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Antiviral Research 35 (1997) 157–165

Antiviral Research

# Cutaneously applied acyclovir acts systemically in the treatment of herpetic infection in the hairless mouse

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Received 18 February 1997; accepted 16 April 1997

**Abstract**

Using the SKH-1 hairless mouse (HM) we have addressed the issue as to whether topically applied acyclovir (ACV) may mediate some of its antiviral actions by a systemic effect. When topically applied in a formulation consisting of polyvinyl alcohol (25% w/v):DMSO:cremophor EL:linoleic acid (63:16:16:5, v/v/v/v), ACV penetrated hairless mouse skin in a concentration-dependent manner and dose-dependently reduced cutaneous herpes simplex virus 1 (HSV-1) KOS infection. Topically applied ACV also effectively reduced the mortality associated with disseminated HSV-2 HG-52 infection. At 1 h following topical application of 1.7% w/v ACV the plasma and skin concentrations of ACV were 5.5 $\eta$moles/ml and 120 $\eta$moles/g. At 1 h following an oral dose of ACV with antiviral efficacy comparable to topically applied ACV (1.7% w/v) the plasma and skin concentrations of ACV were 21.3 $\eta$moles/ml and 51 $\eta$moles/g. These findings imply that when applied topically to the HM, ACV can mediate a portion of its antiviral activity through a systemic mode of action. © 1997 Elsevier Science B.V.

*Keywords:* Herpes; Hairless mouse; Cutaneous; Topical; Acylovir

## 1. Introduction

Both orally and topically delivered acyclovir (ACV) are used clinically to treat herpes simplex virus (HSV) infections. An important consideration in the treatment of cutaneous and mucocutaneous HSV infections is the delivery of adequate amounts of ACV to the site(s) of infection (Spruance and Freeman, 1990). The hairless mouse (HM) cutaneously infected with HSV has been widely employed as an in vivo animal model to study the therapeutic effects of topically administered ACV and other related nucleoside analogs (de Clercq, 1984; Shannon et al., 1985; Lee et al., 1992a,b, 1993). ACV has been shown to be effec-

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*PII* S0166-3542(97)00024-7

tive against cutaneous HSV-1 infection in mice when administered either orally, intraperitoneally or topically (Kern, 1982; Klein, 1982; Collins, 1983; Kern et al., 1986; Lee et al., 1992a,b, 1993). It can effectively prevent lesions localized to the site of infection and zosteriform lesions arising from egression of the virus from spinal ganglia and peripheral nerves, irrespective of the route of administration. The permeation characteristics of HM skin have been well evaluated (Durrheim et al., 1980; Dalvi and Zatz, 1981; Bond and Barry, 1988; Ghanem et al., 1992). Consistent with its therapeutic efficacy following topical application, ACV was found to readily penetrate HM skin, the extent of which depended on the formulation employed (Spruance and Freeman, 1990; Lee et al., 1992a,b, 1993). In their model ACV was cutaneously applied using a transdermal delivery system located about 1.5 cm from the virus inoculation site. The therapeutic efficacy of ACV at the inoculation site was believed to be due to travel of ACV through the systemic circulation (Lee et al., 1992a,b). However, as ACV is a well distributed drug with little protein binding (de Miranda et al., 1991), the possibility of lateral diffusion of ACV though the dermis and subcutaneous space to the primary infection site to evoke a therapeutic effect cannot be ruled out. Thus, we investigated whether or not topically applied ACV might mediate a portion of its antiviral activity through a systemic mode of action in the following manner: (1) We evaluated the penetration of hairless mouse skin by ACV prepared in our formulation; (2) using either HSV-1 KOS mediated cutaneous herpetic infection or HSV-2 HG-52 mediated disseminated systemic infection, we assessed the effectiveness of orally or topically administered ACV; and (3) we measured the blood and skin levels of ACV following both topical and oral administration.

## 2. Materials and methods

### *2.1. In vivo studies*

#### *2.1.1. Virus inoculation*

Hairless SKH-1 mice, 4–8 weeks old originally obtained from Charles River Laboratories, St. Constant, Québec were bred in-house and used in all experiments. They were housed in a 12:12 h light/dark cycle with free access to food and water. Cutaneous infection with HSV-1 strain KOS (initial stocks kindly provided by Dr Curtis Brandt, University of Wisconsin) was initiated by first puncturing the skin of halothane anesthetized mice with a 12 needle punch and subsequently spreading $7.3 \times 10^7$ plaque forming units (pfu) of virus over the puncture site. Mice were inoculated bilaterally on either side of the thoracolumbar region. The minor wounds produced by puncturing mouse skin in this fashion healed within 24 h.

Disseminated systemic infection was initiated by intraperitoneal injection of $5 \times 10^5$ pfu of HSV-2 strain HG-52 (initial stocks were generously supplied by Dr Yves Langelier, Institut de Cancer de Montréal, Montréal, Québec) suspended in 100 $\mu$l of $\alpha$-minimal essential medium.

#### *2.1.2. Measurement of viral disease and drug treatment*

Cutaneous infection: Signs of disease were monitored using a subjective scoring system as follows: No visible infection, score 0; white vesicles, score 1; open vesicles, score 2; brown ulcerating vesicles, score 3; zosteriform ulcerative lesions, score 4.

Systemic infection: Signs of disease such as emaciation and bloating were noted as onset of disease and mortality was recorded.

Drug treatment: The sodium salt of acyclovir (made in-house from the free base supplied by Burroughs Wellcome) was used for all studies. For oral administration, ACV was dissolved in water and either drug or vehicle were administered by gavage at a dose volume of 10 ml/kg body weight. The total daily dose (in the range of 15–250 mg/kg) was administered as three equal doses at 4 h intervals beginning 3 h post-inoculation. Initiation of treatment was for most cases 3 h post inoculation since the goal was to treat primary HSV-1 infection. Oral treatment of primary infection with ACV but delayed at various times post-inoculation was also investigated. For topical treatments, ACV was initially dissolved in

DMSO and subsequently suspended (concentrations ranging from 0.01–1.7% w/v) in a formulation consisting of polyvinyl alcohol (molecular weight range of 30 000–70 000 daltons; Sigma, St. Louis, MO):DMSO:Cremophor EL (BASF, Toronto, Ontario):linoleic acid (Sigma) (63:16:16:5, v/v/v/v). Beginning 3 h post-inoculation, ACV was applied liberally either over the site of virus inoculation (cutaneously) or in the case of HSV-2 systemic infection, on the dorsal flank four times per day at 3 h intervals. The total volume of formulation containing ACV applied to each mouse averaged $\sim 35$ $\mu$l. For all treatment regimens with ACV, the total treatment time was 5 days. In experiments determining the levels of ACV in the skin and plasma following topical application, a known volume of formulation was applied to the skin.

### 2.2. Ex vivo experiments

#### 2.2.1. In vitro skin flux experiments

The in vitro skin flux of ACV was measured using a vertically mounted Franz diffusion cell. Hairless mice were killed by cervical dislocation and dorsal, flank and abdominal skin removed and placed into physiological saline buffered with 100 mM HEPES, pH 7.4. Excess fat and connective tissue were removed and the skin clamped between the donor and receiver chamber (stratum corneum facing the donor chamber side). The receiver bath fluid consisted of 100 mM HEPES buffered physiologic saline pH 7.4 and was maintained at 37°C. 20 $\mu$l of ACV (at various concentrations % w/v) in formulation and spiked with [$^3$H]ACV was spread evenly on the stratum corneum and 0.3 ml samples of receiver chamber buffer withdrawn (with buffer replacement) at various times. The flux of ACV through hairless mouse skin was determined by liquid scintillation spectrophotometry and investigated for up to 24 h.

#### 2.2.2. Determination of plasma levels of ACV

Mice treated either orally or topically with ACV samples spiked with radiolabelled ACV ([$^3$H]ACV, NEN-Dupont) were anesthetized with halothane. Blood was collected via cardiac puncture into heparinized syringes and immediately placed on ice. The plasma was separated by centrifugation (14 000 rpm, 10 min 5°C). A 500 $\mu$l aliquot of the plasma sample was then mixed with 500 $\mu$l of ice-cold methanol, vortexed and centrifuged as described above. The radioactivity of both the pellet and supernatant were determined by liquid scintillation counting, the sum of the radioactivity representing the total radioactivity present in the plasma. The concentration of ACV in the plasma was then calculated according to the dilution of radioisotope employed. In some experiments, the plasma levels were determined by HPLC (HPLC system from Waters, Mississauga, Ontario: controller, model 600 E, pump model 625 LC, sample processor (WISP) model 715 maintained at 10°C, photodiode array detector model 996 connected to system management software (Milenium 2010, version 2.10) and according to published methods (Šalamoun et al., 1987; Mascher and Kikuta, 1992). Initially, ACV from plasma samples was extracted by adding to a 200 $\mu$l sample 40 $\mu$l of water and 20 $\mu$l of 50% TCA. The samples were then vortexed for 30 s and centrifuged at 14 000 rpm for 10 min. A 150 $\mu$l aliquot of the supernatant was added to a tube containing 19 $\mu$l of KOH 1N and then filtered using a polysulfone ultrafiltration membrane ($M_w$ cutoff 10 000). A volume of 50 $\mu$l was then injected onto an HPLC column (C-18, Nova-Pak®, $2 \times 150$ mm) maintained at 30°C. The total run time was 30 min and the gradient conditions were 0–10% of A: 50% acetonitrile in 50 mM ammonium acetate, pH 5.5 and 100–90% of B: 25 mM ammonium formate pH 3.5. The flow rate was set at 0.25 ml/min. The concentration of ACV in plasma samples was determined by comparison to a standard curve. The extraction efficiency was 93% and the limit of detection was 20 $\eta$M.

#### 2.2.3. Determination of skin levels of ACV

Following the application of a finite dose of radiolabelled ACV to the skin over a measured area on the flank, the flank skin was rapidly excised and washed in 2 ml of methanol for a period not exceeding 5 s. The skin was blotted of excess methanol and dried prior to placing in

liquid scintillation fluid-for determination of [$^3$H]ACV. In cases where the skin samples were larger, the skin was first homogenized in physiologic saline and an aliquot taken for determination of [$^3$H]ACV.

## 3. Results

### 3.1. Skin flux of acyclovir

Preliminary investigation indicated that DMSO produced substantial skin irritation upon topical application in the HM. Thus, a non irritating formulation (described above) was developed. The HM skin flux of ACV from the formulation used in these studies was concentration-dependent, with peak skin flux obtained between 0.5 and 1.7% w/v (Fig. 1). At a concentration of 1.7% w/v the skin flux obtained with ACV was 1.5 $\eta$moles/cm$^2$ per h, a value was similar to that obtained for 1.7% w/v clonidine dissolved in DMSO (HM skin flux of clonidine 1.6 $\eta$moles/cm$^2$ per h). Clonidine was used as a standard in our experiments since it has been successfully used in transdermal delivery devices.

### 3.2. In vivo studies

Cutaneous inoculation of HSV-1 KOS in the HM produced cutaneous pathology (white vesicles at inoculation site; 100% of inoculated sides) commencing 2 days post inoculation and evidence of systemic disease (zosteriform lesions distal to inoculation site, 50–60% of inoculated sides) commencing 6–7 days post inoculation. When used on a 5 day treatment regimen, ACV produced a dose-dependent reduction of cutaneous pathology associated with HSV-1 KOS infection whether it was administered topically (skin) or orally (Figs. 2 and 3, respectively). Both the disease localized to the site of inoculation and the formation of zosteriform lesions, were effectively prevented by ACV. Delaying topical treatment with ACV up to 48 h has been shown not to reduce the therapeutic efficacy of ACV (Lee et al., 1992a,b). When the initiation of oral treatment with ACV was delayed by 48 h and subsequently continued for 5 days, a significant therapeutic effect similar in magnitude to that seen with treatment initiation 3 h post-inoculation was observed (Table 1).

Since topically (skin) applied ACV can readily penetrate the skin the effect of topically and orally administered ACV were compared in HSV-2 HG-52 systemically infected hairless mice. The results of these experiments are shown in Fig. 4. Topically applied ACV at 1.7% w/v (approximate total daily dose of 60 mg/kg) produced a significant protection against mortality induced by systemic disseminated HSV-2 HG-52 infection and was comparable in efficacy to 75 mg/kg daily dose administered orally.

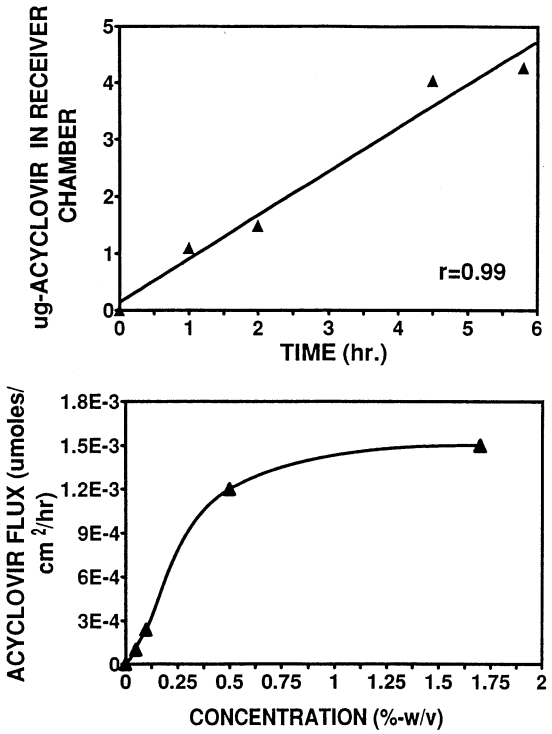


Fig. 1. Skin flux of ACV using HM skin: The time dependent flux of ACV suspended in formulation at a concentration of 1.7% w/v through HM skin is illustrated in the upper panel. The flux was linear for up to 24 h and had a value of $2.3 \pm 0.2 \times 10^{-3}$ $\mu$moles/cm$^2$ per h (mean $\pm$ S.E.M. of six experiments). The concentration-dependence of flux is shown in the lower panel, the flux values ($\eta$moles/cm$^2$ per h) per concentration being 0.05% w/v, 0.10; 0.1% w/v, 0.24; 0.5% w/v. 1.2 and 1.7% w/v, 1.5. The values are the mean of three experiments with the range of values not differing by more than 10% of the mean.

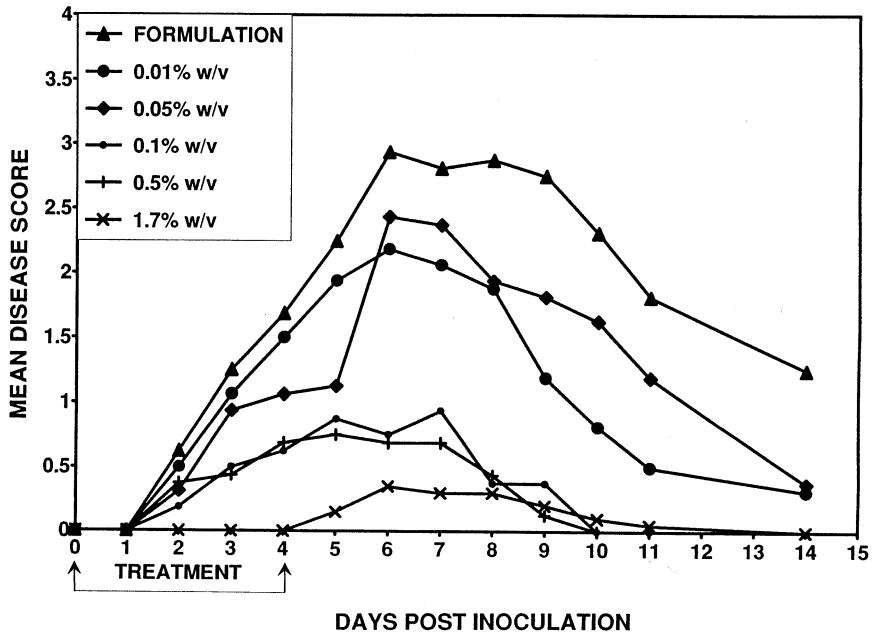


Fig. 2. Reduction of cutaneous HSV-1 KOS pathology by topical ACV: ACV suspended in formulation was topically applied as described in Section 2. A significant reduction of peak disease compared to formulation treated control ($P < 0.05$, ANOVA followed by Student Neuman Keul's post-hoc analysis) was observed at concentrations of 0.05% w/v and above. The reduction of area under the curve (AUC) per concentration was: 0.01% w/v, 21%; 0.05% w/v, 23%; 0.1% w/v, 76%; 0.5% w/v, 78%; and 1.7% w/v, 85%. The $EC_{50}$ for ACV topically was 0.07% w/v. Eight animals were employed per experimental group.

### *3.3. Plasma and skin concentrations of ACV*

Consistent with these antiviral effects for ACV, both topical and oral ACV produced significant plasma and skin concentrations of drug. Following a single topical application of 20 $\mu$l of 1.7% w/v ACV (equivalent to a total body dose of 15 mg/kg), peak plasma levels were attained between 30 and 60 min, remained elevated up to 120 min and then declined (Fig. 5). In the case of a single 100 mg/kg oral dose of ACV, peak plasma levels were attained by 30 min and declined rapidly thereafter (Fig. 5). The plasma levels of ACV 1 h post dosing measured by radioactivity were in good agreement with those determined by HPLC suggesting at least for this time point radioactivity was measuring parent drug and not metabolise (Table 2).

The levels of ACV found in the skin were considerably higher after topical administration when compared to oral administration (Table 3). Following topical administration skin levels were initially high and dropped rapidly, while following oral administration, a peak skin concentration of $52 \pm 18$ $\eta$moles/g was obtained 1 h after dosing and declined thereafter. Using [$^3$H]ACV, it was determined that approximately 50% of the total applied topical dose was shed within 1 h following administration.

## 4. Discussion

Previous studies (Lee et al., 1992a,b, 1993) have concluded that ACV might mediate a portion of its therapeutic efficacy against cutaneous HSV-1 infection in the HM via a systemic mode of action. Using a number of experimental approaches we sought to investigate this premise further. ACV readily penetrated HM skin in a dose-dependent manner when applied in our non-irritating formulation which contained DMSO and linoleic acid, known skin penetration enhancers for ACV (Spruance et al., 1984; Cooper

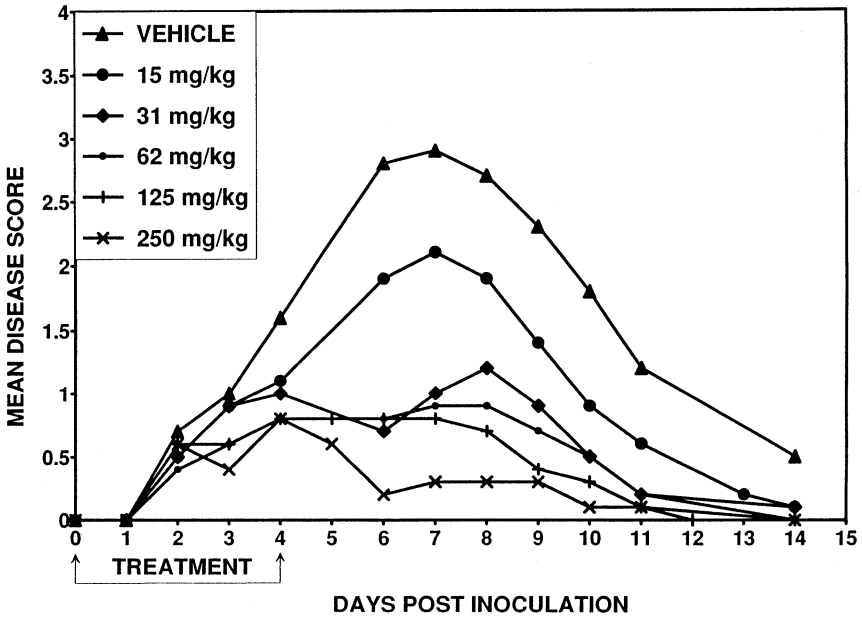


Fig. 3. Reduction of cutaneous HSV-1 KOS pathology by oral ACV: ACV was administered orally, the total daily dose shown in the legend divided into three equal doses per day. A significant reduction of peak disease compared to vehicle treated control ($P < 0.05$, ANOVA followed by Student Neuman Keul's post-hoc analysis) was observed at all doses of ACV. The reduction of AUC per dose was: 15 mg/kg, 34%; 31 mg/kg, 60%; 62 mg/kg, 66%; 125 mg/kg, 69%; 250 mg/kg, 78%. The $ED_{50}$ for ACV orally was 25 mg/kg. Eight animals were employed per experimental group.

et al., 1985). This observation is consistent with other reports of good dermal penetration of HM skin by ACV when suspended in some, but not all formulations (Cooper et al., 1985; Freeman and Spruance, 1986a,b; Freeman et al., 1986; Lee et al., 1992a,b, 1993). The relative permeability of HM skin to ACV is consistent with its dose-dependent reduction of HSV- 1 KOS cutaneous pathology following topical administration. In fact, the extent of skin flux of ACV in this animal model has been shown to be highly correlated with its therapeutic efficacy (Lee et al., 1993). In addition, the ability of ACV to reduce cutaneous pathology is highly correlated with dermal penetration and reduction of cutaneous viral titer in the guinea pig (Spruance et al., 1984; Freeman and Spruance, 1986a,b).

Table 1
Therapeutic efficacy of ACV with oral treatments initiated at varving times post inoculation

| Treatment[a] | Peak mean disease score |
|---|---|
| Control | $3.1 \pm 0.2$ |
| ACV 125 mg/kg; 3 h p.i. | $0.9 \pm 0.3$* |
| Control | $3.6 \pm 0.2$ |
| ACV 125 mg/kg; 24 h p.i. | $1.2 \pm 0.2$* |
| Control | $3.7 \pm 0.2$ |
| ACV 125 mg/kg; 48 h p.i. | $1.7 \pm 0.4$* |
| Control | $3.0 \pm 0.3$ |
| ACV 125 mg/kg; 72 h p.i. | $2.7 \pm 0.4$ |

[a] Treatments were for 5 days as described in Section 2 commencing at the times indicated post inoculation (p.i.); eight animals were employed per experimental group.
* Significantly different, $P < 0.05$, unpaired Student's $t$-test.

Quite possibly as a consequence of the excellent dermal permeability of ACV in the HM when formulated in a medium containing known dermal permeation enhancers such as DMSO and linoleic acid, significant plasma levels of ACV were obtained following topical administration. The retained skin concentrations of ACV, as might be expected, were initially high and then declined rapidly. The diffusion of ACV from der-

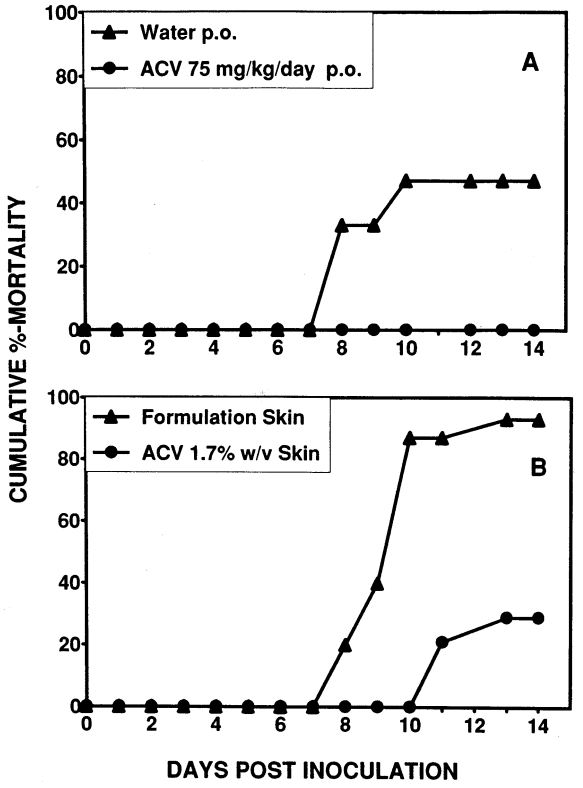


Fig. 4. Reduction of mortality associated with disseminated HSV-2 infection by ACV: Disseminated systemic HSV-2 infection was initiated as described in Section 2. In A, ACV was administered three times per day at a dose of 25 mg/kg for a total daily dose of 75 mg/kg. Mortality in each group was for vehicle treated 52% and ACV treated 0%; significantly different from vehicle treated, $P < 0.05$ $\chi_2$ test with 1 df. In B ACV in formulation was applied to both flanks of the animal as would be in the topical treatment of cutaneous HSV-1 KOS infection. Mortaliy in each group was for formulation treated 92% and ACV treated 25%; significantly different from formulation treated, $P < 0.05$ $\chi_2$ test with 1 df. In each experimental group, 18 mice were employed.

mal reservoirs would be expected to contribute to the plasma levels of ACV following topical administration. However, these observations in the HM are likely not relevant to the clinical situation as approved topical formulations of ACV are suboptimal for drug penetration (Freeman et al., 1986) and the ratio of the treated skin area to the total is extremely small.

The observations concerning the plasma concentrations of ACV following oral dosing are

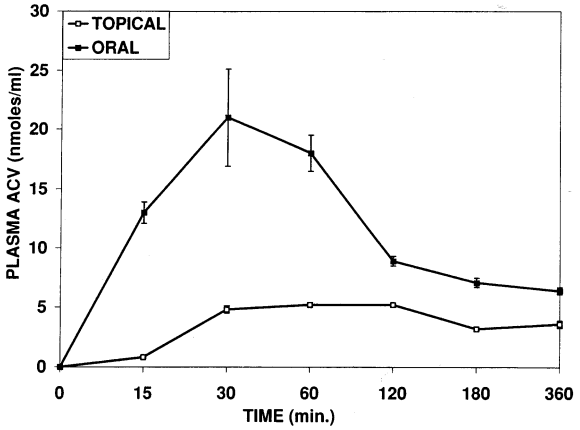


Fig. 5. Concentrations of ACV in the plasma following topical and oral dosing: Plasma levels of acyclovir were determined using [$^3$H]ACV as described in Section 2. The peak plasma concentrations of ACV observed were: following topical administration of 1.7% w/v (at an equivalent dose of 15 mg/kg) $5.5 \pm 0.2$ $\eta$moles/ml; following oral dosing with 100 mg/kg $21.0 \pm 4.1$ $\eta$moles/ml. The results are the mean $\pm$ S.E.M. of six determinations.

consistent with previous pharmacokinetic studies of ACV in the mouse (de Miranda et al., 1991; de Miranda and Good, 1992; Lee et al., 1992a,b, 1993) thus, high plasma levels are achieved following oral dosing which then rapidly decline. Despite this, significant levels of ACV were able to reach the skin and evoke an antiviral effect. A comparison of the ratio of the maximum plasma levels of ACV (Table 2) per finite dose of ACV (mg/kg) on topical (dose of 15 mg/kg; ratio =

Table 2
Plasma concentration of ACV determined by radioactivity and HPLC

| Experiment | Plasma concentration ($\eta$moles/ml) | |
|---|---|---|
| | Radioactivity | HPLC |
| 60 min Post ACV, oral dose 100 mg/kg | $21.3 \pm 4.1$ | $20.2 \pm 1.9$ |
| 60 min Post ACV, topical (total body dose, 15 mg/kg) | $5.5 \pm 0.2$ | $6.3 \pm 1.3$ |

The results are presented as the mean $\pm$ S.E.M. of three determinations.

Table 3
Skin concentration of ACV following either oral or topical administration

| Time following dosing (min) | Skin concentration ($\eta$moles/g skin) | |
|---|---|---|
| | Oral | Topical |
| 30 | $38 \pm 9$ | $142 \pm 33$ |
| 60 | $52 \pm 18$ | $121 \pm 27$ |
| 120 | $12 \pm 1$ | $71 \pm 7$ |
| 240 | $6 \pm 2$ | $55 \pm 6$ |

The results are presented as the mean $\pm$ S.E.M. of five to seven skin samples.

0.37) and oral (dose of 100 mg/kg; ratio = 0.21) administration suggests that both routes will deliver comparable amounts of ACV into the blood.

Delaying treatment with orally administered ACV produced results (Table 1) qualitatively similar to those for delaying topical treatment (Lee et al., 1992a,b). Following a 48 h delay in the initiation of therapy with ACV, a substantial amount of viral replication associated with the untreated primary infection would be expected and the likelihood of viral spread to systemic disease (zosteriform lesion development) would have greatly increased. However, delaying oral treatment with ACV was effective in controlling systemic disease suggesting the requirement of systemic concentrations of ACV for effective therapy of systemic disease. The effectiveness of topical ACV in delay therapy implies that when topically applied ACV cannot solely be acting locally in the skin. Thus, a portion of the antiviral activity of ACV following topical administration is likely mediated through the achievement of effective systemic concentrations.

ACV has been demonstrated to be effective in reducing both the pathology (Kern, 1982; Kern et al., 1986) and associated organ titers of virus in the CNS and periphery following infection with HSV-2 (Kern et al., 1983, 1986). The plasma concentrations of ACV attained following topical administration were found to effectively reduce mortality associated with disseminated HSV-2 HG-52 infection. This observation strongly supports the suggestion that systemic antiviral concentrations of ACV could be achieved following topical administration.

In substantiation of the hypothesis put forward by other investigators concerning the 'systemic' mode of action for topically applied ACV (Lee et al., 1992a,b, 1993), we have clearly shown that ACV can achieve systemic antiviral concentrations following topical application. These findings have important ramifications for the use of not only the HM, but other small animal models for investigating the topical therapy of cutaneous HSV infection in which the treatment area is large enough to produce significant antiviral blood concentrations of drug. Therefore, effective systemic delivery of a topically applied drug might falsely indicate the analysis of its therapeutic potential in small animal models of HSV infection. The possibility of both local and systemic modes of drug action should thus be taken into account in the evaluation of topical anti-HSV drugs when using small animal models.

## Acknowledgements

We gratefully acknowledge the assistance of Nathalie Dansereau for the preparation of viral titers and Giovanni Di Lella for the breeding and care of the hairless mice used in these studies. We also acknowledge Dr. Michael Cordingley for helpful comments concerning the manuscript.

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