in a microtitre plate as described previously [1]. Each antiviral compound, at the appropriate concentration, dissolved in MEM containing 1 or 2 units of antibody against HSV-2 and 4% FBS, was added to respective wells. Plaques were allowed to develop for 72 h before fixation, staining and enumeration [1–3]. In each experiment, toxicity controls (containing test compound and medium only), cell controls (containing medium only), and virus control (containing virus and medium only) were run simultaneously. From dose response curves, the concentration of each compound required to reduce the number of plaques by 50% was determined [1–3].

(ii) Virus yield studies. The reduction in virus yields in the presence of individual drugs and in combination was determined according to the procedure described earlier [2,3]. In combination chemotherapy experiments, MMdUrd and ara-A were used in the ratio of 4 : 3. These concentrations were chosen because MMdUrd in combination with ara-A shows maximum synergistic activity at this ratio [1]. Antiserum was

omitted from the overlay. The medium from samples which had similar treatments were pooled and titrated to determine the amount of virus present. The assay for each drug was carried out in quadruplicate. In all experiments, virus control (virus and medium) and drug control (drug and medium) were also included.

Experimental design

(i) Virus. HSV-2 strains X-265 and MS were used for GH infection. The choice of these two strains was based on the fact that low concentrations of either MMdUrd or ara-A inhibited the replication of HSV-2 strain X-265 in cell cultures; whereas the MS strain was relatively resistant to these drugs [1].

(ii) Initiation of infection. Animals were lightly anesthetized with ether and inoculated intravaginally with 50 μl of virus suspension containing either 2.5×10^5 PFU (mice) or 3.75×10^3 PFU (guinea pigs) of HSV-2 strain X-265. The dose of HSV-2, strain MS was 3.75×10^3 PFU for guinea pigs. An 18 gauge polyethylene catheter attached to a tuberculin syringe was used for inoculation of virus.

(iii) Symptoms of genital herpes in mice and guinea pigs. In both animal models, the first clinical signs of GH infection were erythema, slight swelling and a few vesicles. The infection then progressed to severe erythema, severe swelling, and increased number of vesicles by day 5 to 7. This was followed by coalescing of vesicles, sores, alopecia and partial resolution of lesions in animals that survived beyond day 7 post-infection. In the majority of animals infected with strain X-265, posterior paralysis began on day 5 post-infection with subsequent inability to defecate and urinate. In contrast, posterior paralysis did not occur with strain MS. Perivaginal alopecia was commonly observed in animals inoculated with HSV-2, strain X-265 and was more prominent in mice than in guinea pigs. Recurrence of infection was frequently observed within 30 days in animals infected with strain MS but was not seen in guinea pigs inoculated with strain X-265. Rectal prolapse also occurred in approximately 20% animals infected with strain MS. The symptoms of GH in mice were similar to those reported earlier [16,23].

(iv) Evaluation. Each animal was examined daily for perivaginal alopecia, erythema, swelling, vesicles, sores or ulcers and scabs, paralysis, bleeding and mortality. Alopecia, erythema and swelling were graded from 0 to 4+: 0 (normal), 1+ (slight), 2+ (moderate), 3+ (severe), and 4+ (very severe). For vesicles, grading from 0 to 5+ was used: 0 (no vesicles), 1+ (1 or 2), 2+ (3–5), 3+ (6–8), 4+ (9–12) and 5+ (>12). The scores of these parameters were added together to obtain total lesion score for each animal. The mean lesion score for each group versus days post-treatment was plotted. All scoring and grading was done by the same person for each experiment.

Treatment

For each treatment, animals were lightly anesthetized and 0.05 ml of placebo or drug at the desired concentration was instilled intravaginally using a catheter (18 gauge polyethylene) attached to a tuberculin syringe. An additional amount of drug (0.05 ml) or placebo was also smeared on the external genitalia at each time of treatment. In combination chemotherapy experiments, both MMdUrd and ara-A at desired concentrations were combined into a single preparation. All medications were supplied under

code by the Pharmacy Section, Western College of Veterinary Medicine, University of Saskatchewan. The code was broken after completion of efficacy trials in both animal models. In each experiment, virus control (virus only), placebo (virus and vehicle only) and toxicity controls (drugs only) were run simultaneously. Treatment was initiated 3 h post virus inoculation. Treatment was carried out twice daily (0900 h and 2100 h) for 6 days and once daily (0900 h) for 4 more days.

Assay for virus in vaginal secretions

On days 1, 3, 5 and 7 after virus inoculation, vaginal swabs were taken and placed in 1.0 ml of MEM. Virus titre was determined within 3 h after the swabs were taken. To minimize the interference with virus isolation by residual quantity of drug that may have been present in the samples, two approaches were taken: (i) all swabs were collected 12 h post-inoculation of drugs, and (ii) after infection the indicator cells were washed with medium. Stock solution of virus was serially diluted with MEM and virus yield was determined by infecting RK-13 monolayer cells. The infected cells were incubated for 3 days before staining and enumeration of virus titres [1].

Sensitivity of virus isolated from vaginal secretions to some antiviral drugs

Virus from guinea pigs which failed to respond to treatment, was grown, passaged once in cell culture, titrated, and used for drug sensitivity testing. The sensitivity of each virus isolate to ACV, ara-A, F_3dThd, MMdUrd, MMdUrd-MP, and PFA was determined [1].

Plaque isolation and sensitivity testing of HSV-2, strain MS

Confluent monolayers in a microtitre plate were infected with approximately 10 PFU/well of HSV-2, strain MS. After 72 h of incubation at 37°C and 4% CO_2, the wells containing only 1 plaque were harvested, and each plaque was passaged once to obtain enough virus stock for drug sensitive testing. Drug sensitivity on each plaque isolate was performed as described previously [1].

Statistics

The data for mean day of death was analyzed by the Mann–Whitney U-test. The data for mortality was analyzed by the Fisher exact test and chi square ($P<0.05$). Analysis of variance (ANOVA) and least significant difference (LSD) tests ($P<0.05$) were used for the evaluation of virus yield and mean lesion score data.

Results

Clinical manifestations of primary herpes genitalis

The clinical symptoms of GH in guinea pigs after vaginal inoculation by HSV-2, strains X-265 and MS are quite different (see under symptoms of genital herpes). On the basis of these results, it appears that strain X-265 is considerably more neurotropic

than MS. In contrast, strain MS appears to be more epithelotropic than strain X-265. The symptoms of GH in mice were similar to those reported earlier [16,25]. Our results of GH pathogenesis in guinea pigs are similar to recent reports [15,33] but are at variance with earlier reports [18,19]. The reasons for this discrepancy are not clear at this time.

Comparative efficacy of MMdUrd, ara-A and combination of MMdUrd and ara-A against primary genital herpes

(i) Mice. MMdUrd and ara-A at 5%, 10% and 20% concentration and the combination of 5% MMdUrd and 5% ara-A in derma base was applied twice daily. There were no significant differences in final mortality and the mean day of death between animals treated with drugs and the controls (Table 1). All mice that survived the infection and those that delivered before paralysis, had normal delivery with approximately 10% giving birth to dead fetuses. Mice paralysed prior to delivery could not deliver and the fetuses died in utero. The mean lesions scores (MLS) were significantly lower for 10% ara-A, 20% ara-A and the 5% MMdUrd and 5% ara-A combination treatments compared to the placebo. Comparison of the daily mean lesion scores for different treatments is shown in Fig. 1A. The combination of 5% MMdUrd and 5% ara-A almost completely prevented progression of primary genital herpes and development of lesions and this treatment was as effective as 20% ara-A.

TABLE 1

Comparative efficacy of different treatment regimens on mortality, mean day of death, virus yield and mean lesion scores against primary genital herpes in pregnant mice using HSV-2, strain X-265[a]

Treatment[b]	Day of death (mean)	Mortality (%)	Virus yield[c] (geometric mean)	Mean lesion score[d]
Virus (control)	10.0	100	2.4 ± 0.1	4.7 ± 0.5
Control (virus + placebo)	10.4	88	2.4 ± 0.2	3.8 ± 0.7
5% MMdUrd	10.4	100	1.9 ± 0.4	3.7 ± 0.7
10% MMdUrd	11.1	88	1.7 ± 0.2	3.6 ± 0.6
20% MMdUrd	12.4	88	1.6 ± 0.2	3.5 ± 0.6
5% ara-A	11.0	88	1.6 ± 0.2	2.9 ± 0.5
10% ara-A	14.0	63	1.5 ± 0.2	1.8 ± 0.4
20% ara-A	14.4	63	1.4 ± 0.1	1.1 ± 0.2
5% MMdUrd + 5% ara-A	12.3	50	1.4 ± 0.1	1.2 ± 0.2

[a] Infection was initiated by instillation of 50 μl of virus (2.5×10^5 PFU/ml) in the vagina. This virus dose produced 100% infectivity. Eight mice per group.

[b] Treatment was initiated 3 h post inoculation of virus.

[c] Virus shed ($\log_{10}$ $TCID_{50}$/ml ± S.E.) in the genital tract secretions. Data were analyzed by ANOVA and LSD ($P<0.05$). LSD, $\bar{d} = 0.5$.

[d] Average of the total daily mean lesion scores ± S.E. Data were analyzed by ANOVA and LSD ($P<0.05$). LSD, $d = 1.0$.

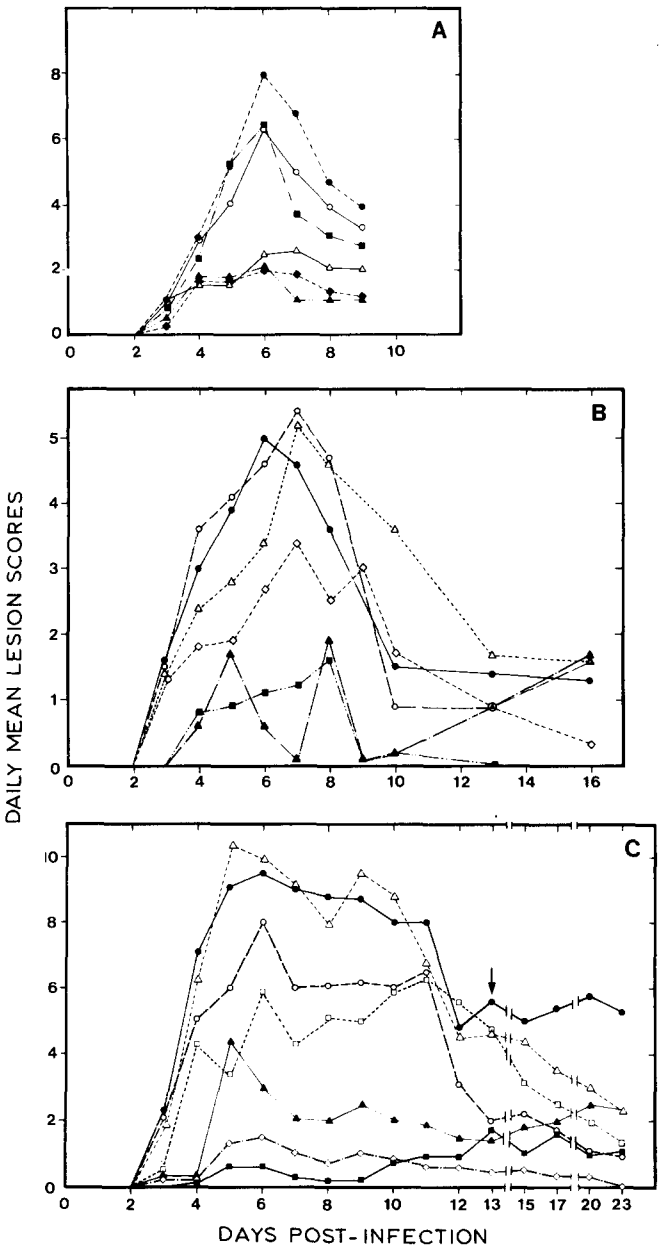

Fig. 1. Effect of various treatment regimens on experimental herpes simplex genitalis in pregnant mice and guinea pigs. Treatment was initiated 3 h after virus infection. (A) Pregnant mice were inoculated intravaginally with 50 μl of 2.5 × 10^3 PFU/ml of HSV-2, X-265. Virus control (●...●), placebo (○——○), 10% ara-A (△——△), 20% ara-A (♦---♦), 20% MMdUrd (■-·-■) and 5% MMdUrd plus 5% ara-A (▲-··-▲). (B) Guinea pigs were inoculated intravaginally with 50 μl of 3.75 × 10^3 PFU/ml of HSV-2, X-265. Virus control (●——●), placebo (○---○), 5% ara-A (□---□), 20% ara-A (▲- . -▲), 20% MMdUrd (△---△) and 5% MMdUrd plus 5% ara-A (■-···-■). (C) Guinea pigs were inoculated intravaginally with 50 μl of 3.75 × 10^3 PFU/ml of HSV-2, MS. Virus control (●——●), placebo (○---○), 5% ara-A (□---□), 20% ara-A (■——■), 5% MMdUrd (△---△), 20% MMdUrd (□---□) and 5% MMdUrd plus 5% ara-A (◇-·-◇).

(ii) Guinea pigs. The results of mean lesions scores are shown in Figs. 1B and C and the other parameters are summarized in Table 2. Treatment with the simultaneous application of 5% MMdUrd and 5% ara-A was also very effective in arresting the progression of GH by both virus strains. Under similar conditions, treatment with 5% ara-A was partially effective; whereas application of 20% ara-A was almost as effective as the combination of drugs. In contrast, treatment with MMdUrd was not effective. However, it was able to prolong the mean day of death of animals inoculated with HSV-2, strain X-265. Mortality in guinea pigs infected with HSV-2, X-265 was also preceded by paralysis in a manner similar to that observed in mice.

Lesions often reappeared within a week to ten days after treatment with MMdUrd or ara-A was stopped in animals infected with strain MS. Interestingly, treatment with the simultaneous application of 5% MMdUrd and 5% ara-A prevented recurrence of infection during the 60-day observation period. In contrast, lesions due to recurrent infection appeared in all other treatment groups from day 13 onward post-infection. The recurrent infections were generally milder and of shorter duration than the primary infection.

Effect of drug treatment on viral shedding in the genital tract

In both animal models, viral shedding from the vagina was detectable 24 h after virus inoculation and animals continued to shed virus until day 5 post-infection. MMdUrd significantly reduced the amount of virus shed in the genital secretions of mice but only had a slight effect on virus shedding in the genital secretions of guinea pigs. In contrast, 5% ara-A, 20% ara-A and the combination of 5% MMdUrd plus 5% ara-A significantly decreased the virus yield from the vaginal secretion in both animal models, infected with HSV-2, strain X-265. However, none of the treatments had a significant effect on virus shedding if the infection was initiated with HSV-2, strain MS. The results of virus yield data are shown in Tables 1 and 2.

Susceptibility of virus isolated from vaginal secretions of guinea pigs to antiviral drugs

In order to determine whether treatment with these antiviral drugs resulted in an alteration of the susceptibility of the virus to each drug, the sensitivity of virus isolated from placebo and drug treated animals was assayed using MMdUrd and ara-A. Furthermore, viruses were analyzed for cross-resistance to four other antiviral agents (ACV, F_3dThd, MMdUrd-MP and PFA), in order to determine whether resistance to one drug would also alter susceptibility to other antiviral drugs. The concentration of each compound that inhibited 50% of plaque formation (ID_{50}) is presented in Table 3. The virus obtained from guinea pigs treated with 20% MMdUrd required 2–4-fold higher concentrations of ara-A, F_3dThd, MMdUrd and MMdUrd-MP. Similarly, the virus isolated from animals treated with 20% ara-A exhibited approximately 2–5-fold resistance to ara-A, MMdUrd, MMdUrd-MP and PFA, but remained sensitive to ACV and F_3dThd. In contrast, the susceptibility of virus isolated from guinea pigs treated with the combination of 5% MMdUrd plus 5% ara-A was not appreciably altered to any of the drugs.

Earlier studies have shown that drug-resistant variants of HSV arise randomly at

TABLE 2

Comparative efficacy of different treatment regimens on mortality, mean day of death, virus yield and mean lesion scores against experimental primary genital herpes in guinea pigs[a]

Treatment[b]	Number of animals	Day of death (mean)	Mortality (%)	Geometric mean virus yield[c]	Mean lesion scores[d]
HSV-2, Strain X-265:					
Virus control	20	10.6	25	2.3 ± 0.1	2.9 ± 0.4
Placebo	20	12.5	30	2.4 ± 0.1	3.0 ± 0.5
20% MMdUrd	14	20.0	29	2.2 ± 0.2	3.1 ± 0.4
5% ara-A	10	16.0	10	1.5 ± 0.2	1.0 ± 0.2
20% ara-A	8	24.0	14	1.3 ± 0.1	0.8 ± 0.2
5% MMdUrd + 5% ara-A	13	-	0	1.3 ± 0.1	0.6 ± 0.2
HSV-2, Strain MS:					
Virus control	12	10.5	17	2.1 ± 0.2	6.8 ± 0.5
Placebo	12	9.8	42	2.1 ± 0.2	4.2 ± 0.6
5% MMdUrd	8	12.5	12.5	1.9 ± 0.2	6.2 ± 0.8
20% MMdUrd	8	12.5	25	1.8 ± 0.2	4.9 ± 0.4
5% ara-A	8	-	0	1.6 ± 0.2	2.0 ± 0.3
20% ara-A	8	6	12.5	1.8 ± 0.2	0.7 ± 0.1
5% MMdUrd + 5% ara-A	12	-	0	1.7 ± 0.2	0.6 ± 0.1

[a] Infection was initiated by instillation of 50 µl of virus (3.75×10^3 PFU/ml) of either strain X-265 or MS in the vagina. The level of infectivity was: strain X-265 (70%) and strain MS (100%).

[b] Treatment was initiated 3 h post-inoculation of virus.

[c] Virus shed ($\log_{10}$ $TCID_{50}$/ml) ± S.E. in the genital tract secretions. Data were analyzed by ANOVA and LSD ($P<0.05$). LSD, $\bar{d}=0.5$ and 0.7 for strains X-265 and MS, respectively.

[d] Average of the total daily mean lesions scores ± S.E. Data analyzed by ANOVA and LSD ($P<0.05$). LSD, $\bar{d}=1$ and 1.3 for strains X-265 and MS, respectively.

TABLE 3

Relative in vitro sensitivities to antiherpes drugs of virus isolated from the vaginal secretions[a] of guinea pigs given different treatments

Drug[c] (μM)	Stock X-265[d]	Treatments[b]			
		Placebo	MMdUrd	ara-A	MMdUrd + ara-A
MMdUrd	18	26	88	59	29
MMdUrd-MP	17	20	110	54	25
ACV	0.4	0.2	0.6	0.1	0.2
F_3dThd	0.2	0.1	0.8	0.1	0.4
ara-A	23	20	56	100	24
PFA	5	10	21	57	23

[a] Infection was initiated by instilling 50 μl containing 3.75×10^3 PFU/ml of HSV-2, X-265 into the vagina of guinea pigs. Virus was isolated on day 5 post-infection.

[b] Treatment was initiated 3 h post-infection. Placebo (derma base).

[c] Concentration (μM) required to cause 50% reduction in plaque development. Amount of virus used was 10 PFU/culture. Antiviral assays were carried out using RK-13 cells.

[d] No treatment.

low frequency, and are always present in a wild type virus population [10,25]. To determine whether emergence of mutants due to drug selection was a factor in treatment failures, a number of plaques of HSV-2, strain MS were cultured in the presence of either MMdUrd or ara-A. The results showed that in virus stocks, virus particles with a wide range of sensitivities are present. Considerable variation in susceptibility (ID_{50}, range 60 μM to >941 μM) to MMdUrd was noticed. Furthermore, ID_{50} of MMdUrd for 50% of the isolates was 2–5-fold higher than ara-A. In contrast, there was less variation in sensitivity to ara-A (ID_{50} range 30–187 μM). For the majority of plaques (16/20), ID_{50} for ara-A was 60 μM or lower. For three isolates (plaques No. 4, 10 and 12) increased resistance to both drugs was evident. ID_{50} values for plaques 4, 10 and 12 were: MMdUrd, 235, 176 and >941 μM and ara-A, 105, 150 and 187 μM respectively. Interestingly, the replication of even these highly resistant isolates was inhibited by each drug at considerably lower concentration when used together. For example, 50% inhibition of plaque formation was observed at 22 μM of ara-A and 58 μM of MMdUrd against isolates 4 and 10 and 88 μM of ara-A and 58 μM of MMdUrd for isolate 12. During treatment with individual drugs, the more resistant isolates are likely to continue to replicate and this may have been responsible for the marginal efficacy of MMdUrd and recurrence of infection observed after ara-A treatment.

Effect of MMdUrd, ara-A and combination of MMdUrd and ara-A on virus production in vitro

One possible reason for recurrence of infection could be that MMdUrd and ara-A when used alone failed to inhibit completely replication of virus; whereas when these

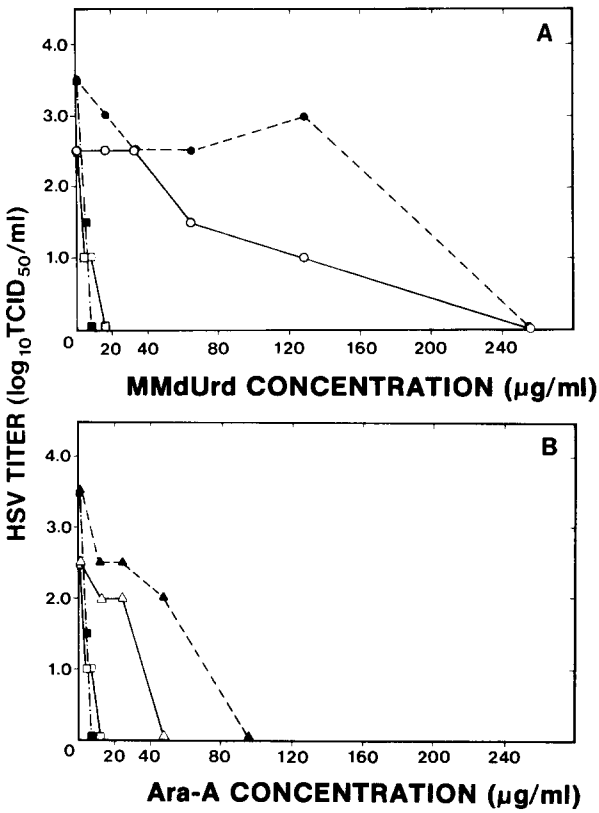

Fig. 2. Effect of antiviral drugs singly and in combination on infectious virus particles yield. (A) MMdUrd (○——○), MMdUrd + ara-A (□——□) after incubation of infected cells for 72 h. After washing of drugs and incubation of cells with fresh maintenance medium for an additional 72 h: MMdUrd (●---●), MMdUrd + ara-A (■-·-■). (B) ara-A (△——△), ara-A + MMdUrd (□——□) after incubation of infected cells for 72 h. After washing of drugs and incubation of cells with fresh maintenance medium for an additional 72 h: ara-A (▲. . .▲), ara-A + MMdUrd (■- . -■). In combination chemotherapy experiments MMdUrd and ara-A were used in the ratio of 4 : 3. The amounts used were: MMdUrd (4, 8, 16, 32 and 64 μg/ml) and ara-A (3, 6, 12, 24 and 48 μg/ml).

drugs were used simultaneously they prevented production of infectious virus particles. To investigate this possibility, the production of infectious virus centres (virus yield) in cell culture after exposure to either MMdUrd or ara-A alone or in combination was determined and the results are shown in Fig. 2. Two interesting observations were made: (i) considerably higher concentration of individual drugs were needed to completely inhibit viral replication and removal of drugs resulted in subsequent viral replication; and (ii) when MMdUrd and ara-A were used together, a substantially lower amount of each drug was needed to inhibit virus production completely and removal of antiviral drugs did not result in an increase in virus yield.

Discussion

Two experimental models of genital herpes (GH) were used to evaluate the efficacy of antiviral drugs because the pregnant mouse, like the pregnant woman is more susceptible to GH [24] and the pathological features of the acute and recurrent GH in female guinea pigs more closely resemble the symptoms of GH in humans [15,18,19,33]. Therefore, the use of two experimental models of GH to test the efficacy of antiviral drugs has benefits, in that although the development of the disease in each animal model is not identical to that in humans, different features of both models do simulate human infections. In the present study, two virus strains used were found to have different pathogenic features as well as different drug sensitivities. Even though the guinea pig reflects the pathological features of GH in women, symptoms of progressive paralysis observed when strain X-265 was used are of rare occurrence in GH patients. Complications such as aseptic meningitis, urinary retention and sacral radiculopathy are observed in patients with primary GH [6] and in this respect, GH symptoms after inoculation with HSV-2, X-265 in these models to some extent resemble clinical symptoms of GH in humans. In contrast, infection with HSV-2, strain MS provides the opportunity for observing the effect of antiviral therapy on recurrent GH. Thus it appears, when using animal models for evaluating the efficacy of antiviral drugs which have potential for use in humans, the choice of the virus strain used for infection is as important as the animal model itself.

The most significant finding in these experiments was the potent inhibitory effect of the 5% MMdUrd and 5% ara-A combination in the treatment of primary GH. Combination chemotherapy was also effective in preventing recurrent infection of GH. One possible reason for the better efficacy of combination chemotherapy may be that this treatment regimen (unlike the single drugs) was effective in preventing the emergence of resistant virus. Most studies to date indicate that the best explanation for the emergence of drug resistant mutants to antiviral drugs is due to selection of the naturally resistant subpopulation of the virus in the presence of drug [10,20,25]. Results of the sensitivity of virus clones to MMdUrd and ara-A also support the fact that drug selection may indeed be a major contributing factor in the emergence of drug resistant variants, observed following antiviral therapy with single drugs. Interestingly, only a very small amount of each drug was needed to inhibit replication of even these highly resistant clones when they were used together suggesting that chances for the emergence of drug resistant mutants due to the process of drug selection are considerably less using this therapeutic regimen. The other possible reason for better therapeutic response when MMdUrd and ara-A are used together, is that the production of infectious virus particles was prevented due to the synergistic interaction between these drugs.

The selective antiviral activity of MMdUrd results from its phosphorylation by herpes virus induced pyrimidine deoxyribonucleoside kinase [35]. 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., unpublished results). Ara-A has been characterized as having a 'multifaceted' mechanism of action [20,22,23,30,31]. The compound, in its di- and triphosphate forms, is an inhibitor of

ribonucleotide phosphate reductase and of terminal deoxynucleotidyl transferase [23]. Ara-ATP has been shown to inhibit preferentially HSV-specified DNA polymerase and this has been suggested as the basis for selective antiviral activity of this drug [23,30]. Thus enhanced antiviral activity observed when MMdUrd and ara-A were used together is most likely due to multiple sites of action of these drugs on the virus.

Despite the fact that MMdUrd and ara-A were almost equipotent in inhibiting proliferation of virus in cell culture [1], only ara-A was effective in altering the clinical course of GH. One interesting observation was that the development of secondary lesions in animals treated with ara-A (when occurred) was delayed. Our results on the efficacy of ara-A in guinea pigs are in agreement with a recent report [27]. However, our data on the efficacy of ara-A in mice does not agree with other published results [16,27]. The only possible explanation is that effective levels of drug were achieved at the site of HSV replication in the genital tissues using this formulation of ara-A. The possible reasons for the marginal efficacy of MMdUrd for the treatment of GH are: (i) inadequate absorption from the epithelial layers of the vagina so that adequate concentrations of the drug were not achieved intracellularly where the virus was replicating; (ii) rapid emergence of drug-resistant mutants because of exposure of virus to low level of drugs; and (iii) reversal of its antiviral activity by high local concentration of endogenous thymidine in the cervix and vaginal secretions. The antiviral activity of acyclovir is reduced because of antagonism due to high concentration of thymidine in mouse vagina [13]. Similarly, if one assumes that a high concentration of thymidine is also present in the guinea pig vagina, the reversal of antiviral activity of MMdUrd would result. It will be interesting to determine levels of thymidine concentration in the vaginal secretion of guinea pigs to support this contention. Further studies on the absorption of these drugs from the vagina are planned after synthesis of labelled drugs. We are also in the process of synthesizing lipophilic derivatives of MMdUrd and their efficacy in the treatment of primary GH infections will be investigated.

In conclusion, we have shown that simultaneous application of 5% MMdUrd and 5% ara-A was effective in the treatment of primary GH. This therapeutic regimen was also effective in preventing recurrent GH, and the emergence of drug resistant virus. It is conceivable that the prevention of recurrences was due to the inhibition of viral replication by early treatment of the primary GH infection, thereby preventing seeding of the ganglia. Thus, the combination of ara-A plus MMdUrd should be considered for use in the treatment of primary GH in human patients.

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