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ANTI-HERPESVIRUS ACTIVITY OF ADENINE ARABINOSIDE ANALOGUES IN TISSUE CULTURE AND A GENITAL INFECTION OF MICE AND GUINEA PIGS*

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Four analogues of adenine arabinoside (ara-A) were compared for activity against herpes simplex virus (HSV) in tissue culture and in a genital infection of mice and guinea pigs. These analogues, 5′-monophosphate (ara-AMP), 5′-valerate ester (ara-AV), 2′,3′-diacetate ester (ara-ADA), and 2′,3′,5′-triacetate ester (ara-ATA) have greater water and lipid solubility and resistance to deamination than ara-A. In mouse embryo fibroblast cells, similar viral inhibitory levels were noted with ara-A, AMP, and ara-AV, while ara-ADA and ara-ATA were 6–10 times less active. In mice infected intravaginally with HSV type 2 (HSV-2), intravaginal treatment with 10% concentrations of each of the compounds beginning 3 h after viral challenge, had no effect on infection rates, titers of virus in vaginal secretions, mortality rates or the mean day of death as compared with placebo-treated controls. In the HSV-2 genital infection of guinea pigs, treatment with 10% vaginal creams or placebo vehicle was initiated 6 or 24 h after viral inoculation. In animals treated at 6 h with ara-A, ara-AMP and ara-AV, there was complete inhibition of viral replication in the vaginal tract and development of external genital lesions. When treatment with these three drugs was delayed 24 h after infection, there was no effect on vaginal virus titers, but lesion severity was reduced by ara-A or ara-AMP therapy. Ara-ATA was ineffective whether begun at 6 or 24 h. The greater solubility in water and lipid as well as the resistance to deamination of ara-AMP and ara-AV did not appear to enhance their antiviral activity over that of ara-A. Additionally, ara-ADA and ara-ATA exhibited less activity both in tissue culture and in the experimental genital infections.

adenine arabinoside analogues genital herpes herpes simplex virus

INTRODUCTION

Systemic administration of adenine arabinoside (ara-A) is effective in the treatment of herpes encephalitis [27], herpes zoster in immunosuppressed patients [26], and in neonatal herpes [28]. Topical ara-A therapy is highly effective in the treatment of herpes keratitis in both experimental animals [17,23] and man [6,16]. In contrast, topical

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ara-A or ara-AMP therapy has not been effective in the treatment of experimental mucocutaneous herpes simplex virus (HSV) infections of animals [2,5,9,11] or against oral or genital HSV infections of humans [1,7,25]. It has been assumed that the failure of topical ara-A in the treatment of cutaneous HSV infections was due to its lack of penetration into the cells of the epidermis where HSV replication is occurring. Biochemical characteristics of ara-A which may in part account for its failure with topical application in dermal HSV infections include: 1) very low solubility in water, 2) low lipid solubility which inhibits penetration into tissues and crossing of cell membranes, and 3) deamination by adenosine deaminase to its chief metabolite 9-β-D-arabinofuranosylhypoxanthine (ara-Hx), which has less antiviral activity than ara-A.

In an attempt to overcome the limitations of ara-A without altering the antiviral activity of the molecule, numerous analogues of the compound have been synthesized [3,19,20,22]. Four of these, 5′-monophosphate (ara-AMP), 5′-valerate ester (ara-AV), 2′,3′-diacetate ester (ara-ADA), and 2′,3′,5′-triacetate ester (ara-ATA) are shown in Fig. 1. The addition of these side chains to the ara-A molecule results in alterations in the physical properties as shown in Table 1. All of the compounds are more soluble in water

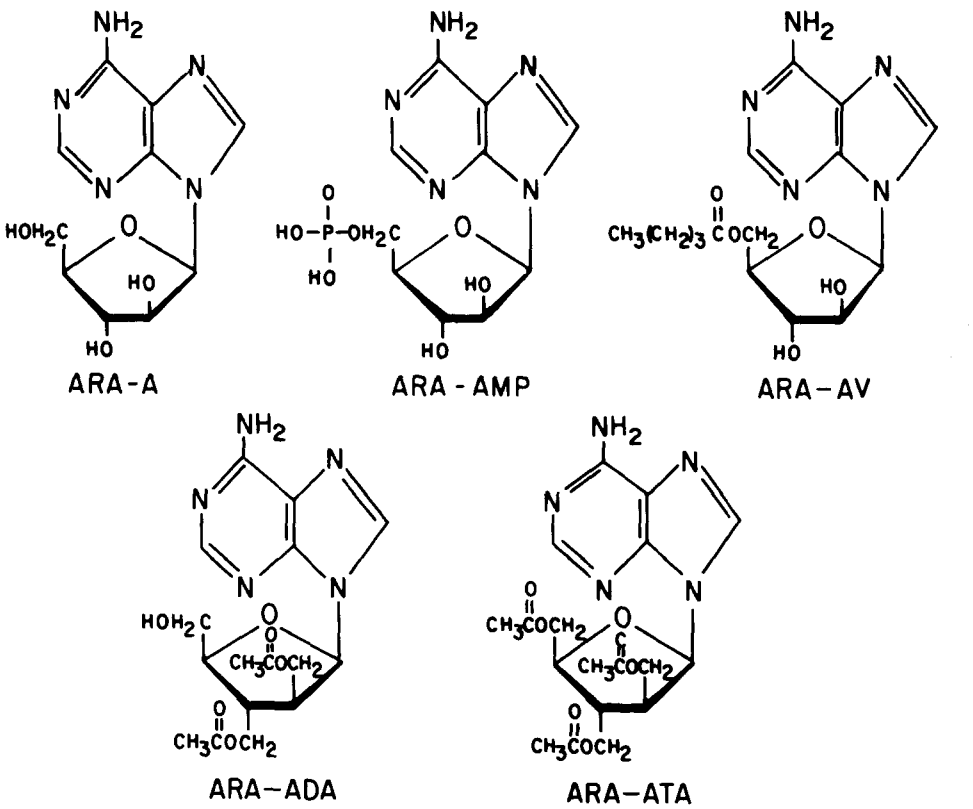

Fig. 1. Chemical structure of ara-A and four analogues showing substitutions with phosphate, valerate, or acetate on the 2′-, 3′- and/or 5′-carbon.

TABLE 1

Properties of ara-A and four analogues

Compound	Molecular weight	Aqueous solubility (mg/ml)	Partition coefficient log Kp[a] (pentanol/H_2O)
Ara-A	285	0.4	-0.48
Ara-AMP	365	100	ND[b]
Ara-AV	355	8.4	1.33
Ara-ADA	351	33.0	0.20
Ara-ATA	393	3.5	0.66

[a] $Kp = \frac{\text{equilibrium concentration in organic phase}}{\text{equilibrium concentration in aqueous phase}}$

[b] Not done.

and have higher partition coefficients in pentanol/water, indicating their greater lipophilicity [3]. The analogues do not exhibit antiviral activity until they are converted to ara-A through either dephosphorylation or deesterification. Importantly, however, either by steric hindrance and/or by the lack of a free 5′-hydroxyl group necessary for the binding of the adenosine deaminase enzyme, the compounds are more resistant to deamination than ara-A [3]. Since it appears that the product of ara-A deamination, ara-Hx, has less antiviral activity than ara-A, resistance to deamination should result in enhanced antiviral activity [3]. In addition, with ara-AV the process of conversion to ara-A results in the production of a deaminase inhibitor, which further inhibits deamination of the ara-A produced [13]. Because deamination occurs only after the analogues have been converted to active ara-A, and since conversion occurs as the molecule is penetrating the skin or mucous membrane, higher levels of ara-A would be expected in tissues where HSV replication is occurring. These analogues, therefore, should have an increased activity in the topical treatment of cutaneous HSV infections.

The purpose of the present study was to compare the anti-HSV activity of ara-A, ara-AMP, ara-AV, ara-ADA and ara-ATA in tissue culture and when applied topically in HSV type 2 (HSV-2) genital infections of mice and guinea pigs.

MATERIALS AND METHODS

Virus, media and cell cultures

The origin and preparation of virus pools, cell cultures and media utilized have been described previously [8].

Antiviral agents

All of the compounds were provided by Parke, Davis and Co. (Ann Arbor, MI) through the Antiviral Substances program of the National Institute of Allergy and Infectious

Diseases (Bethesda, MD). Powder forms were supplied for in vitro testing and 10% vaginal creams with placebo vehicles were provided for animal studies.

Susceptibility of HSV strains in vitro

The sensitivity of four HSV type 1 (HSV-1) and four HSV-2 strains to the ara-A compounds was determined by a 50% plaque reduction assay in mouse embryo fibroblast (MEF) cells. Confluent MEF cell monolayers were inoculated with 20–50 plaque-forming units (p.f.u.) of the appropriate virus and allowed to incubate at 37°C for 1 h. Serial two-fold dilutions of each drug were prepared in twice concentrated Eagle's minimal essential medium (MEM), mixed with an equal volume of a 1% agarose solution and added to the monolayers after incubation. At the appropriate time, monolayers were stained with neutral red and the plaques counted. Susceptibilities are expressed as mM concentrations of drug which inhibited 50% of the plaques compared to the control plates.

Experimental infections

Six to eight week-old female Swiss–Webster mice (Simonsen Laboratories, Gilroy, CA) and 200 g female guinea pigs (Charles River Breeding Laboratories, Inc., Wilmington, MA) were utilized for in vivo studies. Mice were inoculated intravaginally with 10^5 p.f.u. and guinea pigs with 10^4 p.f.u. of the MS strain of HSV-2 2 h after the removal of vaginal secretions with a phosphate-buffered saline (PBS) moistened swab. Groups of 15 mice were treated intravaginally with 0.1 ml of the appropriate drug beginning 3 h after viral inoculation and continued twice daily for 5 days. Groups of 10 guinea pigs were treated with 0.1 ml of drug intravaginally and 0.1 ml of drug applied to the external genitalia beginning at 6 or 24 h after HSV inoculation and continued twice daily for 5 days. The method of viral inoculation and course of these infections were described in a previous publication [10].

Assay for HSV in vaginal secretions

Swabs of vaginal secretions were obtained from infected animals on days 1, 3, 5, 7 and 10 after viral inoculation. Swabs were placed in tubes with 1.0 ml of MEM, vortexed and frozen at -70°C until assayed on fetal lamb kidney (FLK) cells for the presence of HSV. The method of sample collection and assay procedure has been described in detail [10].

Evaluation of lesion severity

Lesions appearing on the external genitalia of guinea pigs were scored daily through 12 days on a 0–4+ scale in 0.5 increments. An explanation of lesion development and score has been described previously [10].

Statistical evaluation

Final mortalities of placebo-treated and drug-treated mice were compared by the Fisher exact test. The virus titer-day area under the curve and lesion score-day area under the curve values were generated through the use of a computer program. These areas

as well as the mean day of death (MDD), were compared utilizing the Mann–Whitney U rank sum test. Placebo-treated groups were compared to untreated controls and drug-treated were compared to placebo-treated groups. For all statistical analysis a P value of 0.05 was considered significant.

RESULTS

Susceptibility of HSV to ara-A, ara-AMP, ara-AV, ara-ADA and ara-ATA in vitro

The sensitivity of four HSV-1 and four HSV-2 strains, including laboratory-passaged strains and recent clinical isolates, to ara-A and the four analogues is presented in Table 2. The 50% inhibitory levels for ara-A, ara-AMP and ara-AV were similar with a mean of 0.017–0.019 mM for HSV-1 and 0.022–0.052 mM for HSV-2. The inhibitory levels for ara-ADA and ara-ATA were 6–10-fold higher: 0.148 and 0.137 mM, respectively, for HSV-1 and 0.219 and 0.198 mM for HSV-2.

Effect of topical treatment with ara-AMP, ara-AV, ara-ADA and ara-ATA on a genital HSV-2 infection of mice

Intravaginal inoculation of mice with HSV-2 results in local viral replication in the vaginal mucosa and an ascending encephalomyelitis and death on days 7–12 after viral challenge [14]. Treatment with 10% vaginal creams or placebo vehicle was initiated 3 h after HSV inoculation and continued twice daily for 5 days. The results of these experiments are shown in Table 3. There were no differences in infection rates, mortality rates, MDD or virus titers in vaginal secretions between drug-treated and placebo-treated mice. We have previously reported similar data for ara-A [9].

Effect of topical treatment with ara-A, ara-AMP, ara-AV or ara-ATA on a genital HSV-2 infection of guinea pigs

In guinea pigs, intravaginal inoculation with HSV-2 results in local viral replication and the development of lesions on the external genitalia beginning on day 4 after infection [10]. Treatment was initiated 6 or 24 h after viral inoculation and continued twice daily for 5 days.

Treatment with ara-A, ara-AMP or ara-AV initiated at 6 h was highly effective in inhibiting viral replication in the vaginal mucosa (Fig. 2), and completely inhibited the development of external lesions (Fig. 3). Treatment with ara-ATA resulted in a slight, but not significant, reduction in virus titers and lesion severity (Figs. 2 and 3). Due to a lack of material, ara-ADA was not tested in this model infection.

When the initiation of therapy was delayed until 24 h after HSV inoculation, no effect on viral replication in the genital tract was observed with any of the drugs tested (Fig. 4). The effect of treatment on lesion severity is shown in Fig. 5. The severity and duration

TABLE 2

Sensitivities of HSV-1 and HSV-2 to ara-A, ara-AMP, ara-AV, ara-ADA and ara-ATA in mouse embryo fibroblast cells[a]

Virus strain	Mean inhibitory concentration (mM)				
	ara-A	ara-AMP	ara-AV	ara-ADA	ara-ATA
HSV type 1					
E-377[b]	0.014	0.017	0.012	0.122	0.123
Wilson[c]	0.011	0.016	0.011	0.152	0.123
HL-34[c]	0.027	0.027	0.031	0.184	0.179
HL-3[c]	ND[d]	0.015	ND	0.135	0.123
Mean ± S.E.M.[e]	0.017 ± 0.005	0.019 ± 0.003	0.018 ± 0.006	0.148 ± 0.013	0.137 ± 0.014
Mean μg/ml	5.0	7.7	6.3	52.0	53.8
HSV type 2					
MS[b]	0.057	0.031	0.027	0.277	0.223
X-79[b]	0.060	0.029	0.019	0.238	0.172
E-196[b]	ND	0.029	ND	0.201	0.227
Heeter[c]	0.038	0.020	0.020	0.160	0.172
Mean ± S.E.M.	0.052 ± 0.007	0.027 ± 0.002	0.022 ± 0.002	0.219 ± 0.025	0.198 ± 0.015
Mean μg/ml	14.7	11.2	7.9	76.8	78.0

a 50% plaque reduction assay, mean of four assays.

b Laboratory-passaged strains.

c Recent clinical isolates.

d Not done.

e S.E.M. = standard error of the mean.

TABLE 3

Effect of topical treatment with 10% ara-AMP, ara-AV, ara-ADA or ara-ATA vaginal creams on an HSV-2 genital infection of mice

Treatment[a]	Infection rate[b]		Mortality rate[c]		MDD	Virus–titer–day[d] (area under the curve)
	No.	%	No.	%		
Ara-AMP						
placebo	13/15	87	11/13	85	9.7	33.5
10% cream	13/15	87	11/13	85	10.6	29.8
Ara-AV						
placebo	9/15	60	6/9	67	12.3	29.6
10% cream	6/15	40	4/6	67	11.5	27.4
Ara-ADA						
placebo	10/12	83	7/10	70	10.6	26.5
10% cream	14/15	93	13/14	93	9.6	33.4
Ara-ATA						
placebo	10/12	83	9/10	90	9.7	32.6
10% cream	12/15	80	10/12	83	10.1	29.4

[a] Treatment begun at 3 h after viral inoculation and continued twice daily for 5 days.

[b] No. infected/No. inoculated.

[c] No. dead/No. infected.

[d] Areas generated through the use of a computer program utilizing the mean virus titer for the group on the days sampled.

of lesions were altered with ara-A and ara-AMP (P 0.05), but not with ara-AV or ara-ATA.

DISCUSSION

In tissue culture, similar inhibitory levels against HSV-1 and HSV-2 were observed with ara-A (0.011–0.060 mM), ara-AMP (0.015–0.031 mM), and ara-AV (0.011–0.027 mM). The inhibitory levels with ara-A and ara-AMP are comparable to those previously reported from our laboratory [9] and others [21]. The di- and tri-acetate esters (ara-ADA and ara-ATA) were 6–10 times less active (0.122–0.277 mM and 0.123–0.227 mM respectively). The reason for the lower activity with ara-ADA and ara-ATA is not fully understood. It is known, however, that none of the analogues exhibit any antiviral effect until they are converted to ara-A [3]. Previous studies have shown that the rates of dephosphorylation of ara-AMP and de-esterification of ara-AV to ara-A are very rapid and complete, whereas the de-esterification of short chain esters such as ara-ADA and ara-ATA is very slow [3,22]. It appears that in tissue culture equal concentrations of active compound were available with ara-A, ara-AMP and ara-AV, while less ara-ADA or ara-ATA was converted

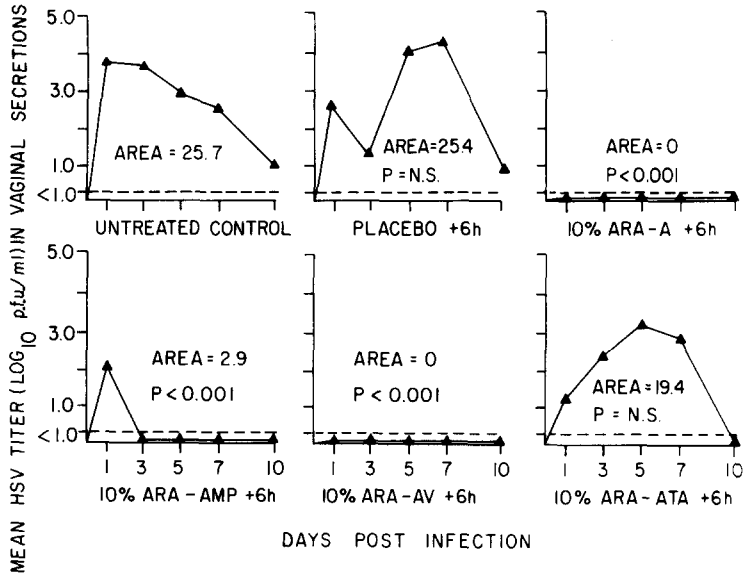

Fig. 2. Effect of topical treatment with ara-A analogues on virus titers in vaginal secretions of guinea pigs inoculated intravaginally with HSV-2. Therapy was initiated 6 h after viral inoculation and continued twice daily for 5 days.

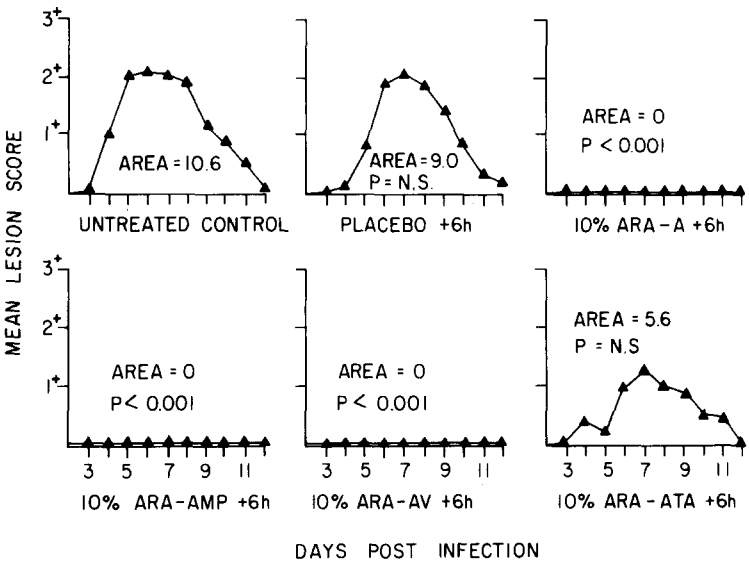

Fig. 3. Effect of topical treatment with ara-A analogues on the severity of genital lesions in guinea pigs inoculated intravaginally with HSV-2. Therapy was initiated 6 h after viral inoculation and continued twice daily for 5 days.

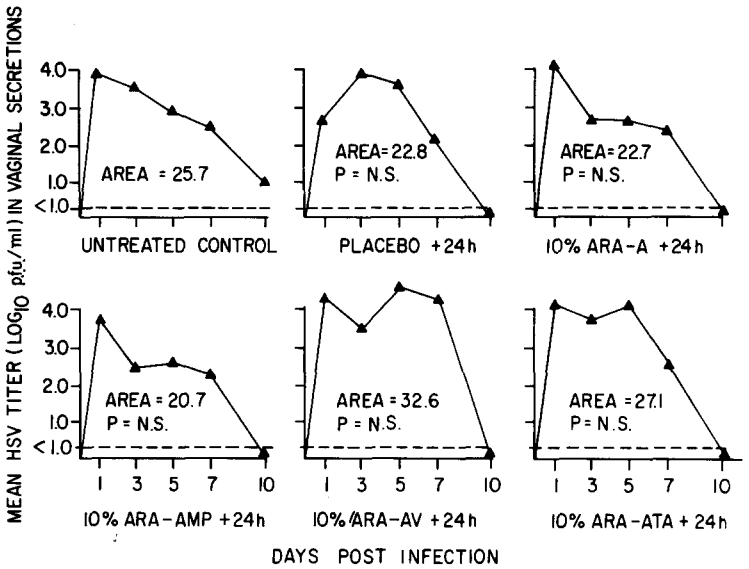

Fig. 4. Effect of topical treatment with ara-A analogues on virus titers in vaginal secretions of guinea pigs inoculated intravaginally with HSV-2. Therapy was initiated 24 h after viral inoculation and continued twice daily for 5 days.

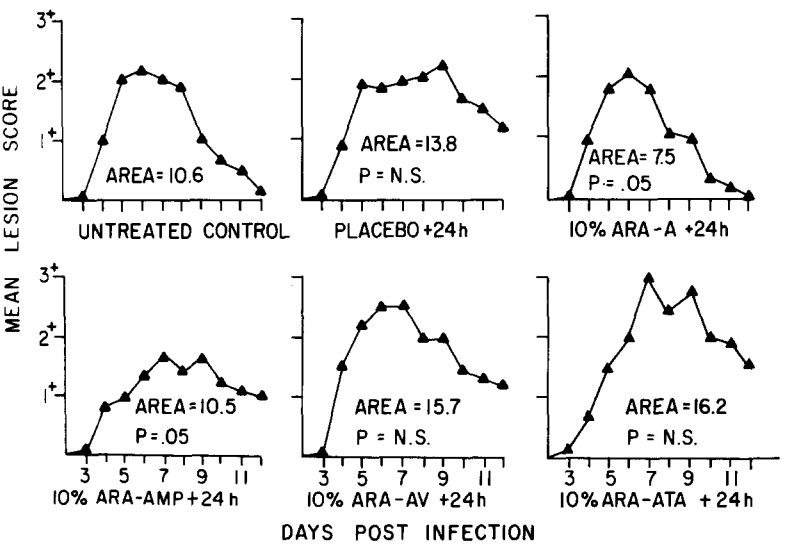

Fig. 5. Effect of topical treatment with ara-A analogues on the severity of genital lesions in guinea pigs inoculated intravaginally with HSV-2. Therapy was initiated 24 h after viral inoculation and continued twice daily for 5 days.

to ara-A. These differences in tissue culture, however, may not be predictive of efficacy with topical application in animals as the slower rate of de-esterification could cause more active ara-A to be released in deeper skin layers.

In the genital infection of mice, none of the compounds was effective in reducing viral replication in the vaginal canal or in reducing subsequent mortality from an ascending encephalomyelitis. This lack of efficacy with topical administration of ara-A and ara-AMP is similar to that reported earlier in genital and other mucocutaneous infections of mice [5,9,11]. In contrast, early therapy (beginning at 6 h) with ara-A, ara-AMP and ara-AV was highly effective against the genital infection of guinea pigs. Virus titers in vaginal secretions were completely suppressed and external lesions did not develop. When therapy was delayed until 24 h after infection, there was no effect on vaginal virus titers with any of the drugs, but treatment with ara-A or ara-AMP did significantly alter lesion severity. Renis [18] reported that intravaginal ara-A therapy in an HSV-2 genital infection of hamsters was effective in reducing vaginal virus titers and mortality and in prolonging the mean survival time if treatment was begun 1 or 24 h after viral inoculation.

Efficacy of ara-A, ara-AMP and ara-AV with early therapy in the guinea pig, but not in the mouse, may be due to species-related differences in penetration of topically applied drug and/or metabolic disposition in genital tissues. Higuchi and coworkers have shown that the permeability coefficients of ara-A analogues in immature guinea pig vaginal tissue are approximately 10 times greater than those of the mouse. On the other hand, the deaminase activity in vaginal tissue of mice and guinea pigs is comparable (W.I. Higuchi, personal communication). The greater efficacy of ara-A, ara-AMP and ara-AV in guinea pigs, therefore, may be due to greater penetration in guinea pig than in mouse genital tissues. The lack of effect with ara-ATA in the guinea pig may be attributable to the greater difficulty in cleaving the short-chain (acetate) esters and/or a lack of esterase activity rather than drug penetration. Using a vaginal membrane chamber model there is 5–10-fold less esterase activity in guinea pig tissue than in the mouse (W.I. Higuchi, personal communication).

It would appear that the efficacy of ara-A and its analogues is related both to drug penetration and to the enzymatic activity in the tissue of the animal being utilized. This hypothesis is supported by studies in experimental animal models which show an increased efficacy of ara-A or ara-AMP by utilizing methods to enhance penetration of topically applied drug or by simultaneous treatment with compounds that inhibit enzymatic degradation of the drugs. For example, the use of cathodal iontophoresis to increase penetration has been shown to enhance the efficacy of topical ara-AMP in HSV-1 and HSV-2 skin infections of hairless mice [12,15]. Additionally, the use of an adenosine deaminase inhibitor has been shown to potentiate the anti-HSV effect of ara-A and ara-AMP in vitro and in vivo [4,24,29].

Although the ara-A analogues tested in this study afforded the biochemical advantages of greater water solubility, lipophilicity and resistance to deamination than that of ara-A, they were no more effective than ara-A against HSV in tissue culture or when applied topically in the experimental genital infections. The results, therefore, fail to confirm the

hypothesis on which the development of these analogues was based, and suggest that effective levels of active drug were still not achieved at the site of HSV replication in the genital tissues of our experimental animals. Tissue penetration and enzymatic activity in those tissues appear to be important determinants of in vivo antiviral activity, and should be considered in the development of additional ara-A analogues.

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